Antimicrobial and anti-biofilm activity of manuka essential oil against *Listeria monocytogenes* and *Staphylococcus aureus* of food origin

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Abstract

The activity of manuka (*Leptospermum scoparium*) essential oil (EO) on biofilms of foodborne *Listeria monocytogenes* and *Staphylococcus aureus* has been studied. Seven strains of *L. monocytogenes* and 7 of *S. aureus* (5 methicillin-resistant) were tested. EO minimal inhibitory concentration (MIC), EO minimal bactericidal concentration (MBC) and biofilm production quantification were determined for each strain by microtiter methods. Moreover, EO Minimum Biofilm Inhibitory Concentration (MBIC) and Minimum Biofilm Eradicating Concentration (MBEC) were determined on 2 *L. monocytogenes* and 3 *S. aureus* that showed the best biofilm production. Finally, on 4 strains out of 5 (2 *L. monocytogenes* and 2 *S. aureus*) EO Biofilm Reduction Percentage (BRP) *vs*. untreated controls was assessed after a treatment with EO sub-inhibitory concentrations. The chemical composition of manuka essential oil was determined by Gas Chromatography-Electron Impact Mass Spectrometry (GC-EIMS). The manuka EO demonstrated good antimicrobial activity: *L. monocytogenes* MIC and MBC were 0.466 mg/ml and 0.933 mg/ml, respectively, whereas *S. aureus* MIC and MBC were 0.233 mg/ml and 0.466 mg/ml, respectively. Furthermore, *L. monocytogenes* showed a MBIC of 0.933 mg/ml and a MBEC in the range of 0.933–1.865 mg/ml, whereas *S. aureus* had a MBIC in the range of 7.461–14.922 mg/ml and a MBEC of 14.922 mg/ml. *L. monocytogenes* revealed no significant BRP after the treatment with manuka EO, whereas *S. aureus* showed a BRP higher than 50% with MIC/2 and MIC/4 EO concentrations. These results provide information for feasible manuka EO applications in food production systems.

Introduction

Biofilms are sessile microbial communities, where microorganisms are attached to surfaces and embedded in an extracellular matrix (Dufour *et al.,* 2012). Biofilm formation improves bacterial persistence and proliferation both in biotic and abiotic habitats. Particularly, in food-processing environments, biofilm may facilitate the spread of spoilage and pathogenic microorganisms, causing contamination of raw materials and food products. This can result in a compromised quality and an increased risk for consumers' health (Vázquez-Sánchez and Rodríguez-López, 2018).

Listeria monocytogenes and *Staphylococcus aureus*, two of the major food-borne pathogens, are biofilm producers (Djordjevic *et al*., 2002; O'Gara, 2007; Mann *et al.,* 2009; Da Silva and De Martinis, 2012). *S. aureus* is well known for being a good biofilm producer (Vázquez-Sánchez and Rodríguez-López, 2018). *L. monocytogenes* is responsible for an important world-wide disease and is usually considered a weak monolayered biofilm producer, although it is known for its capacity to adhere to surfaces and persist in food-processing environments (Takahashi *et al.,* 2009; Oxaran *et al*., 2018).

In food production environments, biofilm formation is usually contrasted with chemical compounds. An improved resistance to common disinfectants is a characteristic feature of microbial biofilms (Chorianopoulos *et al*., 2008). Biofilm resistance is primarily due to the production of extracellular polymeric substances (EPS). Secondarily, antimicrobial substances are also inactivated by physico-chemical interactions and degraded by bacterial extracellular enzymes (Bridier *et al*., 2011). It is important, therefore, to identify active compounds which are successful in contrasting biofilm formation and safe to use in a food production environment.

Manuka (*Leptospermum scoparium*) is a shrub of the Myrtaceae family, original of Australia and New Zealand, but cultivated worldwide, mainly for honey production. Several studies have investigated the antimicrobial properties of manuka essential oil (EO), showing its effectiveness on *L. monocytogenes* and, more often, on *S. aureus* (Harkenthal *et al*., 1999; Lis-Balchin, 2000; Van Klink *et al.,* 2005; Fratini *et al.,* 2019).

The aim of this study was to assess the effect of manuka EO on biofilm production by *L. monocytogenes* and *S. aureus* strains. Particularly, EO minimum inhibitory concentration (MIC), EO minimum bactericidal concentration (MBC) and biofilm production capacity were preliminarily assessed. Subsequently, EO minimum biofilm inhibitory and eradication concentrations (MBIC and MBEC, respectively) and the percentage of biofilm reduction in comparison to that of the corresponding untreated controls were determined on mature biofilms of the tested strains.

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Key words: Manuka essential oil; Biofilm; Listeria monocytogenes; Staphylococcus aureus; Gas Chromatography - Electron Impact Mass Spectrometry.

Aknowledgements: The authors wish to thank Prof. Adriana Ianieri (Food and Drug Department, University of Parma) and Dr. Roberto Fischetti (Experimental Zooprophylactic Institute Lazio-Toscana) for kindly providing the tested bacterial strains.

Contributions: The authors contributed equally.

Conflict of interest: The authors declare no potential conflict of interest.

Funding: this research was funded by Pisa University grants (Fondi di Ateneo).

Received for publication: 14 August 2021.
Accepted for publication: 14 January 2022.

Italian Journal of Food Safety 2022; 11:10039
doi:10.4081/ijfs.2022.10039

Materials and methods

Bacterial strains

A total of 7 *L. monocytogenes* and 7 *S. aureus* strains was tested. *L. monocytogenes* strains were all isolated from animal-based food products. Six *S. aureus* strains were isolated from milk, and 1 was a high biofilm producer reference strain. Five of the 7 *S. aureus* were methicillin-resistant strains. All strains, stored at -80°C, were revitalized subculturing them in Tryptone Soya Broth (Oxoid Ltd., Basingstoke, UK) with 0.6% yeast extract (TSBYE).

Manuka essential oil and its chemical composition

Manuka EO was provided by Flora® (Lorenzana, Pisa) and was extracted from manuka leaves by steam distillation. The EO chemical composition was carried out at the Department of Pharmacy (University of Pisa) by Gas Chromatography-Electron Impact Mass Spectrometry (GC-EIMS), according to Pistelli *et al.* (2017).

Manuka essential oil minimum inhibitory concentration and minimum bactericidal concentration determinations

For each strain, MIC and MBC were performed in TSBYE with 1.5% NaCl and 0.75% glucose ($TSBYE_{gluc+NaCl}$).

A stock dilution of manuka EO was prepared using dimethyl sulfoxide (DMSO) and $TSBYE_{gluc+NaCl}$ (1:3:4) and two-fold serial dilutions were then prepared up to 1:16384. The tests were carried out following Fratini et al. (2019). The MIC value for each strain was determined as the lowest EO concentration without a visible microbial growth.

To determine MBC values, a loopful was taken from the wells corresponding to EO concentrations equal or higher than the MIC, and seeded onto Triptone Soya Agar (TSA, Oxoid). After incubation, MBC value was determined as the lowest EO concentration with no colony growth. Final MIC and MBC were determined as the mode of 3 replicates.

Biofilm production quantification

Biofilm quantification was carried out as described by Stepanović *et al.* (2007), with some modifications. Specifically, after revitalization, strains were prepared in $TSBYE_{gluc+NaCl}$ to enhance the biofilm production (Stepanović *et al*., 2007; Pan *et al.,* 2010). Each bacterial suspension was prepared and spectrophotometrically adjusted to a cell density of approximately 1.5×10^8 CFU/ml. The suspensions were inoculated into the wells of a flat-bottomed cell culture-treated 96-well microtiter plate. For each strain, 200 µl were inoculated in 7 wells. For each plate, 3 wells were inoculated with 200 µl of $TSBYE_{gluc+NaCl}$, as a negative control.

After incubation at 37°C for 72 hours, to obtain a mature biofilm, biofilms were fixed at 60°C for 60 minutes, stained with 2% aqueous crystal violet solution for 20 minutes; after washing, the bound dye was resolubilized with 33% v/v acetic acid for 20 minutes. Afterwards, the optical density (OD) of the wells was measured at 492 nm, using a microtiter-plate reader (Multiskan FC Microplate Photometer, Thermo Fisher Scientific, Ratastie, Finland).

The OD of the negative controls was subtracted from the reading of the inoculated wells. All the trials were carried out in triplicate.

To classify the tested strains in 4 categories (no biofilm producer, weak, medium-strong, and strong biofilm producer), a cut-off value (ODc) and 2 other threshold values (2ODc, 3ODc) were determined as follows: ODc was arbitrarily determined considering the OD of the tested strain with the lowest average OD value among the two species plus 2 standard deviations; the following categories were then considered to classify each strain on the basis of its OD (ODs):

ODs ≤ ODc = no biofilm producer
ODc < ODs < 2ODc = weak biofilm producer
2ODc < ODs < 3ODc = medium-strong biofilm producer
ODs ≥ 3ODc = strong biofilm producer.

Manuka essential oil minimum biofilm inhibitory concentration and minimum biofilm eradicating concentration determinations

For 5 strains (2 *L. monocytogenes* and 3 *S. aureus*) selected on the basis of their biofilm production (the best activities for each species), MBIC and MBEC were determined in agreement with Reiter *et al*. (2013) with some modifications, as detailed below.

All MBIC and MBEC determinations were performed in triplicate and the mode values were considered.

Minimum Biofilm Inhibitory Concentration (MBIC)

For each strain, a bacterial biofilm was produced in the wells of a flat-bottomed microtiter plate, as for biofilm production quantification. The microtiter plate was then washed three times with sterile saline solution using an appropriate micropipette and left to dry for 15 minutes. Two-fold serial dilutions of manuka EO were prepared and added to the corresponding wells. Positive control wells were prepared adding $TSBYE_{gluc+NaCl}$ without EO. Microplates were then incubated at 37°C for 18-24 hours. After incubation, a loopful from every well was seeded on TSA and incubated at 37°C for 18-24 hours. MBIC value was determined as the lowest EO concentration with no bacterial growth on TSA.

Minimum Biofilm Eradication Concentration (MBEC)

After being used for MBIC determination, the microtiter plate was washed in sterile saline. Afterwards, $TSBYE_{gluc+NaCl}$ without EO was added in all wells, and the plate was re-incubated at 37°C for 18-24 hours. Then a loopful from every well was seeded onto TSA and incubated. MBEC value was determined as the lowest EO concentration corresponding to no bacterial growth on TSA.

Manuka essential oil Biofilm Percentage Reduction determinations

Four strains (the best biofilm producers for each species) were also tested for *Biofilm Percentage Reduction* to assess the percentage reduction determined by manuka EO on a mature biofilm produced by the tested strain vs. the corresponding untreated control strains whose biofilm was given as 100% production (0% reduction). Briefly, according to Verma and Maheshwari (2017) with some modifications, in parallel with MBIC determinations, for the chosen strains additional microtiter plates were submitted to the same steps performed for MBIC, using a limited number of EO dilutions for each strain (MIC/2 and MIC/4 for *L. monocytogenes* and from MIC/2 to MIC/16 for *S. aureus*). After MBIC determination, the microtiter plates were washed, and the subsequent steps were carried out as described for biofilm production quantification. The biofilm's biomass dispersion activity of the EO was evaluated comparing the OD value of the treated strain at each dilution to that of the positive control after a 24 hours exposure to the above reported sub-stressing EO concentrations and calculating the OD percentage reduction (BPR).

Statistical analysis

Statistical analysis was performed for BPR results using R v. 4.0.4 software (R Core Team, 2021). A one-way ANOVA was used to determine the significant differences in optical density values determined by the EO dilutions for each tested strain ($P<0.05$), while Tukey HSD test was used for *post-hoc* comparisons.

Results

Manuka essential oil characteristics and composition

The manuka EO chemical composition is reported in Table 1. Sesquiterpenes were the prevalent class of compounds (85.9%), with sesquiterpene hydrocarbons accounting for 50.5% of the total compounds. Among these, *cis*-calamenene represented the bigger portion and was also the overall most represented compound (24.0%). Among oxygenated sesquiterpenes (35.4%), leptospermone and *iso*-leptosper-

mone were the most represented compounds (18.3 and 6.4%, respectively). Flavesone (7.8%), another triketone, represented the majority of non-terpenic derivatives (8.1%).

Manuka essential oil Minimum Inhibitory Concentration and Minimum Bactericidal Concentration

Manuka EO showed a high antimicrobial activity, both on *L. monocytogenes* and *S. aureus*. For all *L. monocytogenes*, MIC and MBC values were the same and corresponded to 0.466 mg/ml and 0.933 mg/ml, respectively. Similarly, MIC and MBC values were the same for all *S. aureus* strains and corresponded to 0.233 mg/ml and 0.466 mg/ml, respectively (Table 2).

Biofilm production quantification

The results of the quantification of biofilm production by the 14 tested strains are reported in Table 2. No *L. monocytogenes* strain showed a strong biofilm production. Indeed, only 1 showed a medium-strong biofilm production, while 5 out of 7 resulted weak producers and 1 was the lowest producer used for the cut-off determination. On the contrary, *S. aureus* strains showed higher biofilm production capacities, with 3 strains being strong producers, 2 medium-strong and 2 weak producers.

Manuka essential oil Minimum Biofilm Inhibitory Concentration and Minimum Biofilm Eradication Concentration

The strains employed were the ones with the best biofilm production. Particularly, for *L. monocytogenes* 2 strains were selected: the medium-high producer, and the weak producer with the highest biofilm production capacity. For *S. aureus*, the 3 strains with a strong biofilm production activity were selected. MBIC and MBEC values against *S. aureus* strains were both markedly higher than those against *L. monocytogenes*, at least 7 times so. Indeed,

Table 1. Chemical composition of manuka essential oil detected by Gas Chromatography - Electron Impact Mass Spectrometry analysis.

Compound	Class	LRI	%
α-cubebene	sh	1351	3.1
α-copaene	sh	1377	5.0
α-guaiene	sh	1440	2.0
γ-muurolene	sh	1480	1.7
β-selinene	sh	1490	3.8
α-selinene	sh	1498	3.5
trans-β-guaiene	sh	1503	1.0
trans-γ-cadinene	sh	1514	1.0
cis-calamenene	sh	1540	24.0
flavesone	nt	1547	7.8
spathulenol	os	1578	1.0
globulol	os	1585	2.4
iso-leptospermone	os	1621	6.4
leptospermone	os	1629	18.3
cubenol	os	1647	1.3
Not known			4.8
Total identified			95.2
Monoterpene hydrocarbons (mh)			0.9
Oxygenated monoterpenes (om)			0,3
Sesquiterpenes hydrocarbons (sh)			50.5
Oxygenated sesquiterpenes (os)			35.4
No terpene derivatives (nt)			8.1

LRI: linear retenction index. Other compounds detected at <1%: α-pinene, *p*-cymene, 1,8-cineol, isoamil 2-methylbutyrate, 3-methyl-3butenyl butanoate, cyclosativene, β-elemene, α-gurjunene, β-caryophyllene, β-copaene, β-gurjunene, α-humulene, *allo*-aromadendrene, α-amorphene, *trans*-cadina-1(2),4-diene, α-calacorene, cis-cadinene ether, viridiflorol, guaiol, 5-*epi*-7-*epi*-α-eudesmol, β-oplopenone, humulene epoxide II, α-muurolol, selin-11-en-4-α-ol, *cis*-calamenen-10-ol, khusinol, cadalene, Z-apritone, 10-norcalamenen-10-one, cyclocolorenone, aristolone, squamulosone, 14-hydroxy-α-muurolene, nootkatone. All these compounds were considered for calculating the total percentage of each class of constituents.

Table 2. Origin and biofilm production capacity of the tested *Listeria monocytogenes* and *Staphylococcus aureus* strains and manuka essential oil Minimal Inhibitory Concentration (MIC), Minimal Bactericidal Concentration (MBC), Minimal Biofilm Inhibitory Concentration (MBIC) and Minimal Biofilm Eradication Concentration (MBEC) values.

Strain	Origin	MIC (mg/ml)	MBC (mg/ml)	Biofilm production	MBIC (mg/ml)	MBEC (mg/ml)
Lm 330	Fermented sausage	0.466	0.933	Weak	-	-
Lm 55	Goat cheese	0.466	0.933	Medium-strong	0.933	1.865
Lm 90	Poultry meat	0.466	0.933	Weak	0.933	0.933
Lm 415	Salmon	0.466	0.933	Weak	-	-
Lm 483	Fermented sausage	0.466	0.933	Weak	-	-
Lm 397	Ovine cheese	0.466	0.933	Weak	-	-
Lm 634	Salmon	0.466	0.933	No production	-	-
Sa ATCC 35556	Reference	0.233	0.466	Strong	7.461	14.922
Sa 852 (MRSA)	Cow milk	0.233	0.466	Strong	14.922	14.922
Sa 916 (MRSA)	Cow milk	0.233	0.466	Strong	14.922	14.922
Sa C21	Cow milk	0.233	0.466	Weak	-	-
Sa 1100 (MRSA)	Cow milk	0.233	0.466	Medium-strong	-	-
Sa 1234 (MRSA)	Goat milk	0.233	0.466	Weak	-	-
Sa 1242 (MRSA)	Sheep milk	0.233	0.466	Medium-strong	-	-

Lm: *Listeria monocytogenes*; Sa: *Staphylococcus aureus*; MRSA: methicillin-resistant.

Table 3. Optical density values of biofilms produced by the tested *Listeria monocytogenes* and *Staphylococcus aureus* strains when treated with sub-inhibitory concentrations of manuka essential oil and Biofilm Percentage Reduction (BPR) in comparison to positive controls.

Essential oil concentration (mg/ml)	Optical Density ± sd (BPR %)			
	Lm 55	*Lm 90*	*Sa ATCC 35556*	*Sa* 852
0.233	0.276±0.020a (12.9)	0.260±0.085a (16.1)	-	-
0.117	0.272±0.094a (13.7)	0.267±0.104a (13.9)	0.193±0.037a (74.0)	0.272±0.029a (75.6)
0.058	-	-	0.312±0.069ab (58.0)	0.549±0.314ab (50.8)
0.029	-	-	0.428±0.103ab (42.4)	0.710±0.279ab (36.4)
0.015	-	-	0.551±0.146bc (25.8)	0.961±0.397ab (13.9)
C+	0.315±0.098a (0)	0.310±0.077a (0)	0.743±0.034c (0)	1.116±0.350b (0)

Lm: *Listeria monocytogenes;* Sa: *Staphylococcus aureus*; BPR: Biofilm Percentage Reduction. For Lm MIC/2 and MIC/4 concentrations were tested, for Sa concentrations from MIC/2 to MIC/16. In each column different letters show significant differences ($p<0.05$).

MBEC against *S. aureus* was approximately 15 mg/ml, while for *L. monocytogenes* it had a maximum value of approximately 2 mg/ml. Results are detailed in Table 2.

Manuka essential oil Biofilm Percentage Reduction

As shown in Table 3, the effect of EO sub-inhibitory concentrations was markedly different in *L. monocytogenes* and *S. aureus*. No significant reduction effect was present at concentrations up to MIC/4 for *L. monocytogenes*, while for *S. aureus* a reduction higher than 50% was registered from MIC/2 to MIC/4 for both strains.

Discussion

The 3 triketones leptospermone, *iso*-leptospermone and flavesone were a major component of the tested EO and represented 32.5% of the total composition. Considering the antimicrobial potential of triketones, these data are also reflected in the MIC and MBC results. Indeed, regarding manuka EO effectiveness against *L. monocytogenes* and *S. aureus*, for both microorganisms the observed MIC and MBC values were well below the 2 mg/ml effectiveness threshold proposed by van Vuuren (2008). There is not much data on the antimicrobial effect of EOs against *L. monocytogenes*. Our MIC and MBC data for *L. monocytogenes* agree with those of Fratini *et al.* (2019) and are approximately 3 times lower than those reported by Harkenthal *et al.* (1999). Various studies have tested the efficacy of manuka EO or its components against *S. aureus,* including some MRSA strains. Several authors observed MIC and MBC values comparable with our findings (Van Klink *et al.,* 2005; Thielmann *et al.,* 2019).

Regarding biofilm production, our data agree with those already reported. Lower results for *L. monocytogenes* are probably due to the low production of extracellular polymeric substances (EPS) by this microorganism (Nilsson *et al*., 2011). On the other hand, *S. aureus* is known to produce many extracellular components and it generates a typical three-dimensional biofilm with many EPS (Vázquez-Sánchez and Rodríguez-López, 2018). This difference in quantity and quality of produced biofilm can also account for the difference in manuka EO efficacy in the inhibition and eradication effects on the biofilm cells produced by *S. aureus* and *L. monocytogenes*, as shown by the MBIC and MBEC results.

As far as the BRP results are concerned, interestingly manuka EO revealed a significant effect in reducing *S. aureus* preformed mature biofilms at very low concentrations. Various dispersal agents of different origin (anti-matrix molecules, dispersal signals, sequestration molecules) are recognized as possible tools to fight microbial biofilms (Sharma et al., 2019) and the effects of some plant components as dispersal agents was demonstrated (Jain and Parihar, 2018). Moreover, the effect of sub-inhibitory doses of EOs in determining different phenotypical changes is already known particularly, they can affect enterotoxins production and antibiotic resistance profile in *S. aureus* (Turchi *et al*., 2018; Turchi *et al.,* 2019). In cases of such sub-inhibitory stresses *quorum sensing* mechanisms could be involved (Bai and Vittal, 2014), particularly in *S. aureus* (Guzzo *et al.,* 2020).

Conclusions

Manuka EO confirmed its good antimicrobial effect both for *L. monocytogenes* and *S. aureus*. However, higher manuka EO doses than MIC/MBC, not easily applicable in food systems, are requested to completely eradicate the bacterial cells embedded in a *S. aureus* mature biofilm. Anyway, the capacity to reduce the biofilm total biomass at very low concentrations could be very useful, considering that manuka EO demonstrated interesting synergistic effects in mixed blends with other EOs also at low concentrations (Fratini *et al*., 2017).

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