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# Antibacterial activity of Oregano, Rosmarinus and Thymus essential oils against *Staphylococcus aureus* and *Listeria monocytogenes* in beef meatballs

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ABSTRACT

Antimicrobial activity of five essential oils (EOs) was investigated up to 72 h against foodborne pathogens (*Staphylococcus aureus*, *Listeria monocytogenes*, *Salmonella enteritidis, Campylobacter jejuni*) through disk diffusion and determination of Minimum Inhibitory Concentrations. The most active EOs were *Thymus vulgaris* and *Origanum vulgare,* followed by *Cinnamomum zeylanicum*, *Rosmarinus officinalis,* and *Salvia officinalis.* The antimicrobial activity of *O. vulgare*, *Rosmarinus officinalis* and *T. vulgaris* was investigated against five enterotoxin producers of *S. aureus* and five *L. monocytogenes* strains, for different amounts of time (up to 14 days), at 4 °C, in meatballs. Concentrations of 2% and 1% restricted the growth of both the pathogens but, as a result of panel tests, altered the meat flavor. The cooked meatballs containing 0.5% of EO were acceptable in terms of taste, and the oils were able to suppress concentrations of $<10^2$ CFU/g of the pathogens, revealing the potential use of *R. officinalis*, *T. vulgaris* and *O. vulgare* as food preservatives at this concentration.



## 1. Introduction

One of the main public health problems, according to the World Health Organization (2002), is food-related diseases, and in particular, foodborne diseases that are the cause of numerous complications and many deaths all over the world. In industrialized countries, their spread is also favored by new lifestyles which have led the entire population to increasingly resort to the catering industry, buying ready-to-eat, ready-to-cook and heat-and-eat foods. To reduce the risks and incidence of foodborne diseases, prevention is fundamental, and international health authorities have directed their attention towards production and conservation of food (WHO, 2002). From the middle of the last century, in order to preserve foods and give them a long shelf life, permitting international trade, synthetic compounds have been used as additives in food production. In recent years, people have expressed strong concerns about the use of these substances as a result of diseases (Fleming-Jones & Smith, 2003) associated with their consumption; this has led to an increased interest in natural substances as food preservatives (Goni et al., 2009; Lv, Liang, Yuan, & Li, 2011). Other studies (Brenes & Roura, 2010; Burt, 2004) have shown that some essential oils (EOs) have strong antioxidant and antimicrobial properties; therefore, they could be used in food production as a possible alternative to synthetic preservative additives, limiting the growth of food pathogens and increasing the shelf life of some foods.

The characteristic that most influences the antimicrobial activity of these natural extracts is their high hydrophobicity, which enables them to cross the bacterial membranes and act directly on them, causing loss of ions and reduction of the membrane potential, loss of function of the proton pumps and ATP depletion (Di

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Pasqua, Hoskins, Betts, & Mauriello, 2006), or damage to proteins, lipids, and organelles present within the bacterial cell (Bakkali, Averbeck, Averbeck, & Idaomar, 2008), causing cell death.

Many authors have performed studies *in vitro* on antibacterial properties on several EOs, (Bakkali et al., 2008; Hyldgaard, Mygind, & Meyer, 2012) finding minimal inhibitory concentrations ($MIC_{100}$ and $MIC_{90}$) values very low (<0.1%) against an initial concentration higher than $10^5$ CFU/mL of many pathogens, such as *Staphylococcus aureus*, *Listeria monocytogenes*, *Salmonella Enteritidis*, *Campylobacter jejuni*, *Escherichia coli*.

Although there is currently very little knowledge regarding the effectiveness of the antibacterial activity of EOs in foods and their mechanisms of interaction with food components (Burt, 2004; Negi, 2012), some authors (Brenes & Roura, 2010; Gutierrez, Barry-Ryan, & Bourke, 2008) have shown that, in food, higher bactericidal concentrations of EO are required than in experimental media, due to the interaction of some EO compounds with those of food, such as meat fat (Hsieh, Mau, & Huang, 2001). These high EO concentrations may lead to exceeding the threshold of acceptability for the taste of food. However, lower concentrations may be used in combination with traditional food preservation techniques, such as refrigeration, modified atmosphere packaging or vacuum packaging, with the aim of suppressing the multiplication of pathogens and aerobic spoilage flora in perishable foods, such as ready-to-eat foods, and ready-to-cook fish and meats, especially minced ones (Chen & Brody, 2013; Nieri, Pesavento, Ducci, Calonico, & Lo Nostro, 2014; Skandamis & Nychas, 2001). In addition, in food in general, the pathogen concentrations are much lower than in experimental media, established often to values higher than $10^5$ CFU/g (Liu & Yang, 2012; Skandamis & Nychas, 2001): in food, *Staphylococcus aureus* rarely exceeds $10^4$ CFU/g (Sergelidis et al., 2012) and *Listeria monocytogenes* $10^2$ CFU/g (De Cesare, Mioni, & Manfreda, 2007; Uyttendaele et al., 2009). Consequently, very low concentrations of EOs might have potentially bactericidal or bacteriostatic effects in food.

The purpose of this study is to evaluate the antimicrobial activity of five essential oils (*Origanum vulgare, Thymus vulgaris, Rosmarinus officinalis*, *Cinnamomum zeylanicum,* and *Salvia officinalis*), classified as Substances Generally Recognized As Safe (GRAS) by the FDA (2013), first, *in vitro*, against *S. aureus, L. monocytogenes, Salmonella enteritidis*, and *Campylobacter jejuni* at optimal growth conditions as culture broth, and then, *in vivo*, in a real food system (only Origanum, Thymus and Rosmarinus) such as raw minced meat experimentally contaminated with *L. monocytogenes* or *S. aureus* and preserved at 4 °C, for different amounts of time up to 14 days. Antibiotic susceptibility was determined for both the pathogens, as virulence factors, and a sensory evaluation of the cooked meat samples was made to evaluate the flavor and organoleptic properties.

The main antibacterial compounds of *O. vulgare* EO are carvacrol and thymol which are present in concentrations close to 15% and 20% (vol/vol) respectively, depending on the chemotype (Burt, 2004; Russo, Galletti, Bocchini, & Carnacini, 1998).

Thymol and carvacrol are also the main antimicrobial constituents of the *T. vulgaris* EO. With concentrations ranging from 10% to 64%, and from 2% to 11% respectively, they represent the monoterpenes with the highest bactericidal power present in the composition of many EOs, due to their phenolic nature (Yanishlieva, Marinova, & Pokorny, 2006).

The antimicrobial properties of the *Cinnamomum zeylanicum* EO are mainly due to the action of two compounds: cinnamaldehyde (concentrations up to 80%), and eugenol (representing about 4%) (Lens-Lisbonne, Cremieux, Maillard, & Balansard, 1987). At high doses, cinnamaldehyde and eugenol can cause serious damage to the bacterial wall leading to cell lysis (Yehouenou et al., 2012).

*Rosmarinus officinalis* EO contains 1.8-cineol (26–51%), camphor (4.9–29%), α-pinene (7–11%), camphene (3.3–12, 8%) and borneol (2.2–12%) (Zaouali, Bouzaine, & Boussaid, 2010). The mechanism of action of these compounds has not yet been fully clarified, and only in the case of 1.8-cineol was it possible to identify a specific activity against the bacterial membrane (Burt, 2004).

The *S. officinalis* EO contains numerous active molecules; the most abundant is α-thujone (1–36.9%), followed by α-pinene (1.2–5.9%), camphene (0.5–5.9%), β - pinene (1.2–5.3%), 1,8-cineole (6.7–20.5%), β-thujone (0.2–28.7%), camphor (3.2–12.3%), bornyl (0.5–7.9%), β-caryophyllene (1.5–15.8%). Among them, sesquiterpens and β-thujone were shown to have the greater antibacterial properties (Lamien-Meda et al., 2009).

This study was aimed at identifying "natural" substances that could eventually replace synthetic additives in food (raw meat and fish, ready-to-eat, ready-to-cook and heat-and-eat) preservation (Faleiro et al., 2003; Hyldgaard et al., 2012) together with hurdle technologies (Barbosa et al., 2009; Chen & Brody, 2013; Davies, 1995; Nieri et al., 2014), and in particular on raw meatballs that could be prepared by food industries and preserved at 4 °C until home cooking.

## 2. Materials and methods

### 2.1. Essential oils

#### 2.1.1. Preparation of dilutions of EOs

The EOs used in this study (Oregano, Cinnamomum, Rosmarinus, Salvia, and Thymus) were extracted by steam distillation method, and purchased from the same retailer (Prodotti Phitocosmetici Dott. Vannucci di Vannucci Daniela e C. Sas). Dilutions of the EO, for disk diffusion assay, were made in sterile glass using distilled water; 0.5% Dimethyl Sulfoxide (DMSO, Carlo Erba Reagenti) was added. The dilutions, in a final volume of 2 mL, were: 25%, 50%, 75%, 100% (vol/vol). Dilutions used for MIC determination were in Mueller Hinton Broth (MHB, Oxoid), concentrations were different for each EO and bacterial species; 0.5% DMSO was added. The addition of DMSO, an aprotic organic solvent belonging to the category of sulfoxides, had the purpose of facilitating the solubilization of EOs in the culture media. All EOs were stored at 4 °C in darkness before use and utilized before the expiration date. EO dilutions were prepared just before the experiments.

Experiments "*in vivo*" were performed adding the suitable volume of EO to the meat without making any dilution and without using DMSO which could be potentially toxic for eukaryotic cells.

### 2.2. "In vitro" experiments

#### 2.2.1. Preparation of microbial suspensions and media

Two different strains of each microorganism were used; one was an ATCC (American Type Culture Collection) strain, and the other was previously isolated from a food product from the Health Sciences Dept. (HSD) BioBank: *S. aureus* (ATCC 25923, HSD 3623), *L. monocytogenes* (ATCC 7644, HSD 3509), *S. Enteritidis* (ATCC 13076, HSD 3657) and *C. jejuni* (ATCC 33291, HSD 3486). All ATCC strains and media were purchased from Oxoid. Before they were used, the pathogens were cultured in Brain Heart Infusion Broth (BHI) for 24 h at 37 ± 1 °C and then streaked on Tryptone Soy Agar (TSA), and incubated at 37 °C for 24 h. Tubes were prepared for each bacterial strain in sterile deionized water, with a turbidity of 0.5 McFarland or 1 McFarland, to perform disk diffusion assays and MIC determination, respectively, using a McFarland standard (bioMérieux). Serial dilutions $10^{-1}$ to $10^{-5}$ of each bacterial suspension were streaked on TSA Petri dishes in order to count the microorganisms