Thymus vulgaris (red thyme) and *Caryophyllus aromaticus* (clove) essential oils to control spoilage microorganisms in pork under modified atmosphere

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Abstract

In recent years, it has been confirmed that essential oils (EOs) exert antimicrobial activity as they are able to inhibit cell growth and inactivate microbial cells. The application of biopreservation strategies by means of EOs opens up interesting perspectives in the food industry, including meat production. The paper aims to evaluate the effects of *Thymus vulgaris* (red thyme) and *Caryophyllus aromaticus* (cloves) EOs on the development of the spoilage population of fresh pork packaged under modified atmosphere (MAP). In particular, the research was focused on *Brochothrix thermosphacta*, a specific spoilage microorganism of fresh meat packed in anaerobic conditions or under MAP. Amongst seven EOs, those that showed the highest antimicrobial activity on 5 *B. thermosphacta* strains *in vitro* were: cloves [minimum inhibitory concentration (MIC) 0.6-2.5 mg/mL], savory (MIC 2.5-5.0 mg/mL), and red thyme (MIC 2.5 to 20 mg/mL). Red thyme and cloves EOs were selected for meat treatment, by increasing the dose at 20 and 40 mg/mL respectively, to take into account the matrix effect that can reduce EO availability. In spite of the minor efficacy observed *in vitro*, 40 mg/mL red thyme EO strongly limited the growth of *B. thermosphacta* in pork samples up to day 6 of storage [below 3.0 Log colony forming unit (CFU)/g, starting from 2.0 Log CFU/g at time 0], and exerted an antimicrobial effect also on the aerobic mesophilic count. Good results were obtained also with 20 mg/mL red thyme EO. The control of *B. thermosphacta* growth through EOs encourages research on alternative methods for extending the shelf life of fresh meat under MAP.

Introduction

Spoilage and pathogenic bacteria are causes of serious concern for the food industry because of fast deterioration of some products and diseases transmitted. Over the years, the food industry has implemented methods of preservation that include, for example, the use of synthetic substances, as well as physical or thermal treatments, and their combination (Gould, 2000). Spoilage microorganisms generate significant economic losses for producers as they decrease shelf life of products (in particular meat and meat products) by forming off-odours, colour changes, and unpleasant tastes (Ercolini *et al*., 2009). In addition, the emergence of bacteria that are resistant to the treatments used to contain their development in food is fostering research on alternative antimicrobial techniques (Rajkovic *et al*., 2009). Biopreservation strategies are a set of processes based on natural and sustainable technologies, aimed at the control of the multiplication of pathogenic or spoilage microorganisms in foods. In this perspective, scientific literature has recently showed an increasing interest in the application of vegetable extracts and essential oils (EOs). EOs are known and used since ancient times for their antioxidant and antimicrobial activity. They may find application in food preservation, shelf life extension, or can be employed as active compounds in packaging materials or in protein-based films ((Oussalah *et al.*, 2004; Zivanovic *et al.*, 2005; Zinaviadou *et al.*, 2009; Tongnuanchan *et al.*, 2012; Tongnuanchan and Benjakul, 2014)). Antimicrobial and antioxidant activity of EOs is due to their chemical composition that includes volatile bioactive compounds able to control pathogenic and spoilage microorganisms (Burt, 2004; Bakkali *et al.*, 2008; Gutiérrez-Larraínzar *et al.*, 2012; Solórzano-Santos and Miranda-Novales, 2012; Gyawali and Ibrahim, 2014).

Brochothrix thermosphacta, a Gram-positive and facultative anaerobic species, is an important spoilage organism in fresh pork stored under modified atmosphere packaging (MAP), together with *Pseudomonas* spp. and lactic acid bacteria. The products of its metabolism mainly cause off-flavours, discoloration, gas production, and a pungent cheesy smell (McClure *et al.*, 1993; Nychas *et al.*, 2008; Gribble and Brightwell, 2012; Casaburi *et al.*, 2014).

The aim of the study was to evaluate the *in vitro* inhibitory activity of seven EOs (*Caryophyllus aromaticus, Mentha piperita* var. *Mitcham, Origanum vulgare, Rosmarinus officinalis, Salvia officinalis, Satureja montana, Thymus vulgaris*) against *Brochothrix thermosphacta* strains isolated from meat products. Moreover, two EOs (*C. aromaticus* and *T. vulgaris)* were selected and tested against *B. thermosphacta* and mesophilic aerobic bacteria (MAB) in fresh pork samples packed under modified atmosphere, to evaluate the possible impact on shelf life extension.

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Materials and Methods

Essential oils

The EOs used for this study were purchased from two Italian companies: Giardino Officinale [Propezzano (TE), Italy] and Zuccari srl (Trento, Italy). Details on the seven EOs are shown in Table 1. EOs were diluted by using sterile phosphate buffered saline (PBS) 10 mM pH 7.4, and Tween 80 (1%) to obtain emulsions with an initial concentration of 40 mg/mL (V/V).

Brochothrix thermosphacta strains

Five strains of *B. thermosphacta* were employed to test the antimicrobial activity of the EOs listed above. Four strains were isolated from meats, and one type strain was purchased from the American Type Culture Collection (Teddington, UK; ATCC 11059). The strains were stored at -80°C in Brain Heart Infusion (BHI; Oxoid Thermofisher, Basingstoke, UK) and glycerol (20% V/V; Sigma Aldrich, St. Louis, MO, USA) to protect bacterial cells from low temperatures damage. Before each test, the bacterial strains were

cultivated overnight at 30°C in BHI Agar (Oxoid Thermofisher), then a single colony of *B. thermosphacta* was inoculated into 1 mL of BHI broth, and incubated at 30°C for 18 h. The cells obtained were centrifuged at 13,000 rpm for 5 min (Eppendorf centrifuge 5415D; Hauppauge, NY, USA), and the pellet was washed three times with PBS 10 mM, pH 7.4. The inocula were standardised at about 10^6 colony forming unit (CFU)/mL, by measuring the absorbance at 600 nm (UV-VIS, Jenway 6305 spectrophotometer; Bibby Scientific Ltd, Stone, UK), and were systematically verified by plate count, by using the selective medium Streptomycin Thallous Acetate Actidione (STAA; Oxoid Thermofisher), with readings after 24 h of incubation at 30°C.

Preparation of fresh pork samples

The experiment was conducted on Italian fresh pork samples collected from *Longissimus dorsi* between the third vertebra and the tailbone. The muscular portions, previously cut into slices, were dissected into pieces with an average weight of 25 g.

Determination of minimal inhibitory concentration

Seven EOs were used to determine the minimum inhibitory concentration (MIC), which is the lowest concentration required to inhibit the growth of the five strains of *B. thermosphacta* after 48 h of incubation at 30°C. MIC was evaluated by applying the microdilution method (CLSI, 2011) on microtitre plates (Oxoid Thermofisher). In each plate a positive control, consisting of 100 µL of inoculum and 100 µL of BHI broth (Oxoid Thermofisher), was added to ascertain the vitality of the strain employed, and a negative control, consisting of 100 µL of BHI broth, was used to ensure sterility of the culture medium.

Fresh pork samples dipped in essential oils emulsions

Each meat sample was dipped in EOs emulsions (20 and 40 mg/mL of cloves or red thyme, respectively) for 60 sec, and was left to dry in sterile Petri dishes under the flow of a microbiological cabinet for about 1 h. Negative controls (non-treated with EOs) were also prepared. Dry samples were placed on polystyrene trays (Aerpack B5, 230×145×26 mm; Coopbox Group Spa, Reggio Emilia, Italy), packed under MAP (70% O, 20% CO_2, 10% N_2) in a heat-sealed PET12/EVOH/PE50 top film (CXTop 64 µ AntiFog; Coopbox Group Spa) and stored at 4°C for subsequent analysis. The amount of EOs employed (20 and 40 mg/mL) refers to the emulsions applied exclusively on the surface of the pork treated; so, the actual amount of EOs referred to the weight of the samples is markedly lower than that applied by dipping.

Microbiological analysis and determination of *Brochothrix thermosphacta* count

Microbiological analyses were performed up to day 13 of storage at times T0, T2, T4, T6, T8, T10, and T13, to determine the count of MAB and *B. thermosphacta* in the treated samples. MAB were enumerated on Plate Count Agar, and *B. thermosphacta* on STAA medium (Oxoid Thermofisher), after incubation at 30°C for 48 h. Microbiological analyses were conducted for 13 days (three days after the expiry date recommended by the manufacturer of pork stored at same conditions in MAP) to detect the possible extension of product shelf life. The MAB count was carried out to obtain information useful to judge the samples deterioration and to assess the products shelf life.

Sensory analysis

The *triangle* test (ISO 4120:2004; ISO, 2004) has been administered to the panelists. This type of test is a procedure for determining whether a perceptible sensory difference or similarity exists between two products. For each taster three samples (coded anonymously), two of which identical and one different, were presented. The three samples were identified by random three digits code and were contemporarily and randomly presented to each judge in plastic dishes. Presentation was random to balance the test and to avoid the *alone* effect. Each taster was asked to identify the different sample. The taste tests were repeated twice in the same day. Pork samples were evaluated at three storage times (T0, T6, T10) in order to detect differences in the judges' EOs perception.

Pork samples, treated with 4% emulsions of red thyme and cloves EOs, together with control samples, were subjected to sensory evaluation by a group of ten panelists of different age and sex. The samples were sectioned into cubes of about 2×2×2 cm and cooked through an electric oven (Air-o-Steam COMBI 6GN 1/1; Electrolux, Stockholm, Sweden) set to 140°C. Pork cubes were cooked for 7 min, reaching 70°C at the product core (temperature control was performed by a thermocouple Velp Scientific VTF digital thermoregulator; Velp, Usmate, Italy).

The aim of the *triangle* test was to verify the tasters' recognition of the samples treated with EOs compared to untreated ones. In addition, a comment section was included for the assessor's remark and the judges were asked to indicate the samples with the most intense aroma.

Statistical analysis

Analyses were run in triplicate. Microbiological counts were converted to Log CFU/g and were subjected to analysis of variance (ANOVA) with a confidence interval of 95%. Means and standard deviations were calculated. Differences among means were evaluated by Tukey test, with significance defined at P=0.05.

Results

Determination of *in vitro* antimicrobial activity of essential oils against *Brochothrix thermosphacta*

Minimal inhibitory concentration values were recorded after 48 h of incubation at 30°C and results are shown in Table 2. The EOs that showed the highest antimicrobial activity *in vitro* were, in order: cloves (MIC 0.6-10.0 mg/mL), savory (MIC 2.5-20.0 mg/mL), and red thyme (MIC 2.5-20.0 mg/mL). Among the effective EOs, the most and the least effective (respectively clove and red thyme) were selected for further trials, and their

Table 1. Description of the seven essential oils studied: plant species, common name, distilled part, providing companies.

Plant species	Common name	Distilled part	Company
Caryophyllus aromaticus	Cloves	Flower	ZU
Mentha piperita var. Mitcham	Peppermint	Leaf	GO
Origanum vulgare	Oregano	Leaf	GO
Rosmarinus officinalis	Rosemary	Leaf	GO
Salvia officinalis	Sage	Leaf	GO
Satureja montana	Winter savory	Flowered plant	GO
Thymus vulgaris	Red thyme	Leaf	ZU

GO, Giardino Officinale [Propezzano (TE), Italy]; ZU, Zuccari srl (Trento, Italy).

concentration was increased at 20 and 40 mg/mL to consider the matrix effect that decreases the availability of bioactive compounds.

Determination of *Brochothrix thermosphacta* and mesophilic aerobic bacteria growth in pork samples treated with essential oils

Counts of *B. thermosphacta* in pork samples are shown in Figure 1. At T0, red thyme EO at both concentrations and 40 mg/mL of cloves EO slightly reduced the count compared to the untreated control. During the storage (13 days at 4°C), red thyme EO controlled the development of *B. thermosphacta*, at both concentrations of 20 and 40 mg/mL. Instead, cloves EO was not able to reduce *B. thermosphacta* growth; still, in some sampling times (T2 and T4) cell growth seemed even encouraged. The 40 mg/mL concentration of red thyme EO was the most effective, as it exerted a bacteriostatic effect on *Brochothrix* during the first six days of storage, and kept the counts significantly lower (P<0.05) than control until the end of storage. In particular, at T2, 3.76 Log CFU/g were detected for the untreated control, and 2.30 Log CFU/g for the samples treated with 40 mg/mL of red thyme EO (P<0.01); at T6, the count in 40 mg/mL of thyme EO treated samples was significantly (P<0.01) lower than the control (about 2.50 Log CFU/g). Until T13, the count values were closer, but still significantly different (P<0.01), with 6.86 Log CFU/g for the untreated control and 6.17 Log CFU/g for the sample treated with 40 mg/mL of red thyme EO. Thyme EO at concentration of 20 mg/mL limited *B. thermosphacta* growth with respect to control only up to T6.

The results for the mesophilic aerobic bacteria are summarised in Figure 2. At T0, there was an immediate and significant (P<0.01) decrease of the aerobic mesophiles in the samples treated with the EOs of red thyme and cloves (40 mg/mL), with 3.08 and 3.30 Log CFU/g respectively, compared to the control (3.72 log CFU/g). The bacteriostatic effect of the EOs was more evident at T2, when 20 and 40 mg/mL of thyme EO and 40 mg/mL of cloves EOs were significantly (P<0.01) effective in containing the growth. The growth reduction with respect to control was maintained until T6, when 40 mg/mL of red thyme EO had the best performance in reducing the count of about 1.6 Log CFU/g. The efficacy of cloves and red thyme EO at the lower concentration was progressively reduced over time up to T13, when only 20 and 40 mg/mL red thyme EOs were still able (P<0.01) to control microbial growth (6.03 and 6.18 Log CFU/g, respectively, with 6.93 Log CFU/g in the control).

Sensory analysis

In the *triangle* test (data not shown), the panelists recognised the different samples (treated with EOs) in the tasting evaluations (α=0.001). The questions, aimed at investigating the flavour perception in the meat sample, revealed that almost all the panelists identified the treated samples (with EOs) as more aromatic.

Discussion

In vitro assays revealed a greater effectiveness of cloves EO than red thyme, while *in situ,* red thyme EO showed a higher ability to control the development of *B. thermosphacta*. This result is not surprising as *in vitro* the medium variables are rather contained, whereas in a complex system such as meat, substrate composition influences the availability of bioactive compounds and the interaction with the microorganism. Moreover, MIC data obtained

Table 2. Minimum inhibitory concentration values (expressed as mg/mL) of seven essential oils against five *Brochotrix thermosphacta* strains after 48 h of incubation at 30°C.

Essential oils	*Brochothrix thermophacta* strains				
	ATCC 11059	B1	B2	B3	B4
Caryophillus aromaticus	0.6	2.5	2.5	10.0	10.0
Mentha piperita Mitcham	2.5	ne	ne	ne	ne
Origanum vulgare	1.3	10.0	10.0	ne	ne
Rosmarinus officinalis	ne	ne	ne	ne	ne
Salvia officinalis	ne	ne	ne	ne	ne
Satureja montana	5.0	5.0	2.5	20.0	20.0
Thymus vulgaris	2.5	ne	20.0	10.0	10.0

ne, not effective at the tested concentrations. Standard deviation was not reported, as the tests repetitions showed no variability.

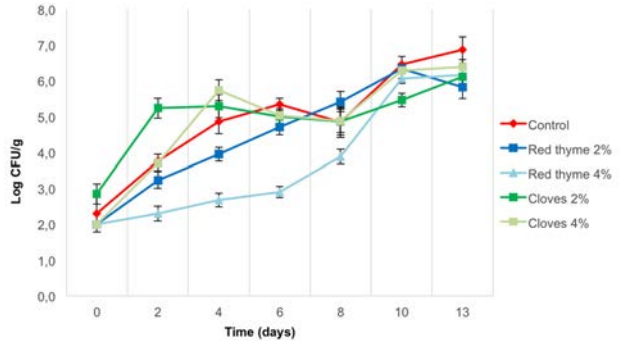

Figure 1. Counts of *Brochothrix thermosphacta* in pork samples under modified atmosphere during thirteen days of storage at 4°C.

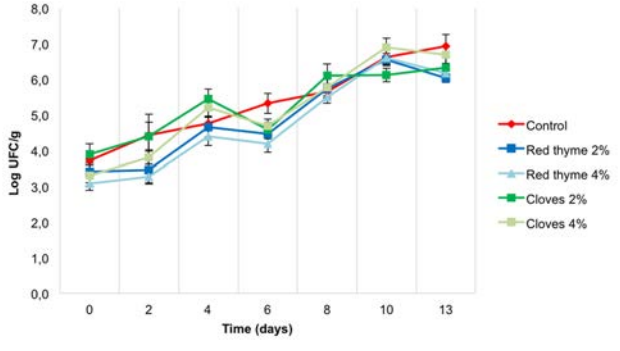

Figure 2. Counts of mesophilic aerobic bacteria in pork samples under modified atmosphere during thirteen days of storage at 4°C.

on specific strains do not necessarily correspond to the behaviour of the species.

Red thyme EO emulsion at 40 mg/mL, applied on the surface of meat samples, was able to contain the growth of mesophilic aerobic and *B. thermosphacta*. This effect is not properly bactericidal but bacteriostatic because it was likely due to a Lag phase extension, as a cell response to the stress caused by phytocomplexes. The compounds that characterise red thyme EO composition are borneol, carvacrol and thymol (Mazzarrino *et al*., 2015). They exert their antimicrobial activity against the cytoplasmic membrane, which is progressively damaged proportionally to the increase of employed EO concentration (Serio *et al*., 2010); furthermore, these compounds disrupt the membrane potential, and produce a loss of ions, adenosine triphosphates (ATP) and small molecules until the cell death (Ultee *et al*., 2000; Di Pasqua *et al*., 2007; Cristiani *et al*., 2007). Cloves EO, mainly composed of eugenol, acts differently compared with red thyme oil because it causes a non-specific membrane permealisation and inhibits the action of certain enzymes such as histidine decarboxylase, amylase, protease and ATPase (Hemaiswarya and Doble, 2009). The great differences in the chemical composition of the two EOs used on meat samples might explain the different degree of *B. thermosphacta* containment during 13 days of storage at 4°C.

Conclusions

In spite of its effectiveness, the amount of red thyme EO (MIC 40 mg/mL) is quite high to be applied on the surface of fresh pork without affecting the sensory profile of the product. However, the association of red thyme EO with other natural preservatives, such as chitosan or other protein-based films, could open up new perspectives for the shelf life extension of meat. The containment of a spoilage bacterium like *B. thermosphacta* through the use of EOs encourages the research on alternative methods for extending the shelf life of fresh meat stored under MAP.

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