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# Antimicrobial effect of Essential Oils against several pathogens and their influence on inhibition of *Salmonella* Enteritidis, *Listeria monocytogenes* and *Staphylococcus aureus* in paste of "alheira" during storage.

Thesis submitted to *Universidade Católica Portuguesa* to obtain the degree of Master in Applied Microbiology.

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Supervisor: Professora Doutora Paula Cristina Maia Teixeira
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*Tese dedicada a minha família*
*Sempre no meu coração*
♡

**Abstract**

Fermented meat products are part of the daily diet in rural areas of Portugal and have become very popular in urban centers. "Alheiras" are traditional, slightly smoked, naturally fermented meat sausages typical of the Northern regions (Trás-os-Montes) in Portugal. Essential oils (EOs), traditionally used as flavoring agents, have been revealing good antimicrobial properties, becoming a good natural alternative to the use of chemical preservatives. The aim of this study was to investigate the antimicrobial effect of some EOs against several pathogens and their influence on inhibition of *Salmonella* Enteritidis, *Listeria monocytogenes* and *Staphylococcus aureus* in "alheira" during storage. First, the *in vitro* antimicrobial effect of 23 EOs against 41 foodborne and spoilage microorganisms was screened by the disc diffusion assay method (21 Gram-positive bacteria, 18 Gram-negative bacteria and two yeasts). Then, the minimum inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) was studied for the EOs that displayed a better antimicrobial activity (i.e. halo > 10 mm) by the Microtiter plate assay. The antimicrobial effect of different concentrations of oregano EO (the EO with the lower MIC) (4%, 1.5%, 0.5%, 0.195% and 0.0975%) was evaluated in paste of "alheira" against *Salmonella* Enteritidis, *L. monocytogenes* and *St. aureus* along 21 days of storage at 4 °C. The pH, water activity values and lactic acid bacteria were also evaluated. At last, sensory assessment was performed.

Results showed that antimicrobial activity was variable, according to EOs used and microorganism. In general, oregano and thyme were the essential oils that showed highest antimicrobial activity and anise, fennel, garlic and ginger were the EOs with lower activity. The lowest minimum inhibitory concentration (0.0244%) against *L. innocua* was observed for Oregano. Oregano and thyme also showed higher MICs, against all microorganisms. Utilization of oregano EO in paste of "alheira" along 21 days of storage at 4 °C resulted in a natural strategy to improve its safety against *S.* Enteritidis, *L. monocytogenes* and *St. aureus*. Although, oregano EO possesses antibacterial properties *in vitro*, their utilization as food antimicrobial agents must be assessed in the food product, in particular in a complex matrix as "alheira". The antibacterial effect varies according to the oregano EO concentration and pathogen used. The results showed that 4% and 1.5% of oregano EO demonstrated the best antimicrobial activity against all the pathogens tested. 0.5% was able to result in ~ 2 log reduction for *S.* Enteritidis, whereas the lowest concentrations used (0.195% and 0.0975%) resulted in ~ 2-3 log reduction after 21 days

for *L. monocytogenes*. Although oregano EO showed anti-listeria properties in low concentrations, the same wasn't verified for *S.* Enteritidis and *St. aureus*. Counts of lactic acid bacteria were ~$10^9$ CFU/ml for all samples and no differences in the values of pH and $a_w$ were detected between samples. The sensory impact of oregano EO at 0.195% in "alheira" by overall acceptability, *just-about-right* scale, consumption trend and potential consumption was evaluated, since the sensorial impact of EOs in foodstuff has been described as a restriction to consumption. The results showed that the concentration have a negative impact on the acceptance of "alheira", because of its very intense flavor.

These results could be interesting for meat industry, helping to ensure the microbiological safety of the products, by meeting the new natural and good health needs of the consumer. However, it is necessary to validate these results in *in situ* production of "alheira", adding the EO as an ingredient, and to evaluate its acceptability by the consumer. It should be also explored the use of EOs in lower concentrations in combination with other treatments.

# Resumo

Os produtos à base de carne fermentada fazem parte da dieta quotidiana nas zonas rurais de Portugal e tornaram-se muito populares nos centros urbanos. Este estudo teve como objetivo avaliar o efeito antimicrobiano de alguns óleos essenciais (OEs) contra vários agentes patogénicos e a sua influência na inibição de *Salmonella* Enteritidis, *Listeria monocytogenes* e *Staphylococcus aureus*. O efeito antimicrobiano *in vitro*, de 23 OEs contra 41 microrganismos isolados de alimentos (21 bactérias Gram-positivas, 18 bactérias Gram-negativas e duas leveduras) foi avaliado por ensaios de difusão do disco. De seguida, a concentração mínima inibitória (CMI) e a concentração mínima bactericida (CBM) foram determinadas para os OEs que mostraram uma maior atividade antimicrobiana (ou seja, halo > 10 mm) através de um ensaio realizado em microplaca. O efeito antimicrobiano de diferentes concentrações de OEs (OE com CMI mais baixo) (4%, 1,5%, 0,5%, 0,195% e 0,0975%) foi avaliado em pasta de "alheira" contra *S.* Enteritidis, *L. monocytogenes* e *St. aureus* ao longo de 21 dias de armazenamento a 4 ° C. O pH, os valores da atividade da água e o nível de bactérias do ácido lático também foram monitorizados. Por fim, foi avaliada a qualidade sensorial da pasta de "alheira" contendo OEs.

Os resultados mostraram que a atividade antimicrobiana foi variável, de acordo com os OEs utilizados e os microrganismos alvo. Em geral, os OEs de orégãos e de tomilho foram os que apresentaram maior atividade antimicrobiana, e os OEs de anis, de funcho, de alho e de gengibre a menor atividade. A menor concentração mínima inibitória (0.0244%) contra *L. innocua* foi observada para o OE de orégão. Os OEs de orégãos e de tomilho também apresentaram maiores CMI contra todos os microrganismos. A utilização do OE de orégãos em pasta de "alheira" ao longo de 21 dias de armazenamento a 4 ° C resultou numa estratégia natural para melhorar sua segurança por redução dos níveis de *S.* Enteritidis, *L. monocytogenes* e *St. aureus*. Embora, o EO de orégãos possua propriedades antibacterianas *in vitro*, a sua utilização como agente antimicrobiano alimentar deve ser avaliada no produto alimentar, em particular numa matriz complexa como a "alheira". O efeito antibacteriano varia de acordo com a concentração de OE de orégãos e patogénico utilizado. Os resultados mostraram que 4% e 1.5% de OE de orégãos apresentaram a maior atividade antimicrobiana contra todos os patogénicos testados. Para a concentração de 0.5% obteve-se uma redução de 2 log para *S.* Enteritidis, enquanto que as concentrações mais baixas utilizadas (0.195% e 0.0975%) resultaram numa redução de ~ 2-3 log para *L. monocytogenes* após 21 dias. Embora o OE de orégãos tenha mostrado propriedades

anti-listeria em baixas concentrações, o mesmo não foi observado para *S.* Enteritidis e *St.* *aureus*. As contagens de bactérias do ácido lático foram ~$10^9$ CFU / ml para todas as amostras e não foram detetadas diferenças nos valores de pH e $a_w$ entre as amostras. O impacto sensorial do OE de orégãos a 0.195% na "alheira" foi avaliado por testes de aceitabilidade geral, numa escala *just-about-right* (JAR). A tendência de consumo e o potencial de consumo foi avaliado, já que o impacto sensorial dos OEs em géneros alimentícios foi descrito como uma restrição ao consumo. Os resultados mostraram que a concentração teve um impacto negativo na aceitação de "alheira", devido ao seu sabor muito intenso.

Estes resultados podem ser interessantes para a indústria da carne, ajudando a garantir a segurança microbiológica dos produtos, atendendo às necessidades naturais do consumidor. No entanto, é necessário validar esses resultados na produção *in situ* de "alheira", adicionando o OE de orégãos como ingrediente e avaliar sua aceitabilidade pelo consumidor. Será também de explorar a utilização dos EOs, em concentrações mais baixas, em combinação com outros tratamentos.

**Palavras-chave:** Patogénicos alimentares; Alheira; Óleos essenciais; Orégãos; Segurança alimentar.

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Cláudia Dias

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# I. Introduction

## 1.1. Alheira - a Fermented meat sausage

Dry-cured sausages are one of the oldest forms of preserving meat and are typical of Mediterranean countries with a dry climate (Spain, France, Portugal and Turkey) (Yilmaz and Velioglu, 2009). In contrast, smoke-cured sausage, or cooked sausage, prevails in countries with a colder weather (Yilmaz and Velioglu, 2009). Fermented meat products are unique and often represented as an element of culinary heritage and identity (Ojha *et al.,* 2015).

Portugal has an excellent "menu" when it comes to traditional fermented meat sausages with unique technological and sensory characteristics such as "alheira", "chouriça", "chouriço", "farinheira", "morcela" and "paio" (Marcos *et al.,* 2016). Although being produced all over the country, these products are predominantly manufactured in the North region (namely in the districts of Vila Real and Bragança, generally defined as Trás-os-Montes) and in the Southern region (Alentejo, comprising the districts of Évora, Beja and Portalegre) (Marcos *et al.,* 2016). In this market, "alheira" is the most representative product in terms of production volume (Patarata *et al.*, 2008). It is produced at different scales, from small units that work more seasonally, to industrial facilities that supply most of the market (Patarata *et al.*, 2008).

The origin of "alheira" goes back to the end of the fifteenth century and it is associated with the presence of Jewish communities in Trás-os-Montes, after they were banned from Castile in 1492 (Ferreira *et al.,* 2006). For the production of "alheira", several meats (duck, turkey, partridge and/or veal) are boiled in water with salt and spices. Bread is thinly sliced and immersed in some of the broth formed during the boiling of the meats and, when it is soft enough, meat in small pieces, spices, olive oil and/or fat drippings are added to the mixture. When everything is completely mixed, the paste is stuffed into pork intestinal or cellulose-based casings and submitted to a dry smoke process, usually for no longer than eight days (Marcos *et al.,* 2016). According to the "Specifications Notebook" (Associação Comercial de Mirandela, 2003), meats should be at least 60% of the total raw material and, of these, 50% should be pork; the bread should not exceed 25% of the total raw materials. The shelf life of "alheira" is about 1 month stored at 4 ºC in air or longer if the sausages are packed under modified atmosphere. "Alheira" should be cooked before consumption by frying, grilling or boiling, according to regional traditions or consumer preferences ("Specifications Notebook", Associação Comercial de Mirandela, 2003). The taste is described as being pleasant, lightly smoked, very particular,

where the garlic taste is noted and the aroma is lightly smoked and described as *sui generis* ("Specifications Notebook", Associação Comercial de Mirandela, 2003).

### 1.1.1. Microbiology of "Alheira"

Fermentation of traditional dry meat sausages relies on natural "contamination" - by environmental microbiota (Albano, 2008). This "contamination" occurs during slaughtering and increases during manufacturing (Albano, 2008). Albano (2008) reviewed that the type of microbiota developed is related to the diversity in formulation, and to the fermentation and ripening practices. Each processing facility has a specific house microbiota, composed of useful microorganisms for the fermentation and flavor of sausage, as well as of spoilage and pathogenic microbiota (Chevallier *et al*., 2006; Benito *et al*., 2007).

Several investigations have established two groups of microorganisms as being mainly responsible for the transformations involved during fermentation and ripening of fermented meat sausages (Albano, 2008; Campelos, 2012; Tremonte *et al.,* 2017). Lactic acid bacteria (LAB), in particular *Lactobacillus* spp., and Gram-positive coagulase negative cocci (CNC), specifically *Staphylococcus* spp. and *Kocuria* spp., are considered technologically fundamental (Talon *et al*., 2007; Di Cagno *et al*., 2008). Total LAB constitutes the major microbiota of the traditional sausages (Campelos, 2012; Greppi *et al*., 2015; Aquilanti *et al*., 2016). LAB usually increase during the very first days of fermentation and then remain constant at 7 - 9 log cfu/g during ripening (Comi *et al.*, 2005; Talon et al., 2007) or they can increase throughout the process and reach a similar final value (Lebert *et al.,* 2007). CNC constitutes the second largest fraction of the microbiota, with a population of 4 - 6 log cfu/g. CNC sometimes grow during the fermentation period or they can grow during ripening (Comi *et al*., 2005) or throughout whole the process (Lebert *et al*., 2007). Normally, is present in the development of color and taste (Ravyts *et al*., 2012; Talon *et al*., 2007). Besides these microorganisms, it has been reported by several authors that fermented dry sausages and other meat products could contain, during processing and in the final product, some of the well-known pathogenic bacteria often associated with meat products, such as *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Salmonella* spp., *Staphylococcus aureus*, *Clostridium* spp. and *Campylobacter* spp. (Siriken *et al*., 2006; Ferreira *et al*., 2006; Ferreira *et al*., 2007; Holck *et al.,* 2017).

Dry fermented sausages are mainly considered as microbiologically relatively safe products; 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 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). Over the past decade in European countries, epidemiological investigations have pointed several foodborne disease outbreaks associated with the consumption of dry fermented sausages (Ammon, *et al.,* 1999; Ethelberg *et al*., 2009; MacDonald *et al*., 2004; Paton *et al*., 1996; Sartz *et al*., 2008 and Schimmer *et al*., 2008). The causative agents in many of these outbreaks have been enterohaemorrhagic *E. coli* (EHEC), a subgroup of Shiga toxigenic *E. coli* (STEC). Other foodborne pathogens, e.g. *Salmonella* spp., have also been implicated as causative agents in dry fermented sausage outbreaks (Bremer *et al.,* 2004; Emberland *et al*., 2006; Kuhn, *et al.,* 2011). This means that many dry-fermented sausages production processes do not adequately maintain the microbial food safety and dry-fermented sausages products in general should be regarded as risk products if no interventions are applied to ensure microbial food safety (Heir *et al*., 2013).

In last years, "alheira" has been characterized as to their chemical and microbiological characteristics (Ferreira *et al.,* 2006; Ferreira *et al*., 2007*;* Albano, 2008*;* Esteves *et al.,* 2006*;* Esteves *et al.,* 2007*;* Esteves *et al.* 2008). Some of these studies related to factors that may influence the safe consumption of this food (Ferreira *et al.,* 2006; Ferreira *et al.,* 2007*;* Albano, 2008).


The microbiota of "alheira" is mainly composed by LAB (Ferreira *et al*., 2007; Albano, 2008; Esteves *et al.,* 2008). Albano *et al.* (2009) observed that LAB constitute the predominant microbial population of "alheira", with particular incidence to *Lactobacillus* spp. and *Enterococcus* spp., which were present in all samples analysed.  Pathogenic organisms, such as *L. monocytogenes*, *Salmonella* spp. and *St. aureus* have already been found in "alheira" (Esteves *et al.,* 2006; Esteves *et al.* 2007; Esteves *et al.* 2008; Ferreira *et al.,* 2006; Ferreira *et al.,* 2007b). According to Esteves *et al.,* (2008) *St. aureus*, *C. perfringens* and *Salmonella* spp. were the most common pathogens, with prevalence rates of 50, 25 and 12.5% respectively. The mean value of *St. aureus* and *C. perfringens* counts were 4.5 and 4.6 log CFU/g respectively. In 19% of the *St. aureus* contaminated samples, this microorganism revealed counts higher than $10^5$ CFU/g, quoted by Bergdoll (1989) as being sufficient to enable staphylococcal enterotoxin production. Forty-one percent of *C. perfringens*-contaminated samples presented values higher than $10^5$ CFU/g, quoted as a foodborne infection dose (Labbe, 1989). Ferreira *et al*., (2007a) found that more than 60% of the lots analyzed were contaminated with *L. monocytogenes* in levels higher than 2.0 log CFU/g (a level in excess of the established microbiological criteria

(EC, 2005)). Esteves *et al.,* (2006, 2008) also detected *Bacillus cereus* and *Yersinia enterocolitica*, but in a low percentage of samples analyzed.

## 1.2. Essential oils

Essential oils (EOs) in aromatic plants are among the most important active constituents of herbs and spices (Krisch *et al.*, 2010). The expression "essential oil" is thought to derive from the name created in the 16th century by Paracelsus von Hohenheim, a Swiss reformer of medicine (Guenther, 1948). Essential oils are secondary metabolites formed by plants, natural liquid extracted from plant material (flowers, buds, seeds, leaves, twigs, bark, herbs, wood, fruits and roots) and are volatile, and characterized by a strong odor (Burt, 2004). About 3000 EOs are known, of which about 300 are used in the industry - mainly for medicine, perfumery or cosmetic (Ghabraie *et al.,* 2016). Their use as flavoring agents in the food industry has been also increasing in order to avoid the use of traditional chemical additives (Ghabraie *et. al.,* 2016).

The main methods to obtain EOs from the plant materials are hydro-distillation (HD), steam distillation, steam and water distillation, maceration, empyreumatic (or destructive) distillation and expression (Burt, 2004). Among these, HD has been the most common approach to extract the EOs from the medicinal herbs/plants (Burt, 2004), where the plant material comes into direct contact with water (Tongnuanchan and Benjakul, 2014).

The differences antimicrobial activity between each oil are usually associated with the different chemical compositions of each EO, that change according to seasons, geographical location of plants and/or the methodology used in EO extraction (García-Díez *et al.*, 2016; Kokkini *et al.* 1996). Table 1 shows the main chemical compounds present in different essential oils. From table 1, it is possible to verify that the prevalent compounds belong to the group of monoterpenes such as limonene and sabinene, and monoterpanoids as linalool and carvone. Thymol and Carvacol are structurally similar among the most studied compounds (Hyldgaard *et al.*, 2012). Carvacrol and thymol are phenolic monoterpenoid and major constituents of oregano and thymol, respectively (Hyldgaard *et al.*, 2012). Depending on the characteristics, the compounds will be different from oil to oil.

The effectiveness of the EOs against a wide range of microorganisms is well documented (Krisch *et al.,* 2010; Guzman *et al.,* 2012). Most studies concerning the antimicrobial mode of action of essential oil constituents have been performed on bacteria, especially in *in vitro* assays.

Gram-negative bacteria such as *E. coli* and *S*. Enteritidis are generally more resistant than Gram-positive bacteria such as *St. aureus*, *L. monocytogenes* and *B. cereus* (Trombetta *et al*., 2005). Table 1 also shows the *in vitro* antimicrobial activity of some EOs, and as observed, the most affected bacteria are *L. monocytogenes*, *Salmonella* spp. and *St. aureus*. The antimicrobial activity of EOs is not assigned to any specific mechanism. There are different locations and / or mechanisms in the microbial cells that can be targeted by the constituents of the EOs. 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. Additionally, these oils can impair a variety of enzyme systems, including the enzymes involved in the energy regulation and synthesis of structural components and inactivate or destroy genetic material, strengthening their antimicrobial activities (Jayasen and Jo, 2013).

**Table 1.** Main chemical composition of Essential oils and *in vitro* antimicrobial activity against spoilage and pathogenic bacteria.

| Essential oil | Main chemical compound | Microbial activity (*in vitro*) | References |
|---|---|---|---|
| **Balm** (*Melissa officinalis*) | (-)-citronellal (40%) | *Salmonella* spp. | Fratianni *et al*., (2010) |
| **Bay** (*Laurus nobilis L.*) | Eucaliptol (58.20%) α-Terpinenyl acetate (19.19%) | *S. enterica* *L. monocytogenes* *St. aureus* | García-Díez *et al.*, (2016) |
| **Carrot (juice)** (*Daucus carrot L.*) | Carotol (20.2%) Sabinene (12.8%) β-caryophyllene (8.0%) | *Campylobacter jejuni* | Calo *et al.*, (2015) |
| **Cinnamon** (*Cinnamomum verum*) | Trans-cinnemaldehyde (68.4%) Limonene (13.2%) | *E. coli* O157:H7 *L. monocytogenes* *S. enterica* | Calo *et al.*, (2015) |
| **Clove** (*Syzygium aromaticum*) | Eugenol (7.5%) | *S. enterica* *L. monocytogenes* *St. aureus* | Calo *et al.*, (2015) |
| **Coriander** (*Coriandrum sativum*) | Linalool (74.6%) | *L. monocytogenes* | Jayasen and Jo, (2013) |
| **Fennel** (*Foeniculum vulgare L.*) | Trans-Anethol (56.4%) | *B. cereus* *B. substilis* *E. coli* *Klebsiella pneumonia* | Calo *et al.*, (2015); Roby *et al.*, (2013) |
| **Garlic** (*Allium sativum*) | Diallyl trisulfide (33.82%) Diallyl Disulfide (18.86%) Diallyl tetrasulphide (10.97%) | *S. enterica* *L. monocytogenes* *St. aureus* | García-Díez *et al.*, (2016) |
| **Marjoram** (*Marjorana hortensis Moench*) | Terpinen-4-ol (20.8%) γ-terpinene (14.1%) | *S. enterica* | Calo *et al.*, (2015) |
| **Nutmeg** (*Myristica fragrans*) | Myristicin (43.35%) Sabinene (23.28 %) | *L. monocytogenes* | García-Díez *et al*, (2016) |
| **Rosemary** (*Rosmarinus offcinalis L.*) | α-Pinene (23.98%) Camphor (22.62%) 1,8-cineole (18.76%) | *L. monocytogenes* | Giarratana *et al*,. (2016) |
| **Oregano** (*Origanum vulgare*) | Carvacrol (47.80%) Thymol (21.41%) γ-terpinene (13.44%) | *S. enterica* *L. monocytogenes* *St. aureus* | Haute *et al*., (2013) |
| **Thyme** (*Thymus vulgaris*) | *p*-cymene (20.61 %) Thymol (55.91 %) | *S. enterica* *L. monocytogenes* *St. aureus* | Haute *et al*., (2013) |

### 1.2.1. Applications

In recent years, consumers demand minimally processed foods. The negative perception of consumers about chemical food additives makes natural methods of preservation and natural preservatives receiving increased attention by the food industry (García-Díez *et al.*, 2016). Nonphytotoxic oils are safe as food additives and certified as "Generally Recognized As Safe" (GRAS), which results in high consumer acceptability (Jayasen and Jo, 2013). Due to the antimicrobial properties and their safety status, EOs are known as good candidates to be used

as food preservatives (Ghabraie *et al*, 2016). However, their application is limited by taste and odor impacts, especially when used at high concentrations (Ghabraie *et al*, 2016). Therefore, it is necessary to determine minimum antimicrobial concentrations that do not cause unacceptable changes in smell and taste. It has been demonstrated that they have antimicrobial activity against pathogenic bacteria at the range of 0.05-0.1% in food systems (Ghabraie *et al*, 2016). Firouzi *et al.* (2007) reported that although *in vitro* work with EOs and their components indicated that compounds such as oregano and nutmeg possessed substantial antimicrobial activity, when used in food systems the amounts required were approximately 1-3% higher, often higher than what would normally be organoleptically acceptable. For example, their use in food for infants has been limited, since the concentration to be used was too high, whereas *in vitro* small concentrations were shown to be sufficient for microbial inhibition (Hyldgaard *et al.,* 2012). Table 2 summarizes results of some studies about the antimicrobial activity of EOs oils applied to different products, in particular to products of meat origin. The authors demonstrated that different EOs significantly reduce microorganisms. However, some of these studies showed that it was not possible to use the oils in the products, since the antimicrobial concentration was too high, changing the smell and taste of the products (Selim, 2010; García-Díez *et al*., 2016).

Nowadays, as already stated, EOs and their components are gaining increased attention because of their relatively safe status, their wide acceptance by consumers and their potential functional and technological uses (Ghabraie *et al.*, 2016). Individual components of EOs are also used as food flavorings (Burt, 2004). A few products that contain EOs are commercialized by the food-additives industry to improve the shelf-life of foods (Burt, 2004). "DMC Base Natural" is a food preservative with 50% essential oils from rosemary, sage and citrus and 50% glycerol (Mendoza-Yepes *et al*., 1997). 'Protecta One' and 'Protecta Two' are combined herb extracts that are classified as GRAS food additives in the US (Cutter, 2000). Thus, as far as we know, there are very few products that make use of EOs, since their *in vitro* activity does not correspond to their *in situ* activity. In many food products, the hydrophobic components of the essential oil are compromised by interactions with components of the food matrix, such as fat (Cava-Roda *et al*., 2010; Rattanachaikunsopon and Phumkhachorn, 2010), starch (Gutierrez *et al*., 2008) and proteins (Kyung, 2011). Furthermore, the antimicrobial effectiveness of EO constituents also depends on pH, temperature (Rattanachaikunsopon and Phumkhachorn, 2010) and the level of microbial contamination (Somolinos *et al*., 2010). Besides all these, safety studies need to be conducted before widespread use of EOs in food preservation, since there

have been reports indicating skin irritation and toxicity in some people who use them frequently (Chivandi *et al.*, 2016).

**Table 2.** Antimicrobial activity (*in situ*) of some EOs on some products.

| Essential oil | Product | Microbial activity (*in situ*) | References |
|---|---|---|---|
| **Balm** (***Melissa officinalis***) | Chicken breast meat | 0.5% oil: Reduction of 50% in *Salmonella* spp. | Fratianni *et al.*, (2010) |
| **Oregano** (***Origanum vulgare L.***) | Ham | 0.05% oil: Reduction of 1.5 log in *L. monocytogenes* | Dussault *et al.*, (2014) |
| **Oregano** (***Origanum vulgare L.***) | Minced meat | *St. aureus*: 0.5% oil: Reduction of 1.5 log 1% oil: Reduction of 1 log 2% oil: Reduction of 2.5 log | Pesavento *et al.*, (2015) |
| **Oregano** (***Origanum vulgare L.***) | Minced meat | *L. monocytogenes*: 0.5% oil: Reduction of 1 log 1% oil: Reduction of 1 log 2% oil: Reduction of 2 log | Pesavento *et al.*, (2015) |
| **Rosemary** (***Rosmarinus offcinalis L.***) | Feta soft cheese | Vancomycin-Resistant Enterococci: 1% oil: Reduction of 6.5% | Selim, (2010) |
| **Rosemary** (***Rosmarinus offcinalis L.***) | Minced meat | *St. aureus*: 0.5% oil: Reduction of 3.5 log 1% oil: Reduction of 3.5 log 2% oil: Reduction of 3.5 log | Pesavento *et al.*, (2015) |
| **Rosemary** (***Rosmarinus offcinalis L.***) | Minced meat | *L. monocytogenes*: 0.5% oil: Reduction of 3 log 1% oil: Reduction of 3 log 2% oil: Reduction of 4 log | Pesavento *et al.*, (2015) |
| **Sage** (***Salvia officinalis***) | Feta soft cheese | *E. coli* O157:H7: 0.5% oil: Reduction of 7 log 1% oil: Reduction of 8.5 log | Selim, (2010) |
| **Sage** (***Salvia officinalis***) | Feta soft cheese | Vancomycin-Resistant Enterococci: 1% oil: Reduction of 7.5 log | Selim, (2010) |
| **Thyme** (***Thymus vulgaris***) | Minced meat | *St. aureus*: 0.5% oil: Reduction of 3 log 1% oil: Reduction of 3.5 log 2% oil: Reduction of 4.5 log | Pesavento *et al.*, (2015) |
| **Thyme** (***Thymus vulgaris***) | Feta soft cheese | *E. coli* O157:H7: 0.5% oil: Reduction of 8 log 1% oil: Reduction of 8.5 log | Selim, (2010) |
| **Thyme** (***Thymus vulgaris***) | Feta soft cheese | Vancomycin-Resistant Enterococci: 0.5% oil: Reduction of 7.5 log 1% oil: Reduction of 8.5 log | Selim, (2010) |
| **Thyme** (***Thymus vulgaris***) | Minced meat | *L. monocytogenes*: 0.5% oil: Reduction of 2.5 log 1% oil: Reduction of 2.5 log 2% oil: Reduction of 3.5 log | Pesavento *et al.*, (2015) |

### 1.3. Objective

The overall objective of this study was to investigate the antimicrobial effect of some EOs against several pathogens and their influence on inhibition of *Salmonella* spp., *L. monocytogenes* and *St. aureus* in "alheira" during storage. In order to achieve the overall objective, specific activities were planed:

1. To determine *in vitro* antimicrobial effect of selected EOs against foodborne and spoilage microorganisms, by the disc diffusion assay method;

2. To determine the minimum inhibitory concentration and minimum bactericidal concentration of 23 EOs against bacteria Gram-negative, Gram-positive (non-spores formers, spores formers and anaerobic spores formers) and yeasts in Microtiter plate assay;

3. To evaluate the influence of oregano essential oil in paste of "alheira" against *Salmonella* spp., *L. monocytogenes* and *St. aureus* along 21 days of storage at 4 ºC.

4. To test the acceptability, by a sensorial analysis, of "alheira" with oregano.

# II.  Materials and methods

## 2.1.  Essential oils

Twenty-three EOs were used in this study. The EOs (plant of origin) were: Anise (*Pimpinella anisum*), Basil (*Ocimum basilicum*), Bay (*Laurus nobilis L.*), Cardamom (*Elettaria cardamomum*) and Fennel (*Foeniculum vulgare*), kindly provided by FRULACT (Gemunde Maia, Portugal); Carrot (*Daucus carrot L.*), Cloves (*Syzygium aromaticum*), Coriander (*Coriandrum sativum*), Cumin (*Cuminum cyminum*), Garlic (*Allium sativum*), Juniper berry (*Juniperus communis*), Marjoram (*Origanum majorana*), Nutmeg (*Myristica fragrans*), Parsley (*Petroselinum crispum*), Peppermint (*Mentha piperita L.*). Oregano (*Origanum vulgare L.*), Rosemary (*Rosmarinus officinalis*) and Sage (*Salvia officinalis*) kindly provided by Ventós Chemical (Barcelona, Spain); Lemon (*Citrus limon*), Garlic (*Allium sativum*), Ginger (*Zingiber officinale*), Oregano (*Origanum vulgare*) and Thyme (*Thymus vulgaris*) by Casa das Essências (Oeiras, Portugal).

## 2.2.  Microorganisms and growth conditions

All strains used in this study are presented in Table 3. All microorganisms were stored at -20 ºC in Tryptic Soy Broth (TSB, Prolabo) with 6 g/l of YE (Lab M) containing 30% (v/v) glycerol (Sigma, Steinheim, Germany), and sub-cultured twice before use in assays.

Each strain was grown on TSA - Tryptic Soy Agar (TSA, Pronadisa, Madrid, Spain) with 6 g/l of Yeast Extract (YE, Lab M) at 37 ºC for 24 h and yeasts in Yeast Malt Agar (YMA, Sigma, Bury, UK) at 25 ºC for 48 h.

**Table 3.** Strains and their source used in this study.

| Microorganisms | Species | Source |
|---|---|---|
| **Gram-positive** | *Bacillus cereus* <br> *Bacillus subtilis* <br> *Bacillus stearothermophilus* <br> *Listeria monocytogenes* SCOTT A <br> *Listeria innocua* 2030c <br> *Staphylococcus aureus* 18N (Methicillin-resistant *Staphylococcus aureus* - MRSA) <br> *Staphylococcus aureus* 2037 M1 (Methicillin-sensitive *Staphylococcus aureus* - MSSA) | ESB culture collection |
| | *Enterococcus faecalis* ATCC 29212 <br> *Staphylococcus aureus* ATCC 29213 | ATCC |
| | *Enterococcus faecalis* DSMZ 12956 <br> *Enterococcus faecium* DSMZ 13590 <br> *Enterococcus flavescens* DSMZ 7370 <br> *Enterococcus casseliflavus* DSMZ 20680 <br> *Enterococcus gallinarum* DSMZ 20628 | DSMZ |
| | *Listeria monocytogenes* L7946 <br> *Listeria monocytogenes* L7947 | McLauchlin, J. *et al.* (1997) |
| **Gram-negative** | *Acinetobacter baumannii* R <br> *Acinetobacter baumannii* S-1 <br> *Acinetobacter baumannii* S-2 <br> *Acinetobacter calcoaceticus* R <br> *Acinetobacter calcoaceticus* S <br> *Clostridium sporogenes* 1.31 <br> *Clostridium sporogenes* 1.34 <br> *Clostridium sporogenes* 1.61 <br> *Clostridium perfringens* 1.16 <br> *Clostridium perfringens* 1.19 <br> *Clostridium perfringens* 1.22 <br> *Klebsiella pneumoniae* <br> *Proteus mirabilis* <br> *Proteus vulgaris* <br> *Pseudomonas aeruginosa* <br> *Salmonella* Braenderup <br> *Salmonella* Enteritidis <br> *Salmonella* Enteritidis 417536 <br> *Salmonella* Enteritidis 545047 <br> *Salmonella* Typhimurium <br> *Yersinia enterocolitica* | ESB culture collection |
| | *Escherichia coli* ATCC 25922 | ATCC |
| | *Yersinia enterocolitica* NCTC 10406 | NCTC |
| **Yeasts** | *Candida albicans* <br> *Saccharomyces cerevisiae* | ESB |

**ESB**: culture collection of Escola Superior de Biotecnologia; **DSMZ**: German Collection of Microorganisms and Cell Cultures; **ATCC**: American Type Culture Collection; **NCTC**: National Collection of Types Cultures – Culture Collection of Public Health England.

**S –** Sensitive to several tested antibiotics;          **R –** Resistant to several antibiotics;

### 2.3. Disk Diffusion Assay (DDA)

Each inoculum was prepared resuspending isolated colonies of each strain, previously cultured on TSA or YMA, in sterile Ringer solution (Lab M) in order to obtain turbidity equivalent to 0.5 in McFarland scale (Biomerieux, Marcy-l'Etoile, France).

The antimicrobial effect of EOs was screened by the disk diffusion assay (DDA) as described by Zaika (1988), with some modifications. Briefly, petri dishes prepared with Mueller-Hinton agar (MHa – Biokar, France), or Tryptose Sulfite Cyclocerine (TSC – Prolabo, Belgium) agar (for *C. perfringens* and *C. sporogenes*) or YMA for yeasts, were dried and 100 µl of standardized inoculum were uniformly spread. Then, filter paper disks (Whatman No. 5 mm diameter) were applied to the surface of the seeded agar plates and 5 µl of each EO was applied to each disk. The plates were kept at 4 ºC for 2 h to allow dispersion and incubated during 18 to 24 hours at 37 ºC for all microorganisms, with the exception of strains of *Clostridium* which were incubated in an anaerobic chamber (Whitley DG250 Anaerobic Workstation) for 48 h at 37 ºC and yeasts that were incubated for 48 h at 25 ºC. The antimicrobial activity was visually evaluated as inhibition zones surrounding the disk and the disk diameter was measured in mm. Inhibition was only considered if the halos were greater than 10 mm, according to García-Díez *et al.*, (2016).

The DDA assay was carried out in triplicate.

### 2.4. Statistical analysis

The comparison of the antimicrobial activity of EOs against each microorganism 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.

### 2.5. Microtiter plate assay (MPA)

The minimum inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) was studied for all EOs. The assay was based on the procedures described in CLSI (2012) using 96-wells microtiter plates. The dilutions of the EOs were established based on the inhibitory profile with the DDA (halos greater than 10 mm). EOs dilutions were prepared directly on the Mueller-Hinton broth (MHb – Biokar, France) in order to obtain in the well each of the followings concentrations: 50%, 25%, 12.5%, 6.25%, 3.125%, 1.56%, 0.78%, 0.39%, 0.195%, 0.0975%, 0.0488%, 0.0244%, 0.0129% and 0.0060%. The inoculum of the target

microorganism was also prepared in MHb (to result in a concentration in the well ca. 6 log CFU/ml). Eighty microliters of MHb, 100 µl with each EO dilution and 20 µl of each microorganism were mixed in each well of the microtiter plates. The plates were covered, incubated during 24 hours and then checked for visible growth in the wells. The MIC was considered the lowest concentration of EO where there was no growth. A negative control without inoculation was included in the test. Since some EOs in the well presented an ambiguous turbidity, the test was complemented with the seeding of a 10µl loop in MHa to confirm the absence of growth. To evaluate the MBC, 10 µl of each well, in which no microbial growth was observed, was spread into MHa and incubated for 24 hours, as described by García-Díez *et al.,* (2016).

## 2.6. Optimization of the protocol of "Inhibitory effect of the selected essential oil (oregano) against selected pathogens in paste of "alheira"

### 2.6.1. Experimental sensorial test

After determination of the MICs by the method described in 2.5, a preliminary experimental sensorial test of acceptability of "alheira" with three different concentrations of oregano essential oil (0.0975%, 0.195% and 0.39%) was performed. The aim was to determine whether it was possible to use these concentrations in an "alheira" and to be pleasant or distasteful to the palate.

### 2.6.2. Determination of the volume and the most suitable concentration of the oil to be used in paste of "alheira"

**A**. The inhibitory effect of the selected essential oil (oregano) at 0.0975% against *L. monocytogenes* L7949 and *S.* Enteritidis was performed. A control without addition of EO was also used. The organisms were sub-cultured twice (24 h at 37 ºC) in 10 ml MHb using a 1% v/v inoculum. An aliquot (250 µl) of each bacterial suspension ($10^7$ cfu/ml) was added to 25 g of paste of "alheira" contained in stomacher bags and 780 µl of EO was also added. After assuring good mixing of the inoculum and EO with the paste (manually massaging of the exterior of the bags), each 25 g were stored at 4 ºC for 7 days. After four hours and 3 and 7 days of storage, samples were analysed for growth of the inoculated strains and LAB bacteria. The pH and $a_w$ was also evaluated.

**B.** The same procedure described in 2.6.2.A) was repeated, using sterilized paste of "alheira" and non-sterilized paste of "alheira", inoculated only with *L. monocytogenes* and with two different volumes of essential oils (in order to reach the same EO concentration - 0.0975%).

**C.** For this experiment different ingredients of "alheira were used (25 g of each): sliced bread, pork and chicken in small pieces, meat cooking broth, sliced bread with cooking broth, olive oil, total mixture, commercial paste of "alheira" and garlic vineyard. The same procedure described in 2.6.2.A) was performed but only for *L. monocytogenes*. For each ingredient, three concentrations of oil were used: 4%, 0.195% and 0.0975%.

## 2.7. Inhibitory effect of the selected essential oil (oregano) against selected pathogens in paste of "alheira"

"Alheiras" produced by an industrial company were used in this study. Before starting, the casing was removed and only the paste of "alheira" was used. In order to ensure a homogeneous sample, paste of different "alheiras" were well mixed together in the same bag.

The inhibitory effect of the selected essential oil (oregano) in different concentrations (4%, 1.5%, 0.5%, 0.195% and 0.0975%) against different pathogens was studied. The microorganisms used were: a cocktail of *L. monocytogenes* (L7946, L7947 and SCOTT A), a cocktail of *S.* Enteritidis (ESB, 405 and 459) and a cocktail of *St. aureus* (18N, 2037 M1 and ATCC 29213). A control without addition of EO was also used. The organisms were sub-cultured twice (24 h at 37 ºC) in 10 ml MHb using a 1% v/v inoculum. An aliquot (2 ml) of each bacterial suspension ($10^7$ cfu/ml) was added to 200 g of paste of "alheira" contained in stomacher bags and 8 ml of each concentration of EO was also added. Each concentration of EO was previously prepared in MHb in order to reach the desired final concentration. After assuring good mixing of the inoculum and EO with the paste (manually massaging of the exterior of the bags), each 200 g was divided in 12 g portions and stored at 4 ºC for 21 days into stomacher bags.

After 4h and 3, 7, 15 and 21 days of storage samples were analysed for growth of the inoculated strains and LAB bacteria. The pH and $a_w$ were also evaluated.

The experimental conditions were: i) not inoculated paste as control; ii) paste inoculated with cocktail of *L. monocytogenes*; iii) paste inoculated with cocktail of *L. monocytogenes* with 4% EO; iv) paste inoculated with cocktail of *L. monocytogenes* with 1.5% EO; v) paste inoculated

with cocktail of *L. monocytogenes* with 0.5% EO; vi) paste inoculated with cocktail of *L. monocytogenes* with 0.195% EO; vii) paste inoculated with cocktail of *L. monocytogenes* with 0.0975% EO. The same was done for cocktails of *S.* Enteritidis and *St. aureus*. Each trial was performed in triplicate.

### 2.8. Microbiological analyses

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

### 2.9. Chemical analyses

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

### 2.10. Statistical analysis

An analysis of variance (two-way ANOVA) was carried out to assess the effects of concentration of EO and time of storage on pathogens. For each time of storage, the comparison of 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.

### 2.11. Sensorial analysis

The sensory evaluation of "alheira" made with oregano EO was carried out by 60 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 to be recruited to like "alheira" and oregano; therefore only 57 consumers were analyzed, since three consumers didn't like "alheira".

Tests were performed in a controlled environment, a room temperature (20 °C), under white fluorescent illumination (6500 K). Recruitment of consumers was made by e-mail or personal invitation among the university staff. Samples were composed by two small balls of paste of "alheira" of approximately 5 g each (cooked in an oven at 180 °C for 15 minutes); then were placed in plastic dishes identified with a three digits 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 made 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 (dislike extremely) to 9 (like extremely) (Lawless and Heymann, 2010). Consumers also evaluated the adequacy of the aroma intensity and flavor 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. It was also asked to the consumers their "willingness to consume" the products, using a scale from 0 to 10 (no and yes, respectively).

### 2.11.1. 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 carried out to Microsoft windows office in 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 attribute intensity ratings to OL for each sample and participant (Popper, 2014). Which are considered significant penalties indicated by at least 20% and which generate OL stands for drop greater than or equal to 1.

# III. Results and discussion

### 3.1. Disk diffusion assay (DDA) and Microtiter plate assay (MPA)

The antimicrobial activity assessed by DDA of the different EOs against foodborne pathogens and microorganisms isolated from spoiled food and from the environment are presented in tables A to F (Appendice). The results showed that in general the antimicrobial activity of the different EOs tested varied and was dependent on the type of oil and type of microorganism.

For Gram-negative bacteria, it was observed that the EOs with higher antimicrobial activity were bay, cloves, oregano (C.E.), oregano (V.) and thyme (Tables A and B). It was demonstrated that *Salmonella* Typhimurium, *E. coli* ATCC 25922, *P. mirabilis* and *Y. enterocolitica* were the most sensitive bacteria to all EOs tested. The three strains of *S.* Enteritidis were sensitive to a lower number of EOs, and presenting smaller inhibition halos. For the other Gram-negative bacteria, oregano (C.E.), oregano (V.) and thyme showed the highest inhibitory effect, although bay, cloves, coriander, cumin and peppermint also demonstrated inhibition, but not so strong (Table B).

The antimicrobial activity of EOs against Gram-positive bacteria is presented in tables C to F (Appendice). The EOs that exhibited the highest inhibitory effect against all Gram-positive bacteria (spores and non-spores formers,), were oregano (C.E.), oregano (V.) and thyme. However, other EOs also demonstrated inhibition, but not so strong, such us bay, cloves, coriander, cumin and rosemary (Table C and D). Among Gram-positive bacteria, *L. innocua* demonstrated to be the most sensitive to most of the EOs. Furthermore, *St. aureus* and *L. monocytogenes* showed to be more sensitive than *Enterococcus*. In table E it is possible to observe that most of the EOs demonstrated large inhibitory halos against Gram-positive bacteria (anaerobic spores formers). Among these bacteria, *C. sporogenes* 1.31 showed to be more sensitive, since it is inhibited by a greater number of EOs. For the Gram-positive bacteria (spores formers) bay, cardamom, cloves, coriander, marjoram, nutmeg, rosemary and sage showed an inhibitory activity, however this was stronger for oregano (C.E.), oregano (V.) and thyme. The highest resistance was observed for *B. cereus*, followed by *B. stearothermophilus;* the most sensitive was *B. subtilis* (Table F).

Yeasts showed to be more sensitive and were inhibited by most of the EOs (big halos of inhibition observed). Coriander, cumin, garlic (V.), marjoram, oregano (C.E.), oregano (V.), peppermint, rosemary and thyme demonstrated the highest activity (Table F). Among yeasts,

*Sac. cerevisiae* was more sensitive than *Candida albicans*, being inhibited by all the EOs, included carrot and ginger.

Statistical analyzes were performed (p < 0.05) for each EO that inhibited several microorganisms. In general, there were significant differences (p < 0.05) for oregano (C.E.), oregano (V.) and thyme in relation to the others EOs.

Results of MIC and MBC of the tested EOs are presented in tables 4 to 10. Through the results obtained in the DDA, all EOs with halos lower than 10 mm were excluded and were not tested in MICs.

Values of MIC and MBC were, on average, higher for Gram-negative microorganisms than for Gram-positive. The absence of inhibition observed in the DDA for EOs was in accordance with the highest MIC and MBC values observed. Conversely the lowest MIC and MBC values of EOs of thyme and oregano (V.) were in accordance with their previously observed strong antimicrobial activity observed in the DDA. The relationship between MIC and MBC (minimal bactericidal concentration) was not variable since most of the EOs act as a bactericide.

**Table 4.** Minimal inhibitory concentration (MIC) and minimal bactericide concentration (MBC) of tested essential oils (EOs) against *Enterobacteriaceae* (results are expressed in % of EO).

| EOs/ Microorganisms | Gram negative (*Enterobacteriaceae*) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | *S.* Braenderup | | *S.* Enteritidis | | *S.* Enteritidis 417536 | | *S.* Enteritidis 545047 | | *S.* Tiphymurium | |
| | MIC | MBC | MIC | MBC | MIC | MBC | MIC | MBC | MIC | MBC |
| Bay | 0.39 | 0.39 | 0.195 | 0.195 | 0.39 | 0.39 | 0.39 | 0.39 | 0.195 | 0.195 |
| Cloves | 0.195 | 0.195 | 0.195 | 0.195 | 0.195 | 0.195 | 0.195 | 0.195 | 0.195 | 0.195 |
| Coriander | 100 | 100 | 1.56 | 1.56 | 1.56 | 1.56 | 1.56 | 1.56 | 0.78 | 0.78 |
| Cumin | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 1.56 | 1.56 |
| Marjoram | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 0.78 | 0.78 |
| Nutmeg | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 12.5 | 12.5 |
| Oregano (C.E) | 0.78 | 1.56 | 1.56 | 1.56 | 0.78 | 0.78 | 0.78 | 0.78 | 0.78 | 1.56 |
| Oregano (V.) | 0.0975 | 0.0975 | 0.0975 | 0.0975 | 0.0975 | 0.0975 | 0.0975 | 0.0975 | 0.0488 | 0.0488 |
| Parsley | 100 | 100 | 50 | 50 | 100 | 100 | 100 | 100 | 100 | 100 |
| Peppermint | 100 | 100 | 0.78 | 0.78 | 1.56 | 1.56 | 1.56 | 1.56 | 0.39 | 0.39 |
| Rosemary | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 0.78 | 0.78 |
| Sage | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 0.78 | 0.78 |
| Thyme | 0.78 | 0.78 | 1.56 | 1.56 | 0.0975 | 0.0975 | 0.0975 | 0.0975 | 0.78 | 0.78 |

Essential oils of bay, cloves, oregano (C.E.), oregano (V.) and thyme presented MICs between 0.0488% and 1.56% for Gram-negative bacteria (Tables 4, 5 and 6). However, the EO that demonstrated a lower inhibitory concentration was the oregano (V.) (0.0975-0.0488%) against all microorganisms tested (Table 4, 5 and 6). The most sensitive microorganisms were *S.* Tiphymurium, *E. coli* and *Y. enterocolitica*, while *S.* Braenderup demonstrated to be the most resistant.

...ibitory concentration (MIC) and minimal bactericide concentration (MBC) of tested ...ainst others *Enterobacteriaceae* (results are expressed in % of EO).

| EOs/ Microorganisms | Others Gram-negative (*Enterobacteriaceae*) | | | | | | | | | | | |
| | *E. coli* ATCC 25922 | | *Klebsiela pneumoniae* | | *Proteus vulgaris* | | *Proteus mirabilis* | | *Yersinia enterocolitica* NCTC 10406 | | *Yersinia enterocolitica* | |
| | MIC | MBC | MIC | MBC | MIC | MBC | MIC | MBC | MIC | MBC | MIC | MBC |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Bay | 0.39 | 0.78 | 0.39 | 0.39 | 0.78 | 0.78 | 0.78 | 0.78 | 0.78 | 0.78 | 0.195 | 0.39 |
| Cloves | 0.195 | 0.195 | 0.195 | 0.195 | 0.195 | 0.195 | 0.0975 | 0.0975 | 0.195 | 0.195 | 0.39 | 0.39 |
| Coriander | 0.78 | 0.78 | 0.78 | 0.78 | 100 | 100 | 0.78 | 0.78 | 1.56 | 1.56 | 0.39 | 0.39 |
| Cumin | 1.56 | 3.125 | 3.125 | 3.125 | 100 | 100 | 1.56 | 3.125 | 3.125 | 3.125 | 0.78 | 1.56 |
| Marjoram | 0.78 | 0.78 | 1.56 | 1.56 | 100 | 100 | 3.125 | 3.125 | 1.56 | 1.56 | 1.56 | 1.56 |
| Nutmeg | 12.5 | 12.5 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 6.25 | 6.25 |
| Oregano (C.E) | 0.78 | 0.78 | 1.56 | 1.56 | 0.78 | 0.78 | 0.78 | 0.78 | 0.78 | 1.56 | 50 | 50 |
| Oregano (V.) | 0.0488 | 0.0488 | 0.0488 | 0.0488 | 0.0975 | 0.0975 | 0.0975 | 0.0975 | 0.0488 | 0.0488 | 0.195 | 0.39 |
| Parsley | 50 | 50 | 100 | 100 | 100 | 100 | 50 | 50 | 25 | 25 | 0.78 | 0.78 |
| Peppermint | 0.39 | 0.39 | 100 | 100 | 0.78 | 0.78 | 0.78 | 0.78 | 0.39 | 0.78 | 0.0488 | 0.0975 |
| Rosemary | 3.125 | 3.125 | 50 | 50 | 100 | 100 | 12.5 | 12.5 | 6.25 | 6.25 | 0.78 | 0.78 |
| Sage | 0.78 | 0.78 | 100 | 100 | 100 | 100 | 1.56 | 1.56 | 1.56 | 1.56 | 0.78 | 0.78 |
| Thyme | 0.78 | 0.78 | 1.56 | 1.56 | 0.78 | 0.78 | 0.78 | 0.78 | 0.78 | 0.78 | 0.78 | 0.78 |

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**Table 6.** Minimal inhibitory concentration (MIC) of tested essential oils (EOs) in other Gram-negative bacteria (results are expressed in % of EO).

| EOs/ Microorganisms | Other Gram negative | | | | | | | | | | | |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| | *Pseudomonas aeruginosa* | | *A. baumanii* R | | *A. baumanii* S-1 | | *A. baumanii* S-2 | | *A. calcoaceticus* R | | *A. calcoaceticus* S | |
| | MIC | MBC | MIC | MBC | MIC | MBC | MIC | MBC | MIC | MBC | MIC | MBC |
| Anise | 100 | 100 | 100 | 100 | 100 | 100 | 25 | 25 | 100 | 100 | 100 | 100 |
| Basil | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| Bay | 0.39 | 0.39 | 0.78 | 0.78 | 0.78 | 0.78 | 0.39 | 0.39 | 0.78 | 0.78 | 0.195 | 0.39 |
| Cloves | 0.195 | 0.195 | 0.195 | 0.195 | 0.195 | 0.195 | 0.195 | 0.195 | 0.195 | 0.195 | 0.195 | 0.195 |
| Coriander | 6.25 | 6.25 | 0.39 | 0.39 | 0.39 | 0.39 | 0.39 | 0.39 | 0.195 | 0.195 | 0.195 | 0.195 |
| Cumin | 100 | 100 | 1.56 | 1.56 | 0.78 | 1.56 | 1.56 | 3.125 | 1.56 | 3.125 | 1.56 | 3.125 |
| Fennel | 100 | 100 | 100 | 100 | 100 | 100 | 25 | 25 | 100 | 100 | 100 | 100 |
| Marjoram | 100 | 100 | 1.56 | 1.56 | 0.39 | 0.39 | 0.39 | 0.39 | 0.39 | 0.39 | 0.39 | 0.39 |
| Nutmeg | 100 | 100 | 100 | 100 | 100 | 100 | 12.5 | 12.5 | 12.5 | 12.5 | 12.5 | 12.5 |
| Oregano (C.E) | 0.78 | 0.78 | 0.39 | 0.78 | 0.39 | 0.39 | 0.39 | 0.39 | 0.78 | 0.78 | 0.78 | 0.78 |
| Oregano (V.) | 0.0975 | 0.0975 | 0.0975 | 0.0975 | 0.0975 | 0.0975 | 0.0975 | 0.0975 | 0.0975 | 0.0975 | 0.0975 | 0.0975 |
| Parsley | 100 | 100 | 50 | 50 | 50 | 50 | 50 | 50 | 100 | 100 | 100 | 100 |
| Peppermint | 0.78 | 0.78 | 0.78 | 0.78 | 1.56 | 1.56 | 0.78 | 0.78 | 0.39 | 0.39 | 0.78 | 0.78 |
| Rosemary | 100 | 100 | 100 | 100 | 0.78 | 0.78 | 0.78 | 0.78 | 0.78 | 0.78 | 0.78 | 0.78 |
| Sage | 1.56 | 1.56 | 100 | 100 | 0.78 | 0.78 | 0.78 | 0.78 | 0.78 | 0.78 | 0.39 | 0.39 |
| Thyme | 1.56 | 1.56 | 0.78 | 0.78 | 0.39 | 0.39 | 0.39 | 0.39 | 0.78 | 0.78 | 0.39 | 0.78 |

In relation to Gram-positive bacteria, there was a greater number of EOs demonstrating low inhibitory concentrations. For the group of non-spore forming bacteria, EOs of bay, cloves, coriander, cumin, marjoram, oregano (C.E.), oregano (V.) rosemary and thyme presented MICs between 0.0244% and 3.125% (Tables 7 and 8). In tables 7 and 8 the bacteria that showed to be more sensitive were the strains of *St. aureus,* strains of *L. monocytogenes* and *L. innocua. Enterococcus* spp. were more resistant.

**Table 7.** Minimal inhibitory concentration (MIC) and minimal bactericide concentration (MBC) of tested essential oils (EOs) against Gram-positive bacteria (results are expressed in % of EO).

| EOs/ Microorganisms | *E. faecalis* ATCC 29212 | | *E. faecalis* DSMZ 12956 | | *E. faecium* DSMZ 13590 | | *E. flavescens* DSMZ 7370 | | *E. gallinarium* DSMZ 20628 | | *E. casseliflavus* DSMZ 20680 | |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| | MIC | MBC | MIC | MBC | MIC | MBC | MIC | MBC | MIC | MBC | MIC | MBC |
| Basil | 25 | 50 | 25 | 25 | 100 | 100 | 100 | 100 | 100 | 100 | 25 | 25 |
| Bay | 0.39 | 0.39 | 0.78 | 0.78 | 0.39 | 0.39 | 0.78 | 0.78 | 0.39 | 0.39 | 0.78 | 0.78 |
| Carrot | 100 | 100 | 0.0975 | 0.0975 | 0.195 | 0.195 | 0.0975 | 0.0975 | 0.39 | 0.39 | 25 | 25 |
| Cloves | 0.195 | 0.195 | 0.39 | 0.39 | 0.195 | 0.195 | 0.0975 | 0.195 | 0.39 | 0.39 | 0.195 | 0.195 |
| Coriander | 1.56 | 1.56 | 0.78 | 0.78 | 0.78 | 0.78 | 0.78 | 0.78 | 1.56 | 1.56 | 1.56 | 1.56 |
| Cumin | 100 | 100 | 50 | 100 | 12.5 | 12.5 | 25 | 25 | 100 | 100 | 50 | 50 |
| Juniper berries | 100 | 100 | 6.25 | 6.25 | 3.125 | 3.125 | 100 | 100 | 100 | 100 | 100 | 100 |
| Lemon | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| Marjoram | 1.56 | 1.56 | 1.56 | 1.56 | 0.78 | 0.78 | 0.78 | 0.78 | 0.78 | 0.78 | 0.78 | 0.78 |
| Nutmeg | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 3.125 | 3.125 | 100 | 100 |
| Oregano (C.E) | 0.78 | 0.78 | 0.78 | 0.78 | 0.78 | 0.78 | 0.39 | 0.39 | 0.78 | 0.78 | 0.78 | 0.78 |
| Oregano (V.) | 0.195 | 0.195 | 0.0975 | 0.0975 | 0.0975 | 0.0975 | 0.0488 | 0.0975 | 0.0975 | 0.0975 | 0.0975 | 0.0975 |
| Parsley | 100 | 100 | 25 | 25 | 100 | 100 | 25 | 25 | 25 | 25 | 100 | 100 |
| Peppermint | 100 | 100 | 6.25 | 12.5 | 12.5 | 25 | 0.78 | 1.56 | 100 | 100 | 100 | 100 |
| Rosemary | 12.5 | 12.5 | 0.78 | 0.78 | 0.78 | 0.78 | 0.78 | 0.78 | 3.125 | 3.125 | 3.125 | 3.125 |
| Sage | 1.56 | 1.56 | 3.125 | 3.125 | 100 | 100 | 1.56 | 1.56 | 100 | 100 | 1.56 | 1.56 |
| Thyme | 1.56 | 1.56 | 0.78 | 0.78 | 1.56 | 1.56 | 0.78 | 0.78 | 0.78 | 0.78 | 0.78 | 0.78 |

**Table 8.** Minimal inhibitory concentration (MIC) and minimal bactericide concentration (MBC) of tested essential oils (EOs) against other Gram-positive bacteria (results are expressed in % in EO).

| EOs/ Microorganisms | *S. aureus* ATCC 29213 | | *S. aureus* 18N (MRSA) | | *S. aureus* 2037 M1 (MSSA) | | *L. monocytogenes* 7946 | | *L. monocytogenes* 7947 | | *L. monocytogenes* SCOOT A | | *Listeria innocua* 2030c | |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| | MIC | MBC | MIC | MBC | MIC | MBC | MIC | MBC | MIC | MBC | MIC | MBC | MIC | MBC |
| Basil | 50 | 50 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 6.25 | 6.25 | 100 | 100 |
| Bay | 0.39 | 0.39 | 0.39 | 0.39 | 0.39 | 0.39 | 0.39 | 0.39 | 0.39 | 0.39 | 0.39 | 0.39 | 0.39 | 0.39 |
| Carrot | 12.5 | 12.5 | 0.195 | 0.195 | 0.39 | 0.39 | 0.79 | 0.79 | 0.39 | 0.39 | 0.39 | 0.39 | 0.0975 | 0.0975 |
| Cloves | 0.195 | 0.195 | 0.195 | 0.195 | 0.0975 | 0.0975 | 0.195 | 0.39 | 0.0488 | 0.0488 | 0.195 | 0.195 | 0.39 | 0.78 |
| Coriander | 1.56 | 1.56 | 0.78 | 0.78 | 0.78 | 0.78 | 0.78 | 0.78 | 0.39 | 0.39 | 0.78 | 0.78 | 0.78 | 0.78 |
| Cumin | 3.125 | 6.25 | 3.125 | 3.125 | 3.125 | 3.125 | 1.56 | 3.125 | 1.56 | 3.125 | 1.56 | 1.56 | 3.125 | 3.125 |
| Juniper berries | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 12.5 | 12.5 | 25 | 25 | 3.125 | 6.25 |
| Lemon | 25 | 25 | 50 | 50 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 3.125 | 3.125 |
| Marjoram | 1.56 | 1.56 | 0.78 | 0.78 | 0.78 | 0.78 | 1.56 | 1.56 | 0.0064 | 0.0064 | 1.56 | 1.56 | 0.78 | 0.78 |
| Nutmeg | 100 | 100 | 100 | 100 | 100 | 100 | 12.5 | 12.5 | 3.125 | 3.125 | 6.25 | 6.25 | 12.5 | 12.5 |
| Oregano (C.E) | 0.78 | 0.78 | 0.78 | 1.56 | 0.78 | 0.78 | 0.78 | 1.56 | 0.39 | 0.39 | 0.78 | 0.78 | 0.78 | 1.56 |
| Oregano (V.) | 0.0975 | 0.0975 | 0.0975 | 0.0975 | 0.0975 | 0.195 | 0.0975 | 0.195 | 0.0975 | 0.0975 | 0.0975 | 0.0975 | 0.0244 | 0.0244 |
| Parsley | 100 | 100 | 25 | 25 | 25 | 25 | 25 | 25 | 50 | 50 | 50 | 50 | 50 | 50 |
| Peppermint | 0.78 | 1.56 | 1.56 | 1.56 | 0.195 | 0.195 | 3.125 | 6.25 | 0.195 | 0.195 | 1.56 | 1.56 | 6.25 | 12.5 |
| Rosemary | 1.56 | 1.56 | 1.56 | 1.56 | 1.56 | 1.56 | 1.56 | 1.56 | 0.0488 | 0.0488 | 0.0975 | 0.0975 | 0.78 | 0.78 |
| Sage | 3.125 | 3.125 | 100 | 100 | 0.78 | 0.78 | 1.56 | 1.56 | 0.0975 | 0.0975 | 1.56 | 1.56 | 0.78 | 0.78 |
| Thyme | 0.78 | 1.56 | 0.78 | 0.78 | 0.78 | 0.78 | 0.78 | 0.78 | 0.39 | 0.78 | 0.78 | 0.78 | 0.39 | 0.78 |

For the spore forming bacteria, bay, basil, cloves, coriander, oregano (CE), oregano (V.) rosemary and thyme were the EOs with the lowest MICs (between 0.0488% and 3.125%) (Table 10) and *B. cereus* was the more sensitive bacteria. For the anaerobic spore formers bacteria almost all EOs demonstrated low inhibitory MICs - from 0.0128% to 3.125% - with the exception of anise, basil, coriander, fennel and nutmeg for which higher MICs were observed (100%) (Table 9).

concentration (MIC) and minimal bactericide concentration (MBC) of tested
thers Gram-positive (anaerobic spores formers) bacteria (results are expressed

Gram positive (anaerobic spores formers)

| EOs/ Microorganisms | Clostridium perfringens 1.16 | | Clostridium perfringens 1.19 | | Clostridium perfringens 1.2 | | Clostridium sporogenes 1.31 | | Clostridium sporogenes 1.34 | | Clostridium sporogenes 1.61 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | MIC | MBC | MIC | MBC | MIC | MBC | MIC | MBC | MIC | MBC | MIC | MBC |
| Anise | 3.125 | 3.125 | 100 | 100 | 1.56 | 1.56 | 1.56 | 1.56 | 100 | 100 | 100 | 100 |
| Basil | 100 | 100 | 1.56 | 1.56 | 1.56 | 1.56 | 1.56 | 1.56 | 1.56 | 1.56 | 1.56 | 1.56 |
| Bay | 0.39 | 0.39 | 0.39 | 0.39 | 0.39 | 0.39 | 0.195 | 0.195 | 0.195 | 0.195 | 0.195 | 0.195 |
| Carrot | 1.56 | 1.56 | 1.56 | 1.56 | 3.125 | 3.125 | 1.56 | 1.56 | 1.56 | 1.56 | 1.56 | 1.56 |
| Cloves | 0.0128 | 0.0128 | 0.0128 | 0.0128 | 0.0128 | 0.0128 | 0.0128 | 0.0128 | 0.0128 | 0.0128 | 0.0128 | 0.0128 |
| Coriander | 1.56 | 1.56 | 0.0128 | 0.0128 | 0.0128 | 0.0128 | 100 | 100 | 0.0128 | 0.0128 | 0.0488 | 0.0488 |
| Cumin | 0.0975 | 0.0975 | 1.56 | 1.56 | 0.0244 | 0.0244 | 3.125 | 3.125 | 1.56 | 1.56 | 0.195 | 0.195 |
| Fennel | 3.125 | 3.125 | 100 | 100 | 3.125 | 3.125 | 3.125 | 3.125 | 1.56 | 1.56 | 3.18 | 3.18 |
| Ginger | 3.125 | 3.125 | 1.56 | 1.56 | 100 | 100 | 100 | 100 | 100 | 100 | 1.56 | 1.56 |
| Juniper berries | 1.56 | 1.56 | 1.56 | 1.56 | 1.56 | 1.56 | 1.56 | 1.56 | 1.56 | 1.56 | 1.56 | 1.56 |
| Lemon | 1.56 | 1.56 | 1.56 | 1.56 | 0.39 | 0.39 | 1.56 | 1.56 | 3.18 | 3.18 | 3.125 | 3.125 |
| Marjoram | 3.125 | 3.125 | 0.0244 | 0.0244 | 0.195 | 0.195 | 0.195 | 0.195 | 1.56 | 1.56 | 3.125 | 3.125 |
| Nutmeg | 0.0128 | 0.0128 | 0.0128 | 0.0128 | 0.0128 | 0.0128 | 100 | 100 | 0.0128 | 0.0128 | 0.0128 | 0.0128 |
| Oregano (C.E) | 0.0128 | 0.0128 | 0.0128 | 0.0128 | 0.0128 | 0.0128 | 0.0244 | 0.0244 | 0.0128 | 0.0128 | 0.0244 | 0.0244 |
| Oregano (V.) | 0.0975 | 0.0975 | 0.0975 | 0.0975 | 0.0975 | 0.0975 | 0.0975 | 0.0975 | 0.0975 | 0.0975 | 0.0975 | 0.0975 |
| Parsley | 1.56 | 1.56 | 1.56 | 1.56 | 1.56 | 1.56 | 1.56 | 1.56 | 1.56 | 1.56 | 1.56 | 1.56 |
| Peppermint | 0.0978 | 0.0978 | 0.0488 | 0.0488 | 0.0975 | 0.0975 | 0.0975 | 0.0975 | 0.39 | 0.39 | 0.78 | 0.78 |
| Rosemary | 0.0128 | 0.0128 | 0.0128 | 0.0128 | 0.0128 | 0.0128 | 0.0128 | 0.0128 | 0.0128 | 0.0128 | 0.0128 | 0.0128 |
| Sage | 1.56 | 1.56 | 0.39 | 0.39 | 0.39 | 0.39 | 0.39 | 0.39 | 1.56 | 1.56 | 0.39 | 0.39 |
| Thyme | 0.0975 | 0.0975 | 0.195 | 0.195 | 0.195 | 0.195 | 0.0975 | 0.0975 | 0.0975 | 0.0975 | 0.0975 | 0.0975 |

**Table 10.** Minimal inhibitory concentration (MIC) and minimal bactericide concentration (MBC) of tested essential oils (EOs) against other Gram-positive (spores formers) bacteria and Yeasts (results are expressed in % of EO).

| EOs/ Microorganisms | Gram Positive (Spores formers) | | | | | | Yeasts | | | |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| | *B. cereus* | | *B. subtilis* | | *B. stearothermophilus* | | *Candida albicans* | | *Saccharomyces cerevisiae* | |
| | MIC | MBC | MIC | MBC | MIC | MBC | MIC | MBC | MIC | MBC |
| Anise | 100 | 100 | 100 | 100 | 100 | 100 | 12.5 | 12.5 | 100 | 100 |
| Basil | 25 | 25 | 50 | 50 | 50 | 50 | 12.5 | 12.5 | 12.5 | 12.5 |
| Bay | 0.39 | 0.39 | 0.78 | 0.78 | 0.78 | 0.78 | 0.195 | 0.195 | 100 | 100 |
| Carrot | 0.39 | 0.39 | 50 | 50 | 0.39 | 0.39 | 100 | 100 | 0.0975 | 0.0975 |
| Cloves | 0.195 | 0.195 | 0.195 | 0.39 | 0.195 | 0.39 | 0.195 | 0.195 | 0.0244 | 0.0488 |
| Coriander | 0.78 | 0.78 | 1.56 | 1.56 | 0.39 | 0.39 | 0.39 | 0.39 | 0.0064 | 0.0064 |
| Cumin | 0.78 | 1.56 | 100 | 100 | 0.78 | 1.56 | 0.78 | 1.56 | 0.78 | 1.56 |
| Fennel | 100 | 100 | 100 | 100 | 100 | 100 | 12.5 | 12.5 | 100 | 100 |
| Juniper berries | 50 | 50 | 100 | 100 | 12.5 | 12.5 | 100 | 100 | 3.125 | 3.125 |
| Lemon | 1.56 | 1.56 | 100 | 100 | 100 | 100 | 25 | 25 | 1.56 | 1.56 |
| Marjoram | 0.78 | 0.78 | 3.125 | 3.125 | 100 | 100 | 0.39 | 0.39 | 0.0064 | 0.0064 |
| Nutmeg | 6.25 | 6.25 | 100 | 100 | 6.25 | 6.25 | 3.125 | 3.125 | 12.5 | 12.5 |
| Oregano (C.E) | 0.39 | 0.39 | 1.56 | 1.56 | 0.78 | 0.78 | 0.195 | 0.195 | 0.195 | 0.195 |
| Oregano (V.) | 0.0488 | 0.0488 | 0.195 | 0.195 | 0.0975 | 0.195 | 0.0488 | 0.0488 | 0.0975 | 0.0975 |
| Parsley | 25 | 25 | 25 | 25 | 50 | 50 | 50 | 50 | 50 | 50 |
| Peppermint | 0.78 | 0.78 | 100 | 100 | 1.56 | 1.56 | 0.195 | 0.195 | 0.195 | 0.195 |
| Rosemary | 0.78 | 0.78 | 50 | 50 | 0.78 | 0.78 | 0.78 | 0.78 | 0.0064 | 0.0064 |
| Sage | 0.78 | 0.78 | 100 | 100 | 0.39 | 0.39 | 1.56 | 1.56 | 0.0975 | 0.0975 |
| Thyme | 1.56 | 1.56 | 1.56 | 1.56 | 0.78 | 0.78 | 0.78 | 0.78 | 0.39 | 0.39 |

Oregano (V.) was the EO that presented the lowest inhibitory MIC, with values between 0.195% and 0.0244%. Oregano (V.) was the one that demonstrated the lowest MICs - 0.0244% against *L. innocua* -, nevertheless the concentration of 0.0975% was demonstrated for the majority of the microorganisms under study. According to these concentrations, this EO can be considered with great inhibitory potential.

Regarding yeasts, these are extremely sensitive, having demonstrated low MICs for most of the EOs (varying between 1.56% and 0.0064%) with the exception of the anise, basil, fennel, juniper berries, nutmeg and parsley that showed higher values between 3.125% and 100% (Table 10). However, *Sac. cerevisiae* was more sensitive than *Candida albicans*. Since most of the EOs have very similar MICs, it is not possible to determine which was the most effective.

Through the analysis of the results obtained for DDA and MPA, it was possible to verify that Gram-positive bacteria are more sensitive than Gram-negative bacteria, which is in accordance with previous reports (Hyldgaard *et al.*, 2012; Nazzaro *et al.*, 2013). Gram-positive bacteria

have only a cell wall that allows hydrophobic molecules to readily penetrate into cells and act on both cell wall and cytoplasm; while Gram-negative bacteria have an outer membrane that contains lipopolysaccharides (LPS) that act as a barrier against macromolecules and hydrophobic compounds, making them more resistant to these same compounds (Nikaido, 1994, 2003).

Regarding the EOs used, it was possible to state that oregano and thyme were the EOs with the greatest inhibitory capacity for all the bacteria used in DDA. The intensity of inhibition was revealed by the mean size of the halos. The MIC and MBC values that were observed in this study were similar to those previously reported in the literature (Garcías-Diaz *et al.* 2016). These results are in agreement with previous publications, which also reported a high antimicrobial activity of oregano and thyme (Semeniuc *et al.*, (2017); Dobre *et al.*, (2011); Maruzzella and Sicurella, (1960)). Erkman and Ozean, (2004) verified that the essential oil of oregano has bacteriostatic and bactericidal effects against bacteria with lower concentrations (0.001% and 0.025, respectively) than other essential oils of spices, being in agreement with the results obtained. Although two oregano oils were used in our study, oregano (V.) has a greater inhibitory capacity, which may be due to extraction mode. These EOs are of different origins and according to Kokkini *et al.* (1996), the extraction of EOs in different seasons of the year produce different amounts of compounds related to each EO. This antimicrobial activity is probably due mainly to its main components: carvacrol for oregano and thymol for thyme**.** Thymol and carvacrol are hydrophobic compounds, which cause functional and structural damages to cytoplasmic membrane (Sikkema *et al.*, 1995). The mode of action of thymol is not fully known, but it is believed to involve the rupture of the inner and outer membrane and the interaction with membrane proteins and intracellular targets, whereas the main mode of action of carvacrol is its ability to position into the membrane, which increases their permeability (Hyldgaard *et al.*, 2012).

Sokovic *et al.* (2010) demonstrated that oregano EO, thyme EO and their principal compounds were the most active against *B. subtilis*, *Staphylococcus epidermidis, St. aureus, S.* Enteritidis, *S.* Tiphymurium, *E. coli, P. mirabilis, Ps. aeruginosa and L. monocytogenes.* In a study carried out by Silva *et al.* (2013) it was also demonstrated that among the essential oils evaluated, the greatest effectiveness was achieved when thyme and oregano, which showed activity against all the tested bacterial strains, were used. Gutierrez *et al.* (2008) showed that *B. cereus, E. coli, L. monocytogenes* and *Ps. aeruginosa* were sensitive to the oregano EO. Regarding yeasts, the results obtained in the present study are in agreement with others studies that showed that

oregano EO exhibited a broad spectrum of activity against *Candida* spp. (Khosravi *et al*., 2011) and that among yeast species, *Sac. cerevisiae* was the most sensitive microorganism against all EOs tested (Çoskun *et al.,* 2016).

## 3.2. Optimization of the protocol of "Inhibitory effect of the selected essential oil (oregano) against selected pathogens in paste of "alheira"

### 3.2.1. Experimental sensorial test

The oregano essential oil was the one presenting lower MICs and was therefore chosen to be used in paste of "alheira" as a control agent of the most prevalent pathogens in this product. However, it was necessary to perform a preliminary sensorial analysis with different concentrations of oregano EO to see if oregano would not change, in an unpleasant way, the flavor of "alheira". It was possible to determine that at 0.39%, the EO flavor prevailed over the "alheira" mass, having a very intense and unpleasant taste; while at 0.195% the taste was pleasant, with some intense flavor to oregano (data not shown). On the other hand, at 0.0975% the presence of oregano was not detected (data not shown).

### 3.2.2. Determination of the volume and the most suitable concentration of oregano EO to be used in paste of "alheira"

In order to optimize the process of add EO in paste of "alheira", several experiments were carried out using different conditions.

First, it was used the lower MIC obtained in the oregano EO (0.0975%). After analyzing the samples over time (4 h, 3 and 7 days), there was no inhibition by the EO for *L. monocytogenes* L7949 and *S.* Enteritidis in samples with oregano EO since the growth of inoculated samples with and without EO were the same (data not shown). The microbiota of paste of "alheira" could have some influence in this result, since paste of "alheira" was not sterilized. Because of this, it was decided to sterilize the paste of "alheira" and use the same conditions used in previous experiment, but only with *L. monocytogenes.* The results obtained were similar, with no inhibition of *L. monocytogenes* in sterilized and non-sterilized paste of "alheira" (data not shown). Therefore, the microbiota of "alheira" may not be the factor that is influencing the absence of inhibition by the oregano EO. "Alheira" is a product with a complex matrix due to its constituents. In many food products, the hydrophobic essential oil constituents are impaired by interactions with food matrix components, such as fats, proteins, water content, antioxidants,

preservatives, pH, salt and other additives that are relevant in the bacterial sensitivity (Burt, 2004). The diffusion rate of active principles of oil and their low vapor pressures can also limit the microorganisms' exposure (Ponce *et al*., 2004).

Since the "alheira" matrix is complex and varied, it was decided to test the inhibitory activity of oregano oil in the different ingredients of "alheira" and in a solution of garlic vine. Three concentrations were used in order to verify if a higher concentration would inhibit the growth of *L. monocytogenes*. Regarding the ingredients, an abrupt inhibition at 4% of oregano EO was observed, while for the remaining concentrations (0.195% and 0.0975%) no difference was found between the EO samples and the control (data not shown). The same was observed with the paste of "alheira", but in the garlic vine the lowest concentrations obtained antimicrobial activity. These results may suggest that the lower concentrations of oregano EO may do not inhibit *L. monocytogenes in situ*, i.e. when in contact with the matrix of "alheira"; but with other matrix such garlic vine the lower concentrations inhibit *L. monocytogenes* (data not shown). On the other hand, the volume added could be not sufficient to mix throughout the paste of "alheira" and thus not inhibit, since different volumes were added, depending on the concentration used (4% volume of 1 ml, 0.195% volume of 244 µl and 0.0975% volume of 122 µl). However, since the garlic vine is a liquid product, the homogenization could become easier, allowing the oregano EO to act in the matrix.

Thus, in the following experiments, different oregano EO concentrations were used, but always using the same volume, in order to try to minimize this potential problem.

### 3.3. Inhibitory effect of the selected essential oil (oregano) against selected pathogens in paste of "alheira"

The counts of cocktail of *S*. Enteritidis, cocktail of *St. aureus* and cocktail of *L. monocytogenes* during 21 days of storage at 4 ºC without and with 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, in the uninoculated control samples there was no growth of *L. monocytogenes, Salmonella* spp., *St. aureus* and *E. coli* (data not shown).

The antimicrobial effect was higher as the concentration of the oregano EO increases. Generally, the addition of EOs in paste of "alheira" decreased the microbial counts of the pathogens tested along the storage time. For all the experiments the concentration of EO used varied for each time ($p < 0.05$).

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After 4h, *S.* Enteritidis was reduced ~3 log at concentrations of 4% and 1.5% and, at lower concentrations, the reduction was lower (Figure 1). After 3 days, only 4% significantly reduced the amount of *S.* Enteritidis. At 4%, *S.* Enteritidis was not detectable after 7 days of storage and at 1.5% *S.* Enteritidis was not detectable after 15 days. The lowest concentrations, only start to show antimicrobial activity against *S.* Enteritidis, after 15 days with 1.5 log reduction with 0.195% and after 21 days with 1 log reduction for 0.0975%.

Briefly, the inhibitory effect of higher concentrations of oregano oil (4% and 1.5%) was observed at the beginning of the storage time, whereas at lower concentrations, this effect took longer, only to be visualized at the end of the time of storage (for 0.195% and 0.0975%), and with smaller logarithmic reduction.
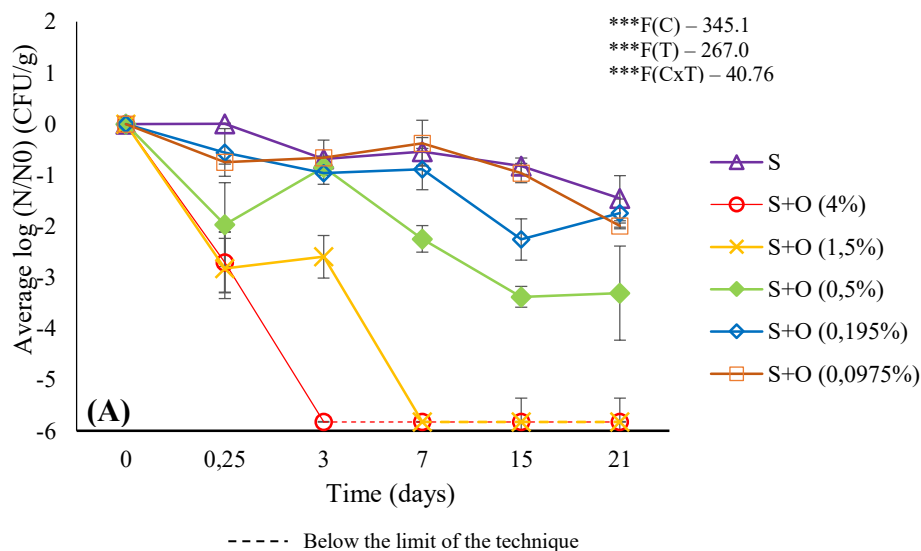


| Concentration (%)/ Time (days) | 0 | 0.25 (4h) | 3 | 7 | 15 | 21 |
|---|---|---|---|---|---|---|
| Controle | - | bcd | bc | bcd | bcde | bcd |
| 4 | - | aef | acdef | adef | adef | adef |
| 1.5 | - | aef | abdef | adef | adef | adef |
| 0.5 | - | abe | bc | abcef | abcef | abcef |
| 0.195 | - | cd | bc | bcd | abcdf | bcd |
| 0.0975 | - | c | bc | bcd | bcde | bcd |

**Figure 1. (A)** 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 paste of "alheira" during 21 days at 4ºC. Results are expressed as average of log (N/N0) (CFU/g) (means ± SD (n = 3)). Two-way ANOVA was performed to determine the influence of concentrations over time (C – Concentration; T – Time; CxT – Concentration x Time). ***Significant at the level $p < 0.001$; The test results are shown with statistic test for each time. One-way ANOVA was performed for each concentration, in each time. Different letters in the same column are significantly different ($p < 0.05$) according to the Turkey-Kramer test.

The activity of different concentrations of oregano EO against *St. aureus* is represented in figure 2. The results obtained after 4h and 3 days were similar to those obtained for *S.* Enteritidis: an immediate reduction occurred at 4% and 1.5% concentrations. However, no reduction was observed at 0.195% and 0.0975%. In the last two points, *St. aureus* was not detectable only at 4%. The lower concentrations showed little inhibitory power and no significant reductions were observed.

Generally, the inhibitory effect of higher concentrations of oregano oil (4% and 1.5%) was observed at the beginning of the storage time, with some oscillations by 1.5%. However at lower concentrations, the reductions was only visualized at the end of the time of storage (for 0.195% and 0.0975%), and with smaller logarithmic reduction.



| Concentration (%)/ Time (days) | 0 | 0.25 (4h) | 3 | 7 | 15 | 21 |
|---|---|---|---|---|---|---|
| Control | - | bc | b | b | bcf | bc |
| 4 | - | adef | adef | acdef | acdef | adef |
| 1.5 | - | adef | bd | b | ab | aef |
| 0.5 | - | bc | bc | b | b | bc |
| 0.195 | - | bc | b | b | bf | bc |
| 0.0975 | - | bc | b | b | abe | bc |
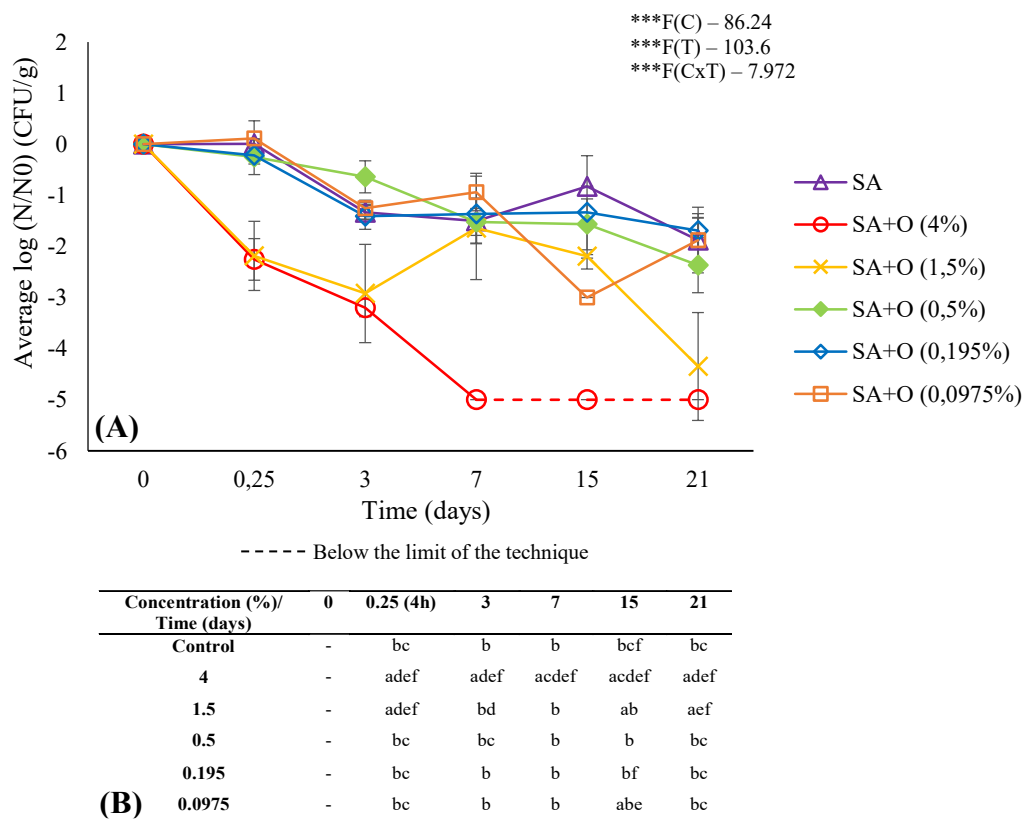
**Figure 2. (A)** The effect of different concentrations of oregano EO (4%, 1.5%, 0.5%. 0.195% and 0.0975%) on the survival of a cocktail of *St. aureus* in paste of "alheira" during 21 days at 4°C. Results are expressed as average of log (N/N0) (CFU/g) (means ± SD (n = 3)). Two-way ANOVA was performed to determine the influence of concentrations over time (C – Concentration; T – Time; CxT – Concentration x Time). ***Significant at the level $p < 0.001$; The test results are shown with statistic test for each time. One-way ANOVA was performed for each concentration, in each time. Different letters in the same column are significantly different ($p < 0.05$) according to the Turkey-Kramer test.

For studies using *L. monocytogenes,* results are presented in figure 3. A gradual decrease of *L. monocytogenes* along time was observed for all the concentrations used. At 4%, there was an immediate reduction after 4 h, of 2.5 log, and 4 log and 5 log at the following times. After 15 days, *L. monocytogenes* was not detectable. For the other concentrations used, the reduction of *L. monocytogenes* was verified more continuously over time and a decrease between ~1 and 2 log was achieved. After 21 days, a reduction of 3 log and 2.5 log when concentrations of 0.195 % and 0.0975%, respectivelly were used.

In general, a significant inibition was observed for all the concentrations of the EO investigated.
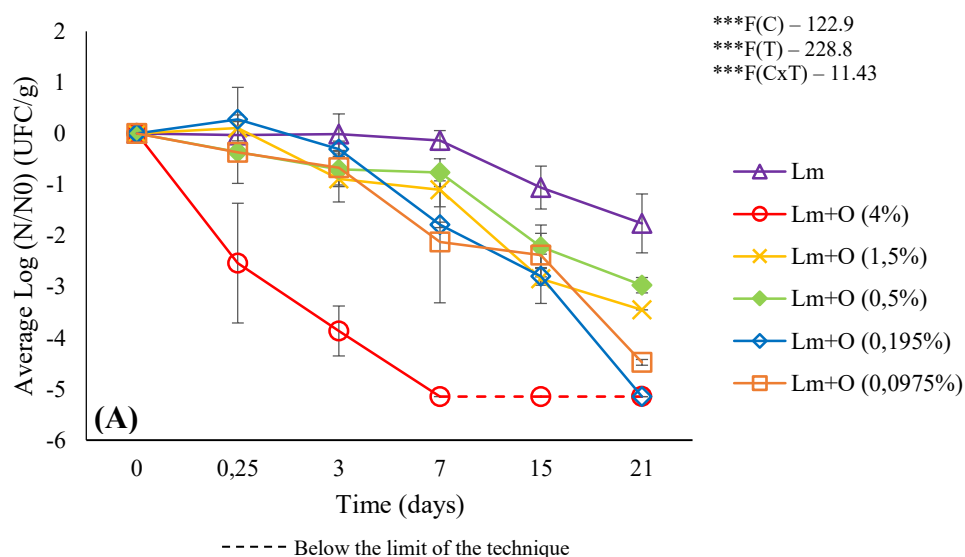


| Concentration (%) / Time (days) | 0 | 0.25 (4h) | 3 | 7 | 15 | 21 |
|---|---|---|---|---|---|---|
| Control | - | b | b | bef | bcdef | bcdef |
| 4 | - | acdef | acdef | acdef | acdef | acd |
| 1.5 | - | b | b | b | ab | abed |
| 0.5 | - | b | b | b | ab | abef |
| 0.195 | - | b | ab | ab | ab | acd |
| 0.0975 | - | b | ab | ab | ab | acd |

**Figure 3. (A)** 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 paste of "alheira" during 21 days at 4ºC. Results are expressed as average of log (N/N0) (CFU/g) (means ± SD (n = 3)). Two-way ANOVA was performed to determine the influence of concentrations over time (C – Concentration; T – Time; CxT – Concentration x Time). ***Significant at the level $p < 0.001$; The test results are shown with statistic test for each time. One-way ANOVA was performed for each concentration, in each time. Different letters in the same column are significantly different ($p < 0.05$) according to the Turkey-Kramer test.

Counts of lactic acid bacteria and pH values are shown in figure 4. Neither LAB counts nor pH values changed significantly (p > 0.05) over time in the presence of EO. For all the other assays with cocktail of each pathogen, the pH values varied between 4.63 and 5.10 and counts of LAB between $10^8$ and $10^9$, with no changes occurring over time (Tables G and H in apenddice). Counts without oil were $10^9$ cfu/g and similar values (with aproximately 0.5 log of difference) were observed in other assays with pathogens and differents concentrations (Tables G, H and I in Apenddice). The presence of essential oils did not influence the growth of LAB present in the samples.
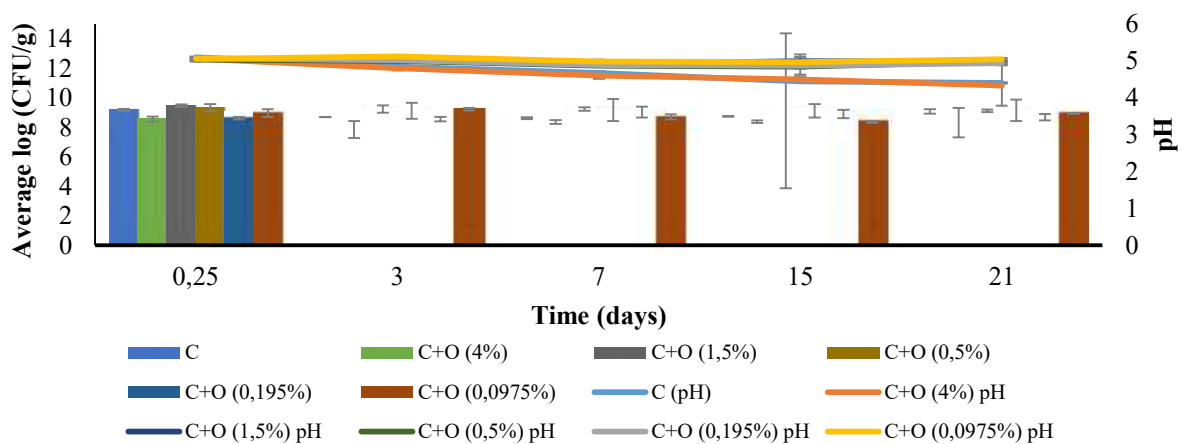


**Figure 4.** Lactic acid bacteria counts and pH values in paste of "alheira" in control, during 21 days of storage at 4ºC. Results are expressed as log CFU/g (expressed as mean ± standard deviation).

Antimicrobial effect of oregano EO against some foodborne pathogens *in vitro* have already been described (Dussault *et al*., 2014); however research on its inhibitory effect on foodstuff is scarce, mainly in traditional meat products (Jayasena and Jo, 2013; Garcías-Diez *et al.,* 2015). Nevertheless, our results are according to some authors. Studies conducted by different authors showed that 1% of oregano EO in minced meat leads to a reduction of 1 log for *S.* Enteritidis, *St. aureus* and *L. monocytogenes* (Skandamis and Nychas, 2001; Barbosa *et al*., 2009; Pesavento *et al*., 2015). Skandamis and Nychas (2001) and Barbosa *et al*. (2009) also showed that 0.9% of oregano EO resulted in 1 log reduction for *S.* Enteritidis. Garcías-Diez, (2015) showed no effect against *S.* Enteritidis with 0.005% of oregano EO in a fermented meat sausage. 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. For *St. aureus* only

the higher concentrations were effective. Regarding the low concentrations, results obtained in the present study are in agreement with those obtained by García-Diez, (2015); 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 pathogen) develops several mechanisms to survive under osmotic stress based mainly on modifications of the internal cell composition such as an accumulation of compatible solutes including and development of a protein-transport system (Stewar *et al*., 2005, Hennekinne *et al*., 2012). The lower antibacterial effect of EOs against *St. aureus* compared to that observed for *S*. Enteritidis and *L. monocytogenes* could be associated to changes on cell membrane composition, the main target of EOs, as already described by García-Diez, (2015).

Different authors performed studies exposing *L. monocytogenes* to different concentrations of oregano EO and reductions in counts were also observed: Dussault *et al*. (2014) applied 0.05% oregano EO on ham and obtained a reduction of 1.5 log after 20 days; Tsigarida *et al*. (2000) with 0.8% in meat observed a reduction of 2/3 log after 14 days of storage at 2 a 10ºC; Pesavento *et al.* (2015) with 0.5% and 2% in minced meat after 14 days achieved reductions between 1.5 log and 2.5 log, similar to those observed in the present experiment. Survival of *L. monocytogenes* was clearly affected by the addition of oregano EO; for all the concentrations investigated, survivors decreased along the storage period.

Through the analysis of figures 1, 2 and 3, 4% of oregano EO was the concentration with greater inhibitory power. However 4% and 1.5% are very high concentrations and couldn't be applied in "alheira" due to its aroma and intense flavor that could change the characteristics of the selected product as reported by Sivropoulou *et al*. (1996). The production of off-flavor or strong odor limits the use of EOs as food preservatives to increase the safety and shelf life of food products (Bajpai *et al.*, 2012, Friedly *et al*., 2009, Sokovic *et al*., 2010; Solorzano Santos and Miranda-Novales, 2012; Tiwari *et al*., 2009).

Regarding lower concentrations (0.5%, 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). Moreover, the matrix used, paste of "alheira", may influence the EO efficiency. As already described, factors present in complex food matrices such as fat content, proteins, water activity, pH, and enzymes can potentially decrease the efficacy of EOs not allowing the oil to spread easily (Burt, 2004; Firouzi *et al*., 2007; Friedly *et al*., 2009). Garcías-Diez, (2015) showed that inhibitory properties of oregano

EO decrease as the level of fat increases, unlike the protein that did not appear to influence the antimicrobial effect of the EO.

### 3.4. Sensorial analysis

The sensory impact of EOs has been reported as one of the most negative aspects of their use (Chouliara *et al*., 2007). Due to this factor, the concentration of 0.1975% was selected to evaluate its sensory acceptance, since higher concentrations had previously been eliminated as not acceptable, as already discussed in section 3.2.1. In the current work, most of the consumers (21%) consume "alheira" once a month.

Analyzing the overall liking (OL), most of the consumers prefer "alheira" without oregano EO than paste of "alheira" with oregano EO, rejecting the null hypothesis ($p < 0.01$) ($t = 8.01$; $df = 56$) (Figure 6 and 7). Most of the consumers commented that sample with oregano EO has a very intense flavor and the after-taste was not pleasant.
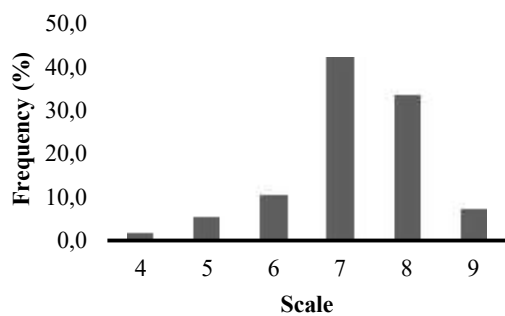


**1-4:** 1.76%; **5:** 5.26%; **6-9:** 92.98%

**Figure 6.** Absolut frequency of overall liking in Sample 789 (without oreganos EO) (1 - 9 = hedonic scale).
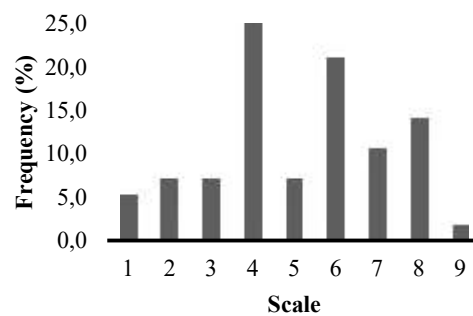


**1-4:** 45.62%; **5:** 7.02%; **6-9:** 47.37%

**Figure 7.** Absolut frequency of overall liking in Sample 382 (0.195% oreganos EO) (1 - 9 = hedonic scale).

In line with the above referred comments of the consumers, 54% and 86% of respondents rated the oregano aroma and flavor too strong, respectively (figure 8, table 11). Corresponding to a mean drop of 2.52 and to a probability $p < 0.01$, the too strong flavor had an important impact on the low acceptance of this sample.
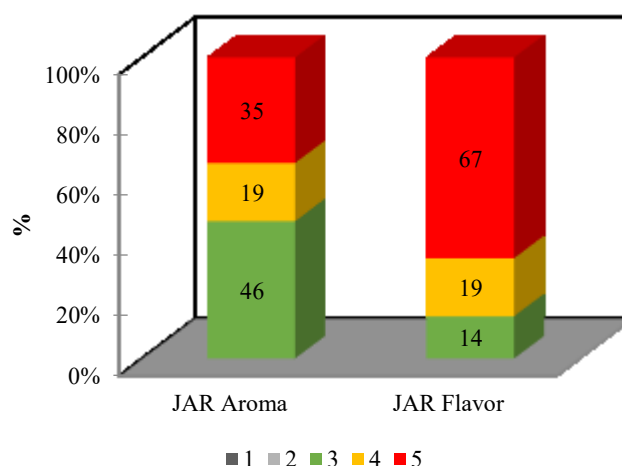


**Figure 8.** Percentages (%) for the JAR (Just-about-right) levels of aroma and flavor (1 - too weak; 2 – weak; 3 - just about right; 4 – strong; 5 - too strong) of sample with oregano EO.

**Table 11.** Penalties of JAR Aroma and JAR Flavor.

| 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 Flavor** | 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 |

According to the results obtained, 0.195% of oregano EO has a negative effect on the consumers' acceptance of "alheira". These results are in accordance with García-Diez, (2015) which states that the application of this type of concentration in a fermented sausage, makes it strong for the taste, being the concentration of 0.05% tested by the author considered strong. The EO concentration used revealed to be not applicable in practice, due to sensory reasons, once only about 25% or less of consumers indicated "will consume it".

# IV.   Conclusions

The current study demonstrated that EOs used had an *in vitro* antimicrobial effect against foodborne pathogens such as *Salmonella* spp., *L. monocytogenes*, *St. aureus* and *E. coli* and also against some spoilage bacteria. Essential oils of oregano and thyme were clearly the ones that demonstrated the greatest inhibitory effect against the different microorganisms. These differences could be associated to several factors such as chemical composition of the EOs or to the specific sensitivity of the target microorganism among others.

Utilization of oregano EO in paste of "alheira" seems to have resulted in an interesting strategy to assure safety against *Salmonella* spp, *L. monocytogenes* and *S. aureus*, but with sensory limitations, that does not allow its use in high concentrations that are those more effective for pathogen inhibition. *In situ* assays of antimicrobial effect against foodborne and spoilage bacteria have been described, although their application in foodstuff is scarce probably to the differences on the antimicrobial effect in food matrix and also to their sensorial impact. So, their utilization as food antimicrobial agents must be assessed in the food product. In this work, it was concluded that high concentrations of oregano (4%, 1.5% and 0.5%), decreased counts of *L. monocytogenes*, *S*. Enteritidis and *St. aureus* present in paste of "alheira". At lower concentrations (0.195% and 0.0975%) the reduction was lower although significant for *L. monocytogenes*. Nonetheless, the use of oregano EO at 0.195% in "alheira" has negative consequences at sensory level, as determined by the sensorial analyses used in the present study. This study maybe considered a starting point for other studies that have now to concentrate on ways to "mask" unpleasant sensorial 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 "alheiras".

## V.    Future works

In spite of some advantages in the food safety that the use of oregano essential oil seems to represent in "alheiras", the following suggestions for future work can be made.
The main suggestions are associated with strategies to mask the unpleasant taste of oil, combined techniques and using the hurdle concept.

Use the oregano EO (at more than one concentration) as an ingredient of "alheira" and add it during the "alheira" production process. By adding the oil into the syrup, this will blend better and undergo the whole process of fermentation and smoking. After, the sensory acceptability of "alheiras", produced with the addition of oregano EO, should be evaluated. Then it would be important to inoculate the pathogens before the fermentation process, as well as the oil, following the possible microbial reduction throughout the process;

Use other oils and use a combination of oils and check their action. Other oils could have different effect on microbial reduction, since they act differently due to their compounds. In addition, possible synergisms between the oils may exist. The use of pressure must continue to be evaluated with different pathogens, different oils and different pressures;

Formulate a microemulsion with the essential oil. Microemulsions would be a possible solution for for better dispersion of the oil, since they are thermodynamically stable. The isotropic mixtures of water, oil, surfactants and co-surfactants are used to improve the loading of the dispersed phase, allowing the diffusion of the oil more easily without great interferences of fats and proteins present in the matrix;

Use High Hydrostatic pressure (HHP) combined with EOs and see a possible synergistic effect.

The applicability of oregano EO in the control of *L. monocytogenes*, *St. aureus* and *S. Enteritidis* in other foods, in particular in ready-to-eat products, should be investigated.  "Alheira" has a very complex food matrix and other types of matrices could be better for the performance of the essential oil.

Although oils are considered GRAS, it is important to assess their toxicity.

# VI. Appendices

**Table A.** Zones of growth inhibition (mm; mean ± standard deviation) showing antibacterial activity of tested essential oils (EOs), against *Enterobacteriaceae* including the disk diameter 6.0 mm.

| EOs/ Microorganisms | Gram-negative (*Enterobacteriaceae*) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | *S.* Braenderup | *S.* Enteritidis | *S.* Tiphymurium | *S.* Enteritidis 459 | *S.* Enteritidis 405 | *E. coli* ATCC 25922 | *Proteus vulgaris* | *Proteus mirabilis* | *Yersinia enterocolitica* NCTC 10406 | *Yersinia enterocolitica* |
| **Bay** | 22.7±6.0$^{cg}$ | 22.3±7.8$^{f}$ | 19.0±1.7$^{ghm}$ | 16.3±1.5$^{befgij}$ | 27.0±1.0$^{b-k}$ | 15.0±2.0$^{ghm}$ | 20.7±1.2$^{efg}$ | 22.0±4.4$^{ghm}$ | 22.7±11.2$^{fl}$ | 39.3±0.6$^{hk}$ |
| **Cloves** | 13.3±1.2$^{eh}$ | 15.3±3.1$^{f}$ | 14.7±2.5$^{ghm}$ | 23.3±0.6$^{acfghij}$ | 20.0±1.0$^{ac-k}$ | 12.3±2.5$^{ghm}$ | 15.7±0.6$^{e}$ | 15.0±4.0$^{ghm}$ | 17.7±4.6$^{fl}$ | 24.3±3.1$^{dh}$ |
| **Coriander** | 10.0±0.0$^{aeh}$ | 11.0±1.0$^{fj}$ | 16.0±0.0$^{ghm}$ | 18.0±1.0$^{befgij}$ | 12.7±0.6$^{abfgk}$ | 17.3±8.4$^{ghm}$ | NI | 14.5±3.5$^{ghm}$ | 19.0±9.6$^{fl}$ | 23.0±7.9$^{dh}$ |
| **Cumin** | NI | NI | 14.0±1.4$^{ghm}$ | NI | 10.5±0.7$^{abfghjk}$ | 12.0±2.8$^{ghm}$ | NI | 13.5±4.9$^{ghm}$ | 13.5±2.1$^{fl}$ | 53.3±21.7$^{bcefikl}$ |
| **Marjoram** | NI | 10.3±0.6$^{fj}$ | 15.5±0.7$^{ghm}$ | 19.7±0.6$^{efgij}$ | 11.0±1.0$^{abfghjk}$ | 14.0±5.3$^{ghm}$ | 11.5±0.7$^{deh}$ | 11.0±1.0$^{ghm}$ | 18.7±6.0$^{fl}$ | 19.0±6.1$^{dhjm}$ |
| **Nutmeg** | NI | NI | 13.5±3.5$^{ghm}$ | NI | NI | 15.7±5.7$^{ghm}$ | NI | 12.7±2.5$^{ghm}$ | NI | 21.0±1.7$^{dhjm}$ |
| **Oregano (C.E)** | 21.0±3.5 | 21.3±8.3$^{f}$ | 40.7±6.0$^{a-fijkl}$ | 26.3±1.5$^{acdfghi}$ | 33.7±3.2$^{a-ehij}$ | 35.0±4.6$^{a-ijkl}$ | 24.3±4.5$^{cfg}$ | 37.0±8.7$^{a-fijkl}$ | 44.0±10.8$^{bcdei}$ | 39.0±1.7$^{hjk}$ |
| **Oregano (V.)** | 26.0±5.3$^{bcg}$ | 55.7±5.1$^{a-eg-j}$ | 39.0±3.6$^{a-fijkl}$ | 31.3±3.2$^{a-eghi}$ | 35.3±0.6$^{a-ehij}$ | 40.3±5.5$^{a-fijkl}$ | 30.3±5.5$^{abcfg}$ | 37.3±6.4$^{a-fijkl}$ | 22.7±3.5$^{fl}$ | 75.7±6.0$^{a-m}$ |
| **Parsley** | 15.0±2.8 | NI | 14.0±5.7$^{ghm}$ | NI | NI | 14.5±4.9$^{ghm}$ | NI | 11.5±2.1$^{ghm}$ | 23.0±8.7$^{fl}$ | 14.3±2.5$^{dhjm}$ |
| **Peppermint** | NI | 11.5±0.7$^{f}$ | 13.0±1.4$^{ghm}$ | 10.7±1.2$^{a-fhj}$ | 15.0±1.0$^{a-gijk}$ | 11.5±0.7$^{ghm}$ | 10.7±1.2$^{adeh}$ | 10.0±0.0$^{ghm}$ | 17.3±2.1$^{fl}$ | 47.7±15.6$^{efhikl}$ |
| **Rosemary** | 10.5±0.7$^{aeh}$ | 10.5±0.7$^{f}$ | 15.3±5.5$^{ghm}$ | 17.0±1.0$^{befgij}$ | 10.3±0.6$^{abfghjk}$ | 16.3±5.5$^{ghm}$ | NI | 12.3±2.5$^{ghm}$ | 24.0±5.0$^{fl}$ | 13.0±1.0$^{adghjm}$ |
| **Sage** | NI | 11.0±1.4$^{f}$ | 12.3±0.6$^{ghm}$ | 11.0±1.0$^{a-fhj}$ | 21.0±1.0$^{ac-ik}$ | 12.5±0.7$^{ghm}$ | 10.5±0.7$^{adeh}$ | 14.0±4.6$^{ghm}$ | 24.0±5.7$^{fl}$ | 15.7±2.5$^{dhjm}$ |
| **Thyme** | 26.3±4.9$^{bcg}$ | 24.3±2.3$^{cdf}$ | 44.3±1.2$^{a-fijkl}$ | 29.0±1.0$^{a-dghi}$ | 32.3±9.6$^{a-ehij}$ | 46.3±1.5$^{a-efijkl}$ | 24.3±3.5$^{cfg}$ | 34.7±7.2$^{b-fijkl}$ | 56.0±16.5$^{a-eg-k}$ | 47.0±4.4$^{efhikl}$ |

Results are expressed as means ± standard deviation (n = 3). Results of some EOs are not presented due to the absence of inhibition for all the microorganisms (<10mm). One-way ANOVA was performed for EOs and microorganisms. Values in same column with different letters are significantly different from each other ($p < 0.05$) according to the Turkey-Kramer test.

**C.E.** → Casa das Essências **V.** → Ventos **NI** → No Inhibition

**Table B.** Zones of growth inhibition (mm; mean ± standard deviation) showing antibacterial activity of tested essential oils (EOs), against Others Gram-negative including the disk diameter 6.0 mm.

| EOs/ Microorganisms | Others Gram-negative | | | | | |
|---|---|---|---|---|---|---|
| | *Pseudomonas aeruginosa* | *A. baumanii* (sensitive) A | *A. baumanii* (sensitive) B | *A. baumanii* (resistant) | *A. calcoaceticus* (sensitive) | *A. calcoaceticus* (resistant) |
| **Anise** | NI | NI | 14.7±2.5[lmr] | NI | NI | 14.0±1.4[jko] |
| **Basil** | NI | NI | 11.0±0.0[lmr] | NI | 10.0±0.0[jn] | 12.0±2.0[jko] |
| **Bay** | 21.0±1.7[f] | 22.0±2.6[hjm] | 26.7±2.0[lm] | 28.0±7.0[dhkl] | 25.7±4.5 | 25.0±6.0[jk] |
| **Cardamom** | NI | NI | 12.0±0.0[lmr] | NI | 11.0±1.4[ij] | NI |
| **Cloves** | 17.0±1.7[f] | 24.0±2.6[hjm] | 19.7±0.6[lm] | 16.3±2.1[ghl] | 19.3±0.6[j] | 20.0±1.0[jko] |
| **Coriander** | 11.5±2.1[fi] | 24.7±5.5[hjm] | 21.0±5.6[m] | 16.3±7.5[ghl] | 33.0±7.8 | 25.0±7.0[jk] |
| **Cumin** | 10.5±0.7[fi] | 21.7±10.4[ghjm] | 35.3±24.0 | 11.7±2.1[aghl] | 35.7±32.5 | 19.7±9.0[jko] |
| **Fennel** | NI | NI | 12.7±1.2[lmr] | NI | NI | NI |
| **Garlic (V.)** | NI | 13.5±4.9[ghjm] | 13.5±2.1[lmr] | 13.0±2.8[ghl] | NI | NI |
| **Marjoram** | NI | 16.0±5.2[ghjm] | 11.7±2.1[lmr] | 11.0±1.0[aghl] | 18.0±3.0[ij] | 17.3±1.5[jko] |
| **Nutmeg** | NI | NI | 27.7±12.0[m] | NI | 18.7±8.5[j] | 20.0±7.2[jko] |
| **Oregano (C.E.)** | 22.3±4.0[f] | 40.0±5.0[defhikl] | 44.7±9.0[abdehijnpq] | 40.0±10.4[b-fh-l] | 48.7±4.9[acgm] | 44.3±12.7[a-iln] |
| **Oregano (V.)** | 52.3±7.5[a-eghi] | 59.3±3.8[a-gikl] | 56.0±3.6[a-fhijknopq] | 68.7±3.2[a-gijk] | 53.3±3.8[acdghlm] | 54.0±3.5[abd-ilmn] |
| **Parsley** | NI | 16.7±10.7[ghjm] | 15.0±7.1[lm] | 13.0±2.8[ghl] | NI | NI |
| **Peppermint** | 10.3±0.6[fi] | 43.7±6.0[a-fikl] | 20.7±4.5[m] | 24.7±4.5[ghl] | 34.7±8.4 | 21.0±6.9[jko] |
| **Rosemary** | NI | 18.7±7.6[ghjm] | 14.7±2.1[lmr] | NI | 18.3±3.2[j] | 26.7±6.7[k] |
| **Sage** | 12.5±2.1[fi] | 15.0±2.0[ghjm] | 14.7±3.2[lmr] | 10.0±0.0[aghl] | 15.7±3.1[ij] | 14.3±2.5[jko] |
| **Thyme** | 27.7±5.9[cdfgh] | 51.3±4.6[a-fikl] | 43.0±7.8[abdhijnpq] | 45.0±5.6[a-fhijk] | 42.3±7.5[a] | 42.0±6.9[abdfghiln] |

Results are expressed as means ± standard deviation (n = 3). Results of some EOs are not presented due to the absence of inhibition for all the microorganisms (<10mm). One-way ANOVA was performed for EOs and microorganisms. Values in same column with different letters are significantly different from each other ($p < 0.05$) according to the Turkey-Kramer test.

**C.E.** → Casa das Essências **V.** → Ventos **NI** → No Inhibition

**Table C.** Zones of growth inhibition (mm; mean ± standard deviation) showing antibacterial activity of tested essential oils (EOs), against Gram-positive including the disk diameter 6.0 mm.

| EOs/ Microorganisms | Gram-positive | | | | | |
|---|---|---|---|---|---|---|
| | *Ent. faecalis* ATCC 29212 | *Ent. faecalis* DSMZ 12956 | *Ent. faecium* DSMZ 13590 | *Ent. flavescens* DSMZ 7370 | *Ent. gallinarium* DSMZ 20628 | *Ent. casseliflavus* DSMZ 20680 |
| **Basil** | $10.5\pm0.7^{lmp}$ | NI | NI | NI | $11.5\pm0.7^{o}$ | $10.0\pm0.0$ |
| **Bay** | $14.0\pm3.5^{lm}$ | $18.0\pm6.0^{i}$ | $19.7\pm6.8^{kn}$ | $21.0\pm1.7^{k}$ | $16.3\pm5.1^{o}$ | $18.3\pm5.6$ |
| **Cardamom** | $10.0\pm0.0^{lmp}$ | NI | $15.0\pm2.8^{kn}$ | $12.0\pm2.8^{k}$ | $15.52\pm2.1^{o}$ | $11.0\pm1.0$ |
| **Carrot** | NI | $31.0\pm11.4^{im}$ | $37.3\pm4.0^{dkl}$ | $33.0\pm14.7^{m}$ | $20.3\pm3.2^{o}$ | $30.3\pm10.7$ |
| **Cloves** | $10.7\pm1.2^{lmp}$ | $17.0\pm4.6^{i}$ | $14.0\pm1.7^{cjkn}$ | $13.0\pm2.6^{k}$ | $12.0\pm1.7^{o}$ | $12.0\pm2.6$ |
| **Coriander** | $12.0\pm1.0^{lmp}$ | $14.0\pm4.2^{i}$ | $20.0\pm10.0^{kn}$ | $14.7\pm4.0^{k}$ | $15.0\pm3.6^{o}$ | $15.0\pm4.6$ |
| **Cumin** | $11.7\pm2.1^{lmp}$ | $14.7\pm8.1^{i}$ | $15.0\pm4.2^{kn}$ | $17.0\pm5.0^{k}$ | $11.0\pm0.0^{o}$ | $14.0\pm6.1$ |
| **Garlic (V)** | $16.5\pm2.1^{m}$ | NI | NI | NI | $11.5\pm0.7^{o}$ | NI |
| **Juniper berries** | $16.3\pm4.0^{m}$ | $19.0\pm0.0^{i}$ | $22.0\pm14.1^{kn}$ | $25.7\pm6.4^{k}$ | NI | NI |
| **Lemon** | $12.5\pm2.1^{m}$ | NI | NI | NI | NI | NI |
| **Marjoram** | $17.3\pm8.7^{m}$ | $14.0\pm6.1^{i}$ | $21.7\pm1.5^{kn}$ | $18.3\pm8.1^{k}$ | $13.3\pm2.3^{o}$ | $18.0\pm6.1$ |
| **Nutmeg** | $17.7\pm9.8^{m}$ | NI | $17.0\pm9.9^{kn}$ | $19.7\pm3.8^{k}$ | $13.0\pm0.0^{o}$ | $23.0\pm18.4$ |
| **Oregano (C.E)** | $32.3\pm5.5^{a-fmo}$ | $30.0\pm5.2^{im}$ | $37.7\pm7.6^{dkl}$ | $31.0\pm2.6$ | $29.3\pm14.7$ | $27.7\pm10.1$ |
| **Oregano (V)** | $50.3\pm5.5^{a-lnop}$ | $56.7\pm4.2^{abcd-hj-n}$ | $68.0\pm4.4^{a-jlmn}$ | $45.3\pm5.0^{abdefhimno}$ | $32.3\pm4.9^{e}$ | $29.3\pm2.5$ |
| **Parsley** | NI | $13.0\pm4.4^{i}$ | NI | $24.7\pm10.3$ | $14.7\pm4.7^{o}$ | $10.0\pm0.0$ |
| **Peppermint** | NI | $14.0\pm1.7^{i}$ | $11.5\pm2.1^{cjkn}$ | $11.7\pm0.6^{ck}$ | NI | NI |
| **Rosemary** | $20.3\pm9.1^{m}$ | $29.7\pm9.9^{i}$ | $24.7\pm5.7^{kn}$ | $18.3\pm4.7^{k}$ | $22.3\pm7.2$ | $20.3\pm7.0$ |
| **Sage** | $12.3\pm2.5^{lmp}$ | $11.0\pm1.0^{bhilmn}$ | NI | $17.3\pm6.7^{k}$ | NI | $12.5\pm3.5$ |
| **Thyme** | $31.0\pm10.6^{a-fmo}$ | $30.3\pm13.1^{im}$ | $45.7\pm9.3^{abd-iklm}$ | $28.3\pm12.7$ | $40.0\pm10.2^{a-jm}$ | $27.3\pm7.4$ |

Results are expressed as means ± standard deviation (n = 3). Results of some EOs are not presented due to the absence of inhibition for all the microorganisms (<10mm). One-way ANOVA was performed for EOs and microorganisms. Values in same column with different letters are significantly different from each other ($p < 0.05$) according to the Turkey-Kramer test.

**C.E.** → Casa das Essências **V.** → Ventos **NI** → No Inhibition

**Table D.** Zones of growth inhibition (mm; mean ± standard deviation) showing antibacterial activity of tested essential oils (EOs), against Others Gram-positive including the disk diameter 6.0 mm.

| EOs/ Microorganisms | Others Gram-positive | | | | | | |
|---|---|---|---|---|---|---|---|
| | *St. aureus* ATCC 29213 | *St. aureus* 18N (MRSA) | *St aureus* 2037 M1 (MSSA) | *L. monocytogenes* 7946 | *L. monocytogenes* 7947 | *L. monocytogenes* SCOTT A | *L. innocua* 2030c |
| Basil | 10.0±0.0[glmr] | 13.0±2.0[klq] | 11.7±1.5[fgkp] | NI | NI | 32.5±10.6[egi-n] | 11.0±0.0[mns] |
| Bay | 21.3±5.9[mr] | 25.0±4.6[klq] | 20.7±3.8[fg] | 19.0±1.7[klq] | 17.0±2.0[lmr] | 24.0±1.0[cejlm] | 22.0±7.8[ns] |
| Cardamom | 10.3±0.6[glmr] | 14.0±0.0[klq] | NI | 13.0±1.4[klq] | 24.5±4.9[lmr] | NI | 12.7±1.5[mns] |
| Carrot | 15.7±2.9[glmr] | 16.7±5.1[klq] | 24.0±1.7[g] | 24.3±12.0[lq] | 38.7±1.5[mr] | 38.0±2.6[bdeg-o] | 30.0±5.6[s] |
| Cloves | 16.3±1.5[lmr] | 17.3±2.5[klq] | 17.7±5.5[fgkp] | 18.7±3.5[klq] | 25.0±5.3[lmr] | 25.3±0.6[cegjlm] | 17.7±2.9[mns] |
| Coriander | 16.0±2.6[lmr] | 18.7±2.5[klq] | 15.3±5.8[fgkp] | 18.3±2.3[klq] | 21.0±4.6[lmr] | 15.0±1.0[a-dfijo] | 21.7±10.2[mns] |
| Cumin | 44.3±26.4[aijq] | 27.3±7.2[klq] | 54.0±32.9[abdehilno] | 21.3±7.8[klq] | 22.7±5.8[lmr] | NI | 21.0±9.6[ns] |
| Garlic (V.) | 16.0±2.6[mr] | 39.0±8.5 | 73.0±0.0[a-ehilmno] | 29.0±11.3 | 25.0±5.7[lmr] | NI | 29.0±14.1 |
| Juniper berries | 11.0±1.4[glmr] | NI | NI | 11.0±1.4[klq] | 21.0±9.0[lmr] | 30.0±1.0[egjklm] | 15.7±2.1[mns] |
| Lemon | 11.3±2.3[glmr] | NI | 13.5±2.1[fgkp] | NI | 10.0±0.0[clmr] | NI | 12.5±2.1[mns] |
| Marjoram | 17.0±6.2[lmr] | 18.0±1.7[klq] | 13.7±1.2[fgkp] | 18.0±2.0[klq] | 16.7±4.2[lmr] | 15.7±0.6[acdfjo] | 15.7±1.2[mns] |
| Nutmeg | NI | 11.0±1.4[klq] | NI | 12.5±0.7[klq] | 31.3±5.9[mr] | 23.3±3.1[cjlm] | 21.0±2.0[ns] |
| Oregano C.E) | 46.7±9.0[acdefijkq] | 53.0±18.0[a-gijnop] | 42.3±3.8 | 43.0±2.6[abdehijnop] | 53.7±16.5[abd-jn-q] | 24.0±1.0[cejlm] | 39.3±6.7[aceijkp] |
| Oregano (V.) | 56.7±3.5[a-fh-kopq] | 52.7±5.0[a-gijnop] | 49.3±2.9[adehi] | 50.7±6.0[a-fhijmnop] | 76.0±6.6[a-knopq] | 48.0±2.6[a-ik-o] | 50.3±1.5[abcefgi-lo-r] |
| Parsley | 30.3±8.0 | 31.7±4.6[kq] | 18.7±10.3[fgp] | 23.0±16.6[lq] | 23.3±3.2[lmr] | 18.0±2.6[acfj] | 24.3±11.0[ns] |
| Peppermint | 28.0±14.7[m] | 21.0±10.4[klq] | 34.3±1.5[g] | 14.5±0.7[klq] | 17.7±8.6[lmr] | 11.0±1.0[a-dfijno] | 14.7±6.4[mns] |
| Rosemary | 22.0±10.5[m] | 26.7±5.7[klq] | 22.3±10.7[fg] | 21.3±4.2[klq] | 14.7±11.8[clmr] | 11.0±1.0[a-dfhijno] | 18.0±9.8[ns] |
| Sage | 12.7±3.8[glmqr] | 11.5±0.7[klq] | 22.7±11.0[fg] | 15.0±0.0[klq] | 26.3±11.8[lmr] | 22.7±5.9[achjlm] | 20.0±4.2[ns] |
| Thyme | 50.0±16.7[a-fhijk] | 56.3±15.0[a-gijmnop] | 50.0±18.8[adehil] | 52.3±13.9[a-fhijmnop] | 62.3±11.6[a-knopq] | 26.0±1.0[cegjlm] | 51.7±10.1[a-gi-lo-r] |

Results are expressed as means ± standard deviation (n = 3). Results of some EOs are not presented due to the absence of inhibition for all the microorganisms (<10mm). One-way ANOVA was performed for EOs and microorganisms. Values in same column with different letters are significantly different from each other ($p < 0.05$) according to the Turkey-Kramer test.

**C.E.** → Casa das Essências **V.** → Ventos **NI** → No Inhibition

**Table E.** Zones of growth inhibition (mm; mean ± standard deviation) showing antibacterial activity of tested essential oils (EOs), against Others Gram-positive (Anaerobic spores formers) and Yeasts including the disk diameter 6.0 mm.

| EOs/ Microorganisms | Gram-Positive (Anaerobic spores formers) | | | | | |
|---|---|---|---|---|---|---|
| | *C. perfringens* 1.16 | *C. perfringens* 1.19 | *C. perfringens* 1.22 | *C. sporogenes* 1.31 | *C. sporogenes* 1.34 | *C. sporogenes* 1.61 |
| **Anise** | $11.5\pm2.1^{nos}$ | NI | $29.7\pm11.6^{pu}$ | $15.0\pm7.1^{mnr}$ | $12.0\pm2.6^{jpqru}$ | $10.0\pm0.0^{pqu}$ |
| **Basil** | NI | $39.5\pm7.8^{t}$ | $44.0\pm0.0$ | $31.0\pm0.0^{mnr}$ | $41.0\pm15.6$ | $37.5\pm6.4$ |
| **Bay** | $34.0\pm4.4^{nos}$ | $28.7\pm7.0^{not}$ | $52.0\pm11.8^{lprs}$ | NI | $41.0\pm15.7^{q}$ | $39.7\pm2.3^{q}$ |
| **Cardamom** | $21.5\pm14.8^{nos}$ | $19.5\pm2.1^{not}$ | $21.5\pm4.9^{epu}$ | $35.3\pm8.5^{mnr}$ | $39.0\pm33.9^{q}$ | $39.0\pm1.4$ |
| **Carrot** | $21.3\pm4.5^{nos}$ | $27.7\pm15.0^{not}$ | $60.7\pm15.6^{dhjlrs}$ | $38.3\pm6.4^{mnr}$ | $49.0\pm13.7$ | $21.3\pm10.2^{qu}$ |
| **Cloves** | $30.0\pm8.7^{nos}$ | $51.7\pm17.5$ | $41.0\pm2.6^{pu}$ | $29.7\pm6.1^{mnr}$ | $31.0\pm5.6^{qu}$ | $32.3\pm11.2^{qu}$ |
| **Coriander** | $18.3\pm10.4^{nos}$ | $31.3\pm15.6^{not}$ | $42.0\pm3.5^{pu}$ | NI | $31.3\pm9.3^{qu}$ | $14.3\pm7.5^{ahm}$ |
| **Cumin** | $19.5\pm6.4^{nos}$ | $26.3\pm14.0^{not}$ | $25.0\pm16.5^{epu}$ | $23.0\pm20.0^{mnr}$ | $50.0\pm22.6$ | $14.3\pm7.5^{gpqu}$ |
| **Fennel** | $12.7\pm2.1^{nops}$ | NI | $43.0\pm16.5^{pu}$ | $14.7\pm4.5^{mnr}$ | $18.3\pm5.7^{jqru}$ | $11.7\pm0.6^{gpqu}$ |
| **Garlic (V.)** | $26.5\pm7.8^{nos}$ | $21.0\pm0.0^{not}$ | $22.5\pm3.5^{epu}$ | $38.0\pm21.2^{nr}$ | $69.0\pm8.5^{k}$ | $34.0\pm5.7^{q}$ |
| **Ginger** | $10.5\pm0.7^{nops}$ | $16.0\pm4.4^{not}$ | NI | $15.0\pm7.0^{mnr}$ | $13.5\pm2.1^{jqru}$ | $20.7\pm16.8^{qu}$ |
| **Juniper berries** | $21.7\pm6.4^{nos}$ | $29.0\pm8.5^{not}$ | $29.3\pm8.0^{pu}$ | $25.0\pm7.0^{mnr}$ | $35.7\pm4.0^{qu}$ | $21.5\pm12.0^{qu}$ |
| **Lemon** | NI | $25.0\pm12.8^{not}$ | $17.7\pm5.5^{cepqu}$ | $13.3\pm3.2^{mnr}$ | $15.3\pm6.8^{jpqru}$ | $11.7\pm1.5^{gpqu}$ |
| **Marjoram** | $26.7\pm17.8^{nos}$ | $26.3\pm15.0^{not}$ | $20.0\pm8.9^{epu}$ | $31.7\pm15.8^{mnr}$ | $33.7\pm19.7^{qu}$ | $15.7\pm7.4^{pqu}$ |
| **Nutmeg** | $34.3\pm20.5^{nos}$ | $32.7\pm4.6^{not}$ | $27.3\pm15.4^{pu}$ | NI | $39.0\pm13.2^{qu}$ | $18.7\pm6.5^{pqu}$ |
| **Oregano (C.E)** | $68.3\pm11.0^{a\text{-}mqr}$ | $74.6\pm5.7^{bcdf\text{-}mqrs}$ | $40.3\pm0.6^{pu}$ | $70.7\pm4.9^{a\text{-}gijklopq}$ | $56.3\pm12.5^{am}$ | $52.7\pm8.4^{ahimnost}$ |
| **Oregano (V.)** | $69.7\pm15.0^{a\text{-}mqr}$ | $73.0\pm19.1^{bcdf\text{-}mqs}$ | $81.3\pm4.6^{adh\text{-}orst}$ | $80.7\pm5.8^{a\text{-}lopq}$ | $84.0\pm0.0^{acdfgik\text{-}ot}$ | $74.3\pm16.7^{acefh\text{-}orst}$ |
| **Parsley** | $48.7\pm10.1^{ahjor}$ | $42.0\pm25.0$ | $51.3\pm18.8^{lqr}$ | $20.0\pm11.8^{mnr}$ | $69.0\pm5.3^{aikmt}$ | $23.7\pm11.5^{qu}$ |
| **Peppermint** | $15.0\pm7.1^{nos}$ | $17.0\pm9.9^{not}$ | $15.0\pm4.4^{cepu}$ | $14.3\pm6.7^{mnr}$ | NI | $15.3\pm7.6^{pqu}$ |
| **Rosemary** | $14.5\pm3.5^{nos}$ | $35.0\pm5.6^{nt}$ | $18.3\pm10.4^{cepu}$ | NI | $46.3\pm19.3$ | NI |
| **Sage** | $13.0\pm3.6^{nops}$ | $30.7\pm9.0^{not}$ | $31.3\pm14.5^{pu}$ | $26.0\pm2.6^{mnr}$ | $24.7\pm4.2^{qru}$ | $19.0\pm4.6^{pqu}$ |
| **Thyme** | $70.7\pm2.1^{a\text{-}mqr}$ | $83.7\pm0.6^{a\text{-}df\text{-}mp\text{-}s}$ | $78.7\pm5.5^{adf\text{-}orst}$ | $78.7\pm8.3^{a\text{-}lopq}$ | $80.3\pm6.4^{afgik\text{-}ot}$ | $69.3\pm15.6^{aefhik\text{-}orst}$ |

Results are expressed as means ± standard deviation (n = 3). Results of some EOs are not presented due to the absence of inhibition for all the microorganisms (<10mm). One-way ANOVA was performed for EOs and microorganisms. Values in same column with different letters are significantly different from each other (p < 0.05) according to the Turkey-Kramer test.

**C.E.** → Casa das Essências **V.** → Ventos **NI** → No Inhibition

**Table F.** Zones of growth inhibition (mm; mean ± standard deviation) showing antibacterial activity of tested essential oils (EOs), against Others Gram-positive (Spores formers) and Yeasts including the disk diameter 6.0 mm.

| EOs/ Microorganisms | Others Gram-positive (Spores formers) | | | Yeasts | |
|---|---|---|---|---|---|
| | *B. cereus* | *B. subtilis* | *B. stearothermophilus* | *Candida albicans* | *Saccharomyces cerevisiae* |
| **Anise** | NI | NI | NI | 16.3±3.2[filnoqrt] | 13.0±1.0[ghjpqtv] |
| **Basil** | 12.0±0.0[hms] | 12.0±0.0[jkn] | NI | 18.0±2.6[fgilnoqrt] | 27.0±2.6[hjp] |
| **Bay** | 25.7±5.1 | 19.3±6.4 | 28.3±7.6[lq] | 38.0±7.0[gil] | 28.0±2.0[hj] |
| **Cardamom** | 13.0±0.0[hms] | 16.5±0.7 | 12.7±3.1[kllq] | 16.7±2.1[fgilnoqrt] | 23.0±2.6[hjpqv] |
| **Carrot** | 26.3±14.0 | 18.7±7.2 | 18.3±6.0[klq] | NI | 13.0±4.2[hjpqv] |
| **Cloves** | 17.0±3.0[hms] | 13.0±2.8[jk] | 16.3±1.5[klq] | 30.3±1.5[fgilnoqrt] | 31.0±7.9[hj] |
| **Coriander** | 23.0±7.2 | 12.7±1.5[jkn] | 23.3±2.1[lq] | 62.0±19.1[a-ehjkmps] | 48.0±21.8[a] |
| **Cumin** | 25.3±7.5 | NI | 31.3±7.8[lq] | 73.0±0.0[a-ehjkmps] | 71.7±2.3[a-fiklmnou] |
| **Fennel** | NI | NI | NI | 16.7±2.1[fgilnoqrt] | 12.7±0.6[ghjpqtv] |
| **Garlic (V.)** | 49.0±9.9[ijkqr] | NI | 36.5±2.1[l] | 73.0±0.0[a-ehjkmps] | 73.0±0.0[afilmnou] |
| **Ginger** | NI | NI | NI | NI | 32.0±14.1[h] |
| **Juniper berries** | 18.0±7.0[hms] | NI | 13.5±3.5[klq] | 11.5±0.7[fgilnoqrt] | 17.0±3.6[hjpqv] |
| **Lemon** | 12.7±2.5[hms] | 10.5±0.7[jkn] | NI | 13.7±1.5[fgilnoqrt] | 23.7±0.6[hjpqv] |
| **Marjoram** | 15.3±0.6[hms] | 13.3±2.1[jkn] | 22.3±1.2[klq] | 73.0±0.0[a-ehjkmps] | 21.0±7.8[hjpqv] |
| **Nutmeg** | 26.3±5.5 | 10.0±0.0[jkn] | 24.3±10.7[lq] | 20.0±8.0[fgilnoqrt] | 23.7±11.4[hjpqv] |
| **Oregano (C.E)** | 43.7±10.5[aeijkpq] | 31.0±3.5[aefghim] | 43.3±14.2[bcdhimnop] | 62.7±10.0[abdehjkmps] | 61.3±6.5[abdeil-ou] |
| **Oregano (V.)** | 31.3±4.0[c] | 31.0±6.6[aefghim] | 61.3±5.7[a-jmnop] | 58.0±2.0[abdhjkmps] | 59.3±4.0[adeil-ou] |
| **Parsley** | 23.3±5.5 | NI | 17.0±3.2[klq] | 22.7±6.0[fgilnoqrt] | 40.7±28.5 |
| **Peppermint** | 16.3±7.7[hm] | NI | 17.3±3.2[klq] | 57.3±32.3[abdhjkmps] | 40.3±12.5[i] |
| **Rosemary** | 18.3±4.2[hms] | 20.3±6.8 | 21.3±1.5[klq] | 54.3±32.3[abdhjkmps] | 50.7±20.7[a] |
| **Sage** | 19.0±5.3[hms] | 12.7±2.3[jkn] | 17.3±8.1[klq] | 16.0±5.6[fgilnoqrt] | 24.3±10.1[hijpq] |
| **Thyme** | 24.3±12.2[aceijkpqr] | 28.0±6.1[afghim] | 54.0±10.1[a-fhijmnop] | 64.0±6.9[abdehjkmps] | 60.7±3.2[adel-ouv] |

Results are expressed as means ± standard deviation (n = 3). Results of some EOs are not presented due to the absence of inhibition for all the microorganisms (<10mm). One-way ANOVA was performed for EOs and microorganisms. Values in same column with different letters are significantly different from each other (p < 0.05) according to the Turkey-Kramer test.

**C.E.** → Casa das Essências **V.** → Ventos **NI** → No Inhibition

**Table G.** Lactic acid bacteria (LAB) counts in paste of "alheira" in *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. Results are expressed as mean ± standard deviation.

| Pathogens / Time | 0.25 (4h) | 3 | 7 | 15 | 21 |
|---|---|---|---|---|---|
| S | 9.13 ± 0.42 | 9.12 ± 0.071 | 9.65 ± 0.073 | 9.51 ± 0.069 | 8.69 ± 0.22 |
| S+O (4%) | 8.58 ± 0.035 | 7.63 ± 0.82 | 8.66 ± 0.54 | 5.23 ± 4.55 | 7.39 ± 0.19 |
| S+O (1.5%) | 9.30 ±0.27 | 9.20 ± 0.59 | 8.99 ± 0.13 | 9.11 ± 0.57 | 8.89 ± 0.48 |
| S+O (0.5%) | 9.45 ± 0.047 | 9.34 ± 0.11 | 9.46 ± 0.058 | 9.15 ± 0.031 | 9.26 ± 0.29 |
| S+O (0.195%) | 8.63 ±0.026 | 9.24 ± 0.074 | 8.94 ± 0.57 | 8.12 ± 0.34 | 9.13 ± 0.11 |
| S+O (0.0975%) | 9.12 ± 0.47 | 9.22 ± 0.067 | 9.10 ± 0.44 | 9.23 ± 0.051 | 9.29 ± 0.54 |
| SA | 8.83 ± 0.30 | 9.45 ± 0.14 | 8.88 ± 0.28 | 8.73 ± 0.49 | 8.99 ± 0.057 |
| SA+O (4%) | 8.30 ± 0.21 | 8.33 ± 0.11 | 8.08 ± 0.036 | 8.24 ± 0.67 | 7.87 ± 0.71 |
| SA+O (1.5%) | 9.20 ± 0.090 | 9.36 ± 0.13 | 8.99 ± 0.22 | 9.11 ± 0.066 | 8.78 ± 0.16 |
| SA+O (0.5%) | 9.34 ±0.060 | 9.32 ± 0.25 | 9.15 ± 0.11 | 9.08 ± 0.090 | 9.18 ± 0.11 |
| SA+O (0.195%) | 8.87 ± 0.10 | 9.25 ± 0.038 | 8.52 ± 0.092 | 8.82 ± 0.36 | 8.45 ± 0.052 |
| SA+O (0.0975%) | 9.07 ± 0.61 | 8.44 ± 0.11 | 8.26 ± 0.40 | 8.20 ± 0.16 | 8.04 ± 0.42 |
| Lm | 8.63 ± 0.10 | 9.30 ± 0.072 | 8.87 ± 0.41 | 8.41 ± 0.0098 | 8.32 ± 0.082 |
| Lm+O (4%) | 8.01 ± 0.14 | 9.18 ± 0.0 | 8.63 ± 0.36 | 8.39 ± 0.11 | 7.70 ± 0.90 |
| Lm+O (1.5%) | 8.26 ± 0.61 | 8.08 ± 0.26 | 8.99 ± 0.13 | 8.60 ± 0.22 | 8.68 ± 0.32 |
| Lm+O (0.5%) | 9.11 ± 0.50 | 9.34 ± 0.62 | 9.23 ± 0.24 | 9.20 ± 0.18 | 9.08 ± 0.23 |
| Lm+O (0.195%) | 9.55 ± 0.066 | 9.08 ± 0.0 | 8.85 ± 0.67 | 8.57 ± 0.53 | 8.28 ± 0.12 |
| Lm+O (0.0975%) | 8.31 ± 0.58 | 8.10 ± 0.41 | 8.13 ± 0.095 | 8.09 ± 0.35 | 7.76 ± 0.21 |

**Table H.** pH in paste of "alheira" in *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. Results are expressed as mean ± standard deviation.

| Pathogens / Time | 0.25 (4h) | 3 | 7 | 15 | 21 |
|---|---|---|---|---|---|
| S | 5.09 ± 0.060 | 4.84 ± 0.021 | 4.67 ± 0.055 | 4.44 ± 0.18 | 4.40 ± 0.021 |
| S+O (4%) | 5.06 ± 0.047 | 4.80 ± 0.070 | 4.60 ± 0.015 | 4.50 ± 0.057 | 4.33 ± 0.051 |
| S+O (1.5%) | 5.05 ± 0.026 | 5.01 ± 0.029 | 4.91 ± 0.01 | 4.99 ± 0.24 | 4.97 ± 0.040 |
| S+O (0.5%) | 5.03 ± 0.032 | 5.00 ± 0.0058 | 4.87 ± 0.046 | 4.84 ± 0.012 | 4.98 ± 0.03 |
| S+O (0.195%) | 5.07 ± 0.017 | 5.01 ± 0.021 | 4.89 ± 0.01 | 4.87 ± 0.029 | 4.94 ± 0.032 |
| S+O (0.0975%) | 5.05 ±0.031 | 5.12 ± 0.055 | 4.98 ± 0.049 | 4.97 ± 0.068 | 5.04 ± 0.16 |
| SA | 5.07 ±0.056 | 4.68 ± 0.057 | 4.72 ± 0.13 | 4.54 ± 0.029 | 4.51 ± 0.14 |
| SA+O (4%) | 5.10 ± 0.01 | 4.74 ± 0.044 | 4.60 ± 0.012 | 4.46 ± 0.021 | 4.32 ± 0.90 |
| SA+O (1.5%) | 5.09 ± 0.017 | 5.03 ± 0.067 | 4.90 ± 0.035 | 4.82 ± 0.021 | 4.89 ± 0.02 |
| SA+O (0.5%) | 4.89 ± 0.021 | 5.03 ± 0.01 | 4.88 ± 0.04 | 4.84 ± 0.11 | 4.96 ± 0.095 |
| SA+O (0.195%) | 5.05 ± 0.021 | 4.99 ± 0.0058 | 4.81 ± 0.012 | 4.88 ± 0.0058 | 5.13 ± 0.16 |
| SA+O (0.0975%) | 5.08 ± 0.035 | 5.10 ± 0.01 | 5.04 ± 0.015 | 4.69 ± 0.015 | 4.64 ± 0.36 |
| Lm | 5.04 ± 0.012 | 4.78 ± 0.076 | 4.72 ± 0.031 | 4.59 ± 0.093 | 4.50 ± 0.071 |
| Lm+O (4%) | 5.12 ± 0.098 | 4.69 ± 0.032 | 4.58 ± 0.01 | 4.47 ± 0.031 | 4.35 ± 0.07 |
| Lm+O (1.5%) | 5.07 ± 0.025 | 5.31 ± 0.34 | 4.81 ± 0.01 | 4.84 ± 0.015 | 5.00 ± 0.045 |
| Lm+O (0.5%) | 4.97 ± 0.036 | 4.92 ± 0.012 | 4.85 ± 0.021 | 4.82 ± 0.01 | 5.01 ± 0.021 |
| Lm+O (0.195%) | 5.05 ± 0.015 | 4.54 ± 0.035 | 4.54 ± 0.032 | 4.41 ± 0.042 | 4.39 ± 0.012 |
| Lm+O (0.0975%) | 5.06 ± 0.015 | 4.73 ± 0.023 | 4.70 ± 0.065 | 4.64 ± 0.074 | 4.73 ± 0.07 |

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