Antimicrobial activity of four essential oils against pigmenting *Pseudomonas fluorescens* and biofilm-producing *Staphylococcus aureus* of dairy origin

Francesca Pedonese,[1] Filippo Fratini,[1] Luisa Pistelli,[2] Federica Maria Porta,[1] Pierluigi Di Ciccio,[3] Roberto Fischetti,[4] Barbara Turchi,[1] Roberta Nuvoloni[1]

[1]Department of Veterinary Sciences, University of Pisa, Pisa; [2]Department of Pharmacy, University of Pisa, Pisa; [3]Food and Drug Department, University of Parma, Parma; [4]Institute for Experimental Veterinary Medicine of Lazio and Tuscany, Pisa, Italy

Abstract

Essential oils (EOs) are mixtures of secondary metabolites of plant origin with many useful properties, among which the antimicrobial activity is also of interest for the food industry. EOs can exert their antimicrobial potential both directly, in food products and active packaging, and indirectly, as sanitizing and anti-biofilm agents of food facility surfaces. Aim of this research was to evaluate the antimicrobial activity of four EOs (bergamot, cinnamon, manuka and thyme) against *Pseudomonas fluorescens* and *Staphylococcus aureus* isolated from milk and dairy products. The chemical composition of EOs was evaluated by Gas Chromatography-Mass Spectrometry analysis. Minimum Inhibitory Concentration values were determined by a microplate method against 9 *Ps. fluorescens* from marketed mozzarella with blue discoloration defect, and 3 biofilm-producing *S. aureus* from milk. Reference ATCC strains were included. Pigment production activity by *Ps. fluorescens* was assessed both in culture and in cheese. EOs of manuka (leptospermone 23%) and thyme (carvacrol 30%, p-cymene 20%, thymol 15%) showed the highest antimicrobial activity against *S. aureus*, MIC values were 0.012%-0.024% and 0.024% v/v, respectively; meanwhile EOs from thyme and cinnamon (cinnamaldehyde 55%) exhibited the best activity against *Ps. fluorescens* with MIC values of 0.098%-0.195% and 0.195%-0.391% v/v, respectively. The antimicrobial activity of these EOs is promising and they could be exploited in the dairy production chain.

Introduction

Essential oils (EOs) are aromatic and volatile mixtures of secondary metabolites obtained from different parts of plants. EOs are composed of many bioactive molecules, mainly terpenes, terpenoids and phenylpropenes, and their actual antimicrobial effect depends on many intrinsic and environmental factors (Hyldgaard *et al.*, 2012). Their use in food industry is aimed to provide flavours and to improve safety and quality of products. They can be employed directly in food, or as components of food packaging (Lucera *et al.*, 2012) and even in sanification of food processing environments and food-contact surfaces due to their antimicrobial and anti-biofilm potential (Valeriano *et al.*, 2012).

The present work was aimed to evaluate the antimicrobial activities of four EOs (*Citrus bergamia* Risso, *Cinnamomum zeylanicum* L., *Leptospermum scoparium* J.R. *et* G. Forst and *Thymus vulgaris* L.) on food-related bacteria: *Pseudomonas fluorescens*, isolated from mozzarella cheese with blue discoloration, and biofilm-producing *Staphylococcus aureus* from milk. Gas chromatography–mass spectrometry (GC-MS) analyses were carried out to perform the chemical characterization of the four tested EOs.

Materials and Methods

Bacterial cultures

A total of 10 *Ps. fluorescens* was used in this study: 9 wild isolates (PS59, PS60, PS63, PS70, PS71, PS231, PS249, PS251 and PS282) from commercial samples of spoiled mozzarella cheeses with blue pigmentation and the reference strain *Ps. fluorescens* ATCC 13525. The wild isolates, provided by Istituto Zooprofilattico Sperimentale Lazio-Toscana (Pisa), were phenotypically identified by API 20 NE (bioMérieux, Marcy-l'Etoile, France). Moreover, the identification was confirmed genetically according to Scarpellini *et al.* (2004). After DNA extraction by boiling at 95°C for 10 min, the species-specific PCR was performed using the KAPA Taq ReadyMix PCR kit (Kapa Biosystems, Boston, MA, USA).

The pigmentation capacity of pseudomonads was studied in mozzarella cheese and in a lab-made minimal nutritive broth used for the cultivation and maintenance of *Pseudomonas fluorescens* and other microorganisms (Yeast Extract Glucose Broth, YEGB, https://www.bioworld.com/productinfo/3_43_287_688/10174/Yeast-Extract-Glucose-Broth.html). YEGB had the following composition: glucose 1.5 g/L, di-potassium hydrogen orthophosphate 5.2 g/L, potassium dihydrogen phosphate 3.18 g/L, magnesium sulfate 0.12 g/L, yeast extract 0.5 g/L, ammonium chloride 0.54 g/L. Marketed citric mozzarella samples were inoculated with a final count of about 10^2 CFU/mL in preservation liquid. Samples were stored at 10°C for 72 hours in their packaging and then opened, maintained at the same temperature and observed daily up to 10 days. *Pseudomonas* quantitative determination on Pseudomonas Agar with CFC Supplement (Oxoid, Basingstoke, UK) was carried out to exclude the presence of pseudomonads in the preservation liquid. As for YEGB assay, each isolate wasgrown overnight at 25°C in YEGB, and ten-fold serial dilutions in sterile saline solution were prepared. Ten µL of the dilutions were seeded, in 5 different Eppendorf tubes per isolate, to obtain final counts of pseudomonads ranging from 10^1 to10^5 CFU/mL. Each tube, of 1.5 mL declared capacity, was filled to the top with 1.8 mL of YEGB to maximize the surface of broth culture exposed to air. Eppendorf tubes were left open, incubated at 25°C and observed daily up to 7 days.

Among biofilm-producing *S. aureus*, 3 strains, isolated from raw milk, came from the collection of Food and Drug Department, Parma (PA1 and PA2) and from that of Veterinary Science

Correspondence: Francesca Pedonese, Department of Veterinary Sciences, University of Pisa, Viale delle Piagge 2, 56124 Pisa, Italy.
Tel: +39-050-2216707 - Fax: +39-050-2210654.
E-mail: francesca.pedonese@unipi.it

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Department, Pisa (PI1) and their biofilm-producing capacity had been previously assessed following Di Ciccio *et al.* (2015), the other 2 were reference strains, *S. aureus* ATCC 35556 (strong producer) and *S. aureus* ATCC 12600 (intermediate producer). *S. aureus* PA1 and *S. aureus* PA2 had been previously characterized as methicillin-resistant (MRSA) (Di Ciccio *et al.*, 2016); *S. aureus* PI1 was a methicillin-susceptible, enterotoxin C producer (Pedonese *et al.*, 2014).

Essential oils

EOs (from Flora s.r.l., Lorenzana, Pisa, Italy) of bergamot (*C. bergamia* Risso, Cb), cinnamon bark (*C. zeylanicum* L., in 40% ethanol, Cz), manuka leaves (*L. scoparium* J. R. *et* G. Forst, Ls) and thymus (*T. vulgaris* L., Tv) were used.

Gas chromatography-mass spectrometry analysis

The chemical composition of EOs was determined by using Gas Chromatography-Electron Impact Mass Spectrometry (GC-MS). Analyses were performed with a Varian CP-3800 gas chromatograph equipped with a DB-5 capillary column (30 m × 0.25 mm; coating thickness 0.25 µm) and a Varian Saturn 2000 ion trap mass detector. Analytical conditions were those reported by Fratini *et al.* (2014). The identification of the constituents was based on comparison of retention times with those of pure authentic samples, comparing their retention indices relative (LRI) to the series of *n*-hydrocarbons, by computer matching against commercial libraries (NIST 98 and ADAMS) (Adams, 1995) and homemade library mass spectra, built up from pure substances, known oils and MS literature data (Stenhagen *et al.*, 1974).

Minimal inhibitory concentration determination assay

MIC values of EOs, *i.e.* the lowest concentration that inhibits the visible microbial growth, were determined following Wiegand *et al.* (2008) with minor modifications. The assay was performed in sterile 96-well microtiter plates using 10 µL of bacterial *inoculum* and 190 µL of each EOs dilution. EOs dilutions were prepared in Tryptone Soy Broth (Oxoid) supplemented with dimethyl sulfoxide (DMSO) to a final ratio of 1:3:4 (v/v) and a two-fold dilution series was performed from 1/8 to 1/16384. For the bacterial *inoculum* an overnight broth culture of each microorganism, spectrophotometrically (Ultrospec 2100 Pro, Amersham Biosciences, Buckinghamshire, GB) adjusted at 550 nm at about 1.5 x 10^8 CFU/mL, was used. Positive and negative controls, and for Cz also ethanol control,

Table 1. Chemical composition of essential oils detected by gas chromatography-mass spectrometry analysis.

Compound	Class	LRI	Cb	Cz	Ls	Tv
α-pinene	mh	940	1.1		1.7	1.1
Sabinene	mh	978	1.1			
β-pinene	mh	981	6			
Myrcene	mh	993				1.3
α-phellandrene	mh	1006		1.1		
α-terpinene	mh	1019				1.5
p-cymene	mh	1026				20.2
o-cymene	mh	1026		2.5		
Limonene	mh	1032	30.8			
β-phellandrene	mh	1033		4.4		
γ-terpinene	mh	1062	7.4			8.3
Linalool	om	1102	12.5	5		7.4
Camphor	om	1148				1
4-terpineol	om	1180				1.8
(Z)-cinnamaldehyde	nt	1223		1.2		
Linalool acetate	om	1260	34.9			
(E)-cinnamaldehyde	nt	1274		55		
Thymol	om	1290				15.3
Carvacrol	om	1301				29.7
α-cubebene	sh	1351			3.2	
Eugenol	pp	1361		4.6		
α-copaene	sh	1376		1.6	4.8	
α-gurjunene	sh	1410			1	
β-caryophyllene	sh	1418		10.2	2.4	4.8
Aromadendrene	sh	1441			1.8	
(E)-cinnamyl acetate	nt	1449		1.1		
cis-muurola-3,5-diene	sh	1450			5	
α-humulene	sh	1456		2.8		
trans-cadina-1(6),4-diene	sh	1470			3.7	
γ-muurolene	sh	1477			1.1	
β-selinene	sh	1485			4.8	
trans-muurola-4-(14),5-diene	sh	1494			1	
Viridiflorene	sh	1495			5.3	
trans-calamenene	sh	1522			15.2	
δ-cadinene	sh	1523			4.5	
α-calacorene	sh	1542			6.3	
Caryophyllene oxide	os	1582		1.4		
iso-leptospermone	os	1623			5.6	
Leptospermone	os	1631			22.9	
Cubenol	os	1647			1.1	
Benzylbenzoate	nt	1766		1.8		
Unknown			0.3	3.2	0	0.1
Mh			48.4	10.3	2.7	34.4
Om			49.2	6.2	0.2	58.1
Sh			1.8	14.6	64.9	6.5
Os			0	1.6	31.7	0.4
Nt			0.3	59.5	0.5	0.5
Pp			0	4.6	0	0
Total (unknown excluded)			99.7	96.8	100.0	99.9

LRI, linear retenction index; Cb, *Citrus bergamia*; Cz, *Cinnamomum zeylanicum*; Ls, *Leptospermum scoparium*; Tv, *Thymus vulgaris*; mh, monoterpene hydrocarbons; om, oxygenated monoterpenes; sh, sesquiterpenes hydrocarbons; os, oxygenated sesquiterpenes; nt, no terpene derivatives; pp, phenilpropanoids. Other compounds were detected at <1%. They were: for Cb, α-thujene, myrcene, p-cymene, (E)-β-ocimene, terpinolene, α-terpineol, 1-octanyl acetate, neral, geranial, α-terpinyl acetate, neryl acetate, geranyl acetate, trans- α-bergamotene, (E)-β-farnesene, β-bisabolene, bergaptene; for Cz, α-thujene, α-pinene, camphene, benzaldehyde, β-pinene, α-terpinene, p-mentha-2,4(8)-diene, 4-terpineol, α-terpineol, (Z)-ocimenone, humulene epoxide-II, tetradecanal; for Ls, β-pinene, myrcene, p-cymene, limonene, 1,8-cineole, γ-terpinene, isopentylisovalerate, methylbutanoate, cyclosativene, β-elemene, β-copaene, α-neoclovene, alloaromadendrene, α-amorphene, α-muurolene, α-bulnesene, (E,E)-α-farnesene, δ-amorphene, trans-γ-cadinene, cadina-1,4-diene (cubenene), flavesone, (E)-nerolidol, spathulenol, caryophyllene oxide, globulol, viridiflorol, valerianol; for Tv, α-thujene, camphene, β-pinene, 1-octen-3-ol, 3-octanone, α-phellandrene, cis-sabinene hydrate, terpinolene, borneol, p-cymen-8-ol, α-terpineol, cis-dihydrocarvone, verbenone, methylcarvacrol, linalool acetate, isobornyl acetate, carvacrol acetate, α-humulene, (E)-β-farnesene, γ-muurolene, viridiflorene, trans-γ-cadinene, δ-cadinene, caryophyllene oxide. All these compounds were considered for calculating the total percentage of each class of constituents.

were included. The microplates were incubated at 25°C (pseudomonads) and 37°C (staphylococci) for 24 hours avoiding evaporation. The assay was made in triplicate and the mode was determined.

Results

Confirmation of identification and pigment production by *Pseudomonas fluorescens*

All isolates were phenotypically identified as *Ps. fluorescens* with an API identification percentage range of 99.7%-99.9% and a T index range of 0.87-0.97. Species-specific PCR results confirmed that all the isolates belonged to the species *Ps. fluorescens*. They were able to produce blue pigment in mozzarella cheese and in its preservation liquid, beginning from 2 days after the package opening, and more abundantly at the end of the trial. The pigmentation production was evident for all isolates except for PS63 and PS282. The lack of pigmentation was confirmed for these 2 isolates also in YEGB. All the other isolates showed a visible pigmentation at all dilutions with YEGB method beginning from 48 hours of incubation. The isolation of no-pigment producing *Ps. fluorescens* from *blue* mozzarella cheeses indicates that cheese samples were contaminated with different strains.

Essential oils composition

GC-MS profile revealed the presence of 7, 13, 18 and 11 different compounds with a percentage higher than 1% for Cb, Cz, Ls and Tv, respectively (Table 1). The most representative compounds mainly included in monoterpenes and sesquiterpenes were linalool acetate (34.9%) and limonene (30.8%) for Cb, leptospermone (22.9%) and trans-calamenene (15.2%) for Ls and carvacrol (29.7%), p-cymene (20.2%) and thymol (15.3%) for Tv. Cz showed the non-terpene derivative (E)-cinnamaldehyde (55%) together with the sesquiterpene β-caryophyllene (10.2%), as main compounds.

Determination of minimal inhibitory concentration against *Pseudomonas fluorescens* and *Staphylococcus aureus*

Overall, the tested biofilm-producing *S. aureus* showed to be more sensitive than *Ps. fluorescens*. Particularly, as shown in Table 2, very promising MIC values were obtained for Ls (0.012% v/v for 4 strains out of 5, among which the MRSA, and 0.024% v/v for the remaining one), which resulted even better than Tv (0.024% v/v). Cz evidenced an intermediate effect (0.049%-0.098% v/v), while Cb was poorly effective (≥ 0.781% v/v). *Ps. fluorescens* growth was not affected by Cb and Ls, which were active only at high concentrations. Also Tv and Cz showed higher MIC values than those obtained for *S. aureus* (0.098%-0.195% and 0.195%-0.391% v/v, respectively). Ethanol solvent had negligible effect on the antimicrobial activity of Cz EO (MIC: 3.125% v/v).

Discussion

The tested microorganisms, belonging to a pathogenic, *S. aureus*, and a spoilage species, *Ps. fluorescens*, have been chosen for their relevance for dairy industry. *S. aureus* is one of the most frequent pathogens associated with milk and dairy outbreaks (De Buyser *et al*., 2001). In recent years biofilm-producing and methicillin-resistant *S. aureus*, such as the wild ones tested in this study, have been recovered from food of animal origin, in which milk and dairy products (Parisi *et al.*, 2016; Basanisi *et al.*, 2017). Thus, the role of food-producing animals and food products as vectors of *S. aureus*, MRSA included, in the food chain is of great concern. Pseudomonads are involved in the spoilage of many chilled food of animal origin; in milk and dairy products they are responsible for off-odours, off-flavours, decreased cheese yields, discolorations (Arslan *et al.*, 2011). The species *Ps. fluorescens* is the main causative agent of blue discoloration of fresh cheeses (Martin *et al.*, 2011) and of mozzarella cheese, in particular (Cenci Goga *et al.*, 2014). In this study, *Ps. fluorescens* isolated from blue mozzarella cheese were studied. Concerning the pigment production, the use of YEGB, which is also available on the market for bacterial cultivation and maintenance purposes, could be a cheap and simple tool for food industry to monitor the presence of pigmenting pseudomonads, either in the production environments or in products, even if the test requires previous isolation of microorganisms.

Antimicrobial activity of EOs has been documented since the mid-twentieth century (Burt, 2004). Many studies have focused on the antimicrobial properties of different EOs against *S. aureus*, comprising MRSA. Particularly thyme and cinnamon and their major constituents often showed interesting results. However the tested bacterial strains were generally reference strains or clinical isolates (Smith-Palmer *et al.*, 1998; Jia *et al.*, 2011; Boskovic *et al.*, 2015; Reyes-Jurado *et al.*, 2016). Regarding our study, we evaluated the antimicrobial activity of four EOs (bergamot, cinnamon, manuka and thyme) against wild food strains, isolated from milk and dairy products, of *Ps. fluorescens* and biofilm-producing *S. aureus*, MRSA included, in addition to reference strains. As to manuka, the antimicrobial properties of its honey have been studied

Table 2. Minimal inhibitory concentration of four essential oils against the tested *Pseudomonas fluorescens* and *Staphylococcus aureus*.

	EOs MIC (% v/v)			
	Cb	Cz	Ls	Tv
Ps. fl. PS59	3.125	0.195	3.125	0.195
Ps. fl. PS60	3.125	0.195	3.125	0.195
Ps. fl. PS63	3.125	0.391	3.125	0.195
Ps. fl. PS70	3.125	0.391	3.125	0.098
Ps. fl. PS71	3.125	0.391	3.125	0.195
Ps. fl. PS231	3.125	0.391	3.125	0.195
Ps. fl. PS249	3.125	0.391	3.125	0.098
Ps. fl. PS251	3.125	0.391	3.125	0.098
Ps. fl. PS282	3.125	0.391	3.125	0.195
Ps. fl. ATCC 13525	3.125	0.391	3.125	0.098
S.a. PA1	0.781	0.049	0.012	0.024
S a. PA2	1.563	0.049	0.012	0.024
S.a. PI1	1.563	0.049	0.012	0.024
S.a. ATCC 35556	1.563	0.098	0.024	0.024
S.a. ATCC 12600	1.563	0.049	0.012	0.024

Eos, essential oils; Cb, *Citrus bergamia;* Cz, *Cinnamomum zeylanicum*; Ls, *Leptospermum scoparium*; Tv, *Thymus vulgaris*; S.a., *Staphylococcus aureus*; Ps.fl., *Pseudomonas fluorescens*. Results are the mode of three independent trials.

more than that of its EO (Mandal and Mandal, 2011). Generally, in our study we observed that the EO resulted to be much more active on Gram positive bacteria than on Gram negative, as reported also by Maddock-Jennings *et al.* (2005); in addition, van Klink *et al.* (2005), documented in particular the remarkable antimicrobial effect of EO major compounds, β-trichetones, on MRSA. Moreover, we recently found in another study (Fratini *et al.*, 2017) very interesting MIC results for manuka EO, comparable to those obtained with our biofilm-forming *S. aureus*, on different *S. aureus* strains from milk (methicillin-susceptible and not biofilm-producing strains). Data about bergamot EO are scanty: a study performed by Fisher and Phillips (2006) using different Gram positive and negative microorganisms as target bacteria showed that Cb EO was more effective against the Gram-positive bacteria. *S. aureus* was less affected, if compared with *Listeria monocytogenes* and *Bacillus cereus*. More recently, Marotta *et al.* (2016) evidenced a strain-dependent activity of bergamot EO on *L. monocytogenes* from food samples. In our research Cb EO was effective on *S. aureus* only at high concentrations.

Overall, it is known that Gram-negative bacteria are more resistant to the EOs effect, due to the hydrophilic lipopolysaccharides contained in the outer membrane, which create a barrier against EOs hydrophobic antimicrobial compounds (Hyldgaard *et al.*, 2012). Generally, *Ps. fluorescens* sensitivity to EOs has been poorly investigated. Outtara *et al.* (1997) reported that cinnamon EO was more active than thyme EO on *Ps fluorescens* isolated from beef, whereas we found a higher activity of Tv EO. Also, we found MIC values of about 1-2 µL/mL and 2-4 µL/mL, for Tv and Cz EOs respectively, against *Ps. fluorescens* ATCC 13525. Similarly, Mith *et al.* (2014) found that *Ps. fluorescens* ATCC 13525 was more resistant than other Gram positive and Gram-negative reference strains to the different EOs tested and among which cinnamon and thyme, with MICs ranging from 1 to 1.5 µL/mL.

Conclusions

The need for natural alternatives to synthetic chemicals is increasing in food industry, as well as the consumers request for natural products. Our study provides interesting data about EOs sensitivity of food-related wild microorganisms, which may be useful in implementing new solutions for sanification of food facilities surfaces and/or for direct use in food and packaging, particularly in the dairy production chain.

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