

Antimicrobial activity of essential oils against *Staphylococcus aureus* in fresh sheep cheese

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Abstract

Essential oils (EOs) are aromatic oily liquids extracted from different parts of specific plants, well known especially for their aromatic and antibacterial properties. Nowadays, EOs are exploited in the food sector mainly for their aromatic properties. Thanks to their antimicrobial activity, however, they could also be used as additives to increase the safety and the shelf-life of food products. Aim of this study was to assess the antimicrobial activity of *Thymus vulgaris* L. oil and of *Origanum vulgare* L. oil against *Staphylococcus aureus* both *in vitro* and on fresh cheese, and to determine whether the use of EOs can modify the microbiological and/or chemical-physical properties of the products. The antimicrobial activity against *S. aureus in vitro* was assessed by preparation of the aromatogram (diffusion in agar test), minimum inhibitory concentration test and minimum bactericidal concentration assessment. Raw sheep milk was experimentally contaminated with a strain of *S. aureus* ATCC 25922 and was used to produce three types of fresh cheese: without EOs, with thyme and oregano EOs (both EOs at a concentration of 1:1000). The samples were analysed on the day of production, after three and seven days. The results obtained from the tests showed that the concentration of *S. aureus* and the counts of lactic flora remained unchanged for all types of cheese. Even the chemical-physical parameters were constant. The results of inhibition tests on the cheese disagree with those relating to the *in vitro* tests. Most likely this is due to the ability of EOs to disperse in the lipids the food: the higher the fat content is, the lower the oil fraction will be able to exert the antimicrobial activity.

Introduction

Food safety is a well-known topic all over the world and it involves everyday millions people who are affected by contaminated and/or poorly preserved food. The World Health Organization (WHO) defines this issue as one of the most widespread problems for the consumers' health and one of the main causes of economic losses for the companies (WHO, 2007). Nowadays consumers are very interested in the quality of food products and they are informed by the mass media on emergencies and on scandals related to food and on diseases attributable to them. Also the presence of some synthetic preservatives in the food worries consumers. As a matter of fact, the interest about the replacement of these synthetic substances with natural antimicrobial compounds, such as extracts and essential oils (EOs) which are effective and non-toxic, is increasing (FDA, 2011). EOs are aromatic oily liquids extracted from different parts of specific plants, well known from ancient times especially for their aromatic and antibacterial properties. So far, EOs have been exploited in the food sector mainly for their former feature, *i.e.* their aromatic properties. Thanks to their antimicrobial activity, however, they could also be used as additives in order to increase the safety and shelf-life of food products. This perspective looks tempting, when compared to side effects, such as migraine and allergies caused by synthetic additives (EFSA, 2014).

The multiple components of EOs play an important role in determining their antimicrobial activity (Burt, 2004). An important feature of the components of EOs is their hydrophobicity which allows them to enter the cell membrane of the bacteria causing structural and functional damages as the leakage of ions and molecules, provoking their death (Burt and Reinders, 2003; Burt, 2004; Goni *et al.*, 2009). EOs are antimicrobial which do not cause phenomena of resistance thanks to the multiple mechanisms of action. They have a level of security published by the US Food and Drug Administration (FDA, 2011). This means that they are generally recognised as safe for the consumer without limitations. In order to allow the use of EOs as additives, it is necessary to ascertain that the organoleptic properties of the food products are unchanged or even improved in order to preserve the food characteristics.

Aim of this study was to assess the antimicrobial activity of *Thymus vulgaris* L. oil and of *Origanum vulgare* L. oil against *Staphylococcus aureus*. The study was performed both *in vitro* and on fresh sheep cheese, in order to check whether the use of these EOs can modify the chemical-physical properties of the products.

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Materials and Methods

In vitro antimicrobial tests

The antimicrobial activity against *S. aureus* was assessed *in vitro* by preparation of the aromatogram (diffusion in agar test), minimum inhibitory concentration (MIC) test and minimum bactericidal concentration (MBC) assessment.

Aromatogram (agar diffusion test)

The aromatogram is a qualitative method to test the antimicrobial activity of a substance against a particular microorganism. This method has been prepared using essential oils of *Thymus vulgaris* L. and *Origanum vulgare* L. The strain used was *S. aureus* ATCC 25922. Little sterilised disks of blotting paper saturated with 10 µL of these EOs were placed on the surface of a Müeller Hinton plate count agar previously spreaded with *Staphylococcus aureus* inoculum. After a latency period at 37°C±1 for 24 h, the diameter of the inhibition halo against the strain of the *S. aureus* was measured with a caliber (Tajkarimi *et al.*, 2010).

Minimum inhibitory concentration

MIC is a quantitative test that exploits the dilutions techniques. This test was carried out in two phases: the first phase involved the preparation of the bacterial suspension (inoculum) with the strain of *S. aureus* and later the determination of the MIC. This procedure was performed for *Thymus vulgaris* L. and for *Origanum vulgare* L. EOs. The inoculum was prepared with a series of dilutions in

saline solution starting from a known concentration (0.5 McFarland) until a suspension of *S. aureus* equal to 10^3 CFU/mL was reached; the concentration was confirmed with seeding by inclusion on Baird-Parker plates of RPF agar (BP RPF). For the determination of MIC nine tubes were filled with Mueller Hinton Broth (MHB) as liquid broth, Tween 80 as surfactant, in order to have different concentrations of tested OEs the dilutions method was used (32, 16, 8, 4, 2, 1, 0.5, 0.25, 0.125 μ L/mL) to obtain a final volume of 2 mL. Then, in each tube 100 μ L of inoculum of *S. aureus* previously prepared were poured. A negative control was also set up, consisting of a tube containing only the broth (MHB), Tween 80 and 100 μ L of inoculum. After a latency period at $37^\circ\text{C}\pm 1$ for 24 h the turbidity was checked in order to assess the lowest concentration of the tested essential oil, and its capability of inhibiting the development of *S. aureus*.

Minimum bactericidal concentration

After the MIC test, 100 μ L of solution were taken from each of the test tubes in which turbidity was absent. From both *Thymus vulgaris* L. and *Origanum vulgare* L. 100 μ L of suspension were taken from the tubes with 4, 8, 16 and 32 μ L/mL concentration, for planting on Blood Agar plates. Subsequently, after a latency period at $37^\circ\text{C}\pm 1$ for 24 h the plates were examined to determine the lowest concentration (μ L/mL) of EOs which could eliminate 100% of the *S. aureus*.

Antimicrobial tests on matrix

For the antimicrobial tests on matrix raw milk sheep was used. The milk was initially checked for the presence of coagulase-positive staphylococci, as required by ISO 6888-2:1999 (ISO, 1999) and the enumeration of mesophilic lactic acid bacteria (LAB) was performed as required by ISO 15214:1998 (ISO, 1998). The presence of *S. aureus* was < 1 cfu/mL, while the concentration of LAB was 1.7×10^4 CFU/mL. The identification of LAB was performed using miniaturised biochemical tests (API 50CHL Biomerieux®; bioMérieux, Marcy l'Etoile, France) and isolated colonies were confirmed by biomolecular method (PCR). The DNA was extracted from bacterial colonies by boiling and later subjected to PCR analysis using the primers and reaction conditions reported in literature (Fortina *et al.*, 2003). The two methods have provided similar results: all colonies examined were found to be belonging to the species *Lactobacillus paracasei*. The experimental contamination with *S. aureus* was performed inoculating 5 mL bacterial suspension (about $\times 10^5$ CFU/mL) in 5 L of raw milk. The final concentration for *S. aureus* was verified by seeding by inclusion on BP-RPF and it was equal to 1.2×10^2 CFU/mL. The contaminated milk was used to produce three different types of fresh cheese: white (without

addition of EOs), thymus (with addition of thymus EOs in a concentration equal to 1:1000) and finally a type of cheese oregano (with addition of oregano EOs in a concentration equal to 1:1000). The cheeses were analysed in three stages: on the date of production (T0), after 3 days (T1) and after 7 days (T2) from the date of production. The cheeses obtained were analysed for *S. aureus* and LAB. The determination of fat in dry matter (fat DM) was also carried out, together with that of fat as it is (fat II), protein, humidity and non-fat dry (NFD) through technology near infrared reflectance spectroscopy (NIRS) by Foodscan (Foss-Electric), as required by ISO 21543/IDF 201:2006 (ISO, 2006).

Results and Discussion

From the tests carried out *in vitro*, the EOs in the study were shown to possess respectively an MIC of 4 μ L/mL and an MBC of 8 μ L/mL. Through the aromagram, halos of inhibition equal to 18 mm for the two EOs were obtained. The results of tests on matrix have not shown substantial differences in the concentration of *S. aureus* and LAB both in white and in the types thymus and oregano in the three times considered (Tables 1 and 2). In Table 3 the results of the determinations of fat DM, fat II, protein, humidity and NFD of all three types of cheese are shown; essentially there were no

significant differences.

Conclusions

Through the three tests performed *in vitro* the actual antimicrobial power against the *S. aureus* of the EOs of thymus and oregano has been proven. From the results obtained from the tests of inhibition on matrix it is however clear that the concentration of *S. aureus* remains almost unchanged at the three times of analysis for all types of cheeses produced (Table 1). Such a situation occurs also in general for the enumeration of LAB in which we note only an increase in the cheese produced without the addition of EOs at T2 (Table 2). The chemical-physical parameters as well remained largely constant (Table 3). As reported in literature, the results of the inhibition tests on different matrices disagree with those relating to the tests *in vitro* (Burt, 2004). Using EOs at higher concentrations in food than in laboratory media could be due to the more complex growth environment in food: microbial cells have a greater protection from antimicrobial agents. The fat in food could form a protective coat around bacteria, as well as against antimicrobial agents. Furthermore, the transfer of EOs to the active site in bacteria could be hampered due to the reduced water content in food compared to laboratory media (Smith-Palmer *et al.*, 2004).

Table 1. *S. aureus* concentration in the three types of cheese at different times.

Cheese type	T0 (ufc/g)	T1 (ufc/g)	T2 (ufc/g)
White	2.4×10^3	1.6×10^3	1.4×10^3
Thymus	1.1×10^3	2.7×10^3	2.3×10^3
Oregano	1.2×10^3	2.2×10^3	1.9×10^3

T0, date of production; T1, after 3 days from the date of production; T2, after 7 days from the date of production.

Table 2. Lactic acid bacteria concentration in the three types of cheese at different times.

Cheese type	T0 (ufc/g)	T1 (ufc/g)	T2 (ufc/g)
White	2.6×10^5	6.9×10^5	5.6×10^6
Thymus	1.0×10^5	7.5×10^5	9.5×10^5
Oregano	2.1×10^5	2.4×10^5	8.8×10^5

T0, date of production; T1, after 3 days from the date of production; T2, after 7 days from the date of production.

Table 3. Average values of the physical parameters of the three types of cheese.

Cheese type	Fat DM (%)	Fat II (%)	Protein (%)	Humidity (%)	NFD (%)
White	46.2 ± 0.96	19.8 ± 1.56	18.3 ± 1.37	56.6 ± 3.07	23.1 ± 0.92
Thymus	47.2 ± 0.02	20.9 ± 1.22	19.3 ± 1.61	55 ± 3.41	23.5 ± 1.49
Oregano	47.5 ± 0.68	21 ± 0.99	19.1 ± 1.04	55 ± 2.57	23.3 ± 0.55

DM, dry matter; fat II, fat as it is; NFD, non-fat dry.

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