Evaluation of the antibacterial activity of bergamot essential oils on different *Listeria monocytogenes* strains

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Abstract

Essential oils are aromatic and volatile substances extracted from plants and characterized by antimicrobial activity. The aim of the present study was to evaluate the antibacterial activity (agar disc-diffusion method) of seven different bergamot essential oils (BEOs) on eight *Listeria monocytogenes* strains. Minimal inhibitory concentration (MIC) of most efficient BEOs was estimated. Extremely variable results for agar disc-diffusion method for *L. monocytogenes* strains were reported. One of the tested microorganisms resulted insensible to all the BEOs; 3 strains showed an inhibition from weak to null and the remaining 4 a variable susceptibility. Among the BEOs tested, one showed a strong activity against four pathogenic strains. Four BEOs revealed weak, moderate or null activity in all the 7 sensitive strains, while for two oils only a weak or no activity was reported. MIC values were 0.625 µL/mL for the most efficient BEO, 2.5 and 5 µL/mL for the other samples that showed moderate inhibition. Experiment results are significantly related to the strains tested ($P<0.01$), rather than the BEO employed ($P>0.01$). In conclusion, we can consider BEO as a natural technological hurdle for *Listeria monocytogenes* in combination with other preservation strategies. Finally, this study underlines the necessity to evaluate the antimicrobial activity of EOs on a significant strains number of the same bacteria.

Introduction

The interest in natural methods that can make food safer, avoiding the use of chemical preservatives or additives, has increased the study on these products (Giarratana *et al.,* 2013, 2016; Klein *et al.,* 2013; Moreira *et al.,* 2005; Muscolino *et al.,* 2016). In this regard, essential oils (EOs), aromatic oily liquids obtained from plant materials, are well known for their preservative properties. These substances and their components are used in food, pharmaceutical and cosmetic industries, for their antibacterial, antifungal, antiviral, nematocidal, anti-carcinogenic and antioxidant properties (Frassinetti *et al.,* 2011; Giarratana *et al.,* 2014, 2015a, 2015b; Rota *et al.,* 2008; Silva-Angulo *et al.,* 2015). Bergamot essential oil (BEO), extracted from the peel of *Citrus bergamia Risso*, is characterized by several of these properties (Navarra *et al.,* 2016; Pernice *et al.,* 2009; Sicari *et al.,* 2015; Trombetta *et al.,* 2010). *C. bergamia Risso* is a typical fruit of southern Italy and its production is limited to the Ionian Sea coastal areas of Reggio di Calabria province (Sicari et *al.,* 2015). BEO antibacterial and antiseptic activity is related to the presence of well-recognized antimicrobial compounds (Fisher and Phillips, 2006; Navarra *et al.,* 2016; Pernice *et al.,* 2009). These substances can be distinguished in volatile (*e.g.* limonene, linalool and linalyl acetate) and non-volatile (*e.g.* bergamottin, citroptene and bergaptene) components (Salvo *et al.,* 2016). The major active EOs components are phenols, terpenes, aldehydes and ketones, whose action is performed against the cytoplasmic membrane of target microorganism cells (Hyldgaard *et al.,* 2012). The hydrophobicity is also an important characteristic, which enables EOs to accumulate in cell membranes causing an increase of permeability until cell death (Moreira *et al.,* 2005; Silva-Angulo *et al.,* 2015). For all these reasons EOs employ in food technology is a concrete prospective for undesirable microbial flora control. The aim of this study was to evaluate the in vitro antimicrobial activity of different BEOs against several *Listeria monocytogenes* strains.

Materials and Methods

Essential oils collection

For this study, seven different samples of *Citrus bergamia Risso* essential oil (BEO) were tested. Among these, five were collected from local producers from Reggio Calabria district (BEO*a-e*), while, the remaining two from commercial products (BEO*f*: Mystic moments, Fordingbridge, UK; BEO*g*: Erboristeria magentina, Poirino, Italy).

Bacterial cultures

Eight *Listeria monocytogenes* strains were tested: five from seafood samples (wild types) and three from American Type Culture Collection (ATCC) (Table 1). Working cultures were prepared by inoculating a loopful from the frozen stock (-80°C), on tryptic soy broth (Biolife, Milan, Italy) +0.6% yeast extract (YE) (Biolife) and then incubated at 37±0.5°C for 24 h, in order to achieve an OD_{600} of 1.2, corresponding to 10^9 colony forming unit/mL (SmartSpec Plus; Bio-Rad, Milan, Italy).

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Determination of antimicrobial activity

BEOs antibacterial activity was evaluated by agar disc diffusion method. Broth cultures strains were spread with a sterile swab on the surface of TSA (Biolife) + 0.6% YE agar plates. Sterile 6-mm paper disc impregnated with 15 µL of each tested BEO*a-g* were placed on the surface of inoculated plates. Two disks impregnated one with 15 µL of Streptomycin (50 µg/mL) (Biolife) and one with 15 µL of sterile distilled water as positive and negative control were used respectively. Plates were incubated at 37±0.5°C for 24 h. Microbial inhibition was visually evaluated as the diameter of the inhibition zones surrounding the disks, including them, and recorded in millimeters according to NCCLS (2015). The antimicrobial activity of BEO was divided into three ranges according to Rota *et al*. (2008): weak activity (inhibition zone ≤12 mm), moderate activity (12 mm<inhibition zone<20 mm) and strong activity (inhibition zone ≥20 mm).

Determination of minimal inhibitory concentration with broth dilution assay

BEOs with an antimicrobial activity from moderate to strong were tested for minimal inhibitory concentration (MIC) according to a modified NCCLS/CLSI standard method (NCCLS/CLSI, 2015). Serial two-fold dilutions of each BEO were made in a concentration ranging from 5 to 0.31 µL/mL in 10 mL sterile test tubes containing trypticase soy broth with 0.6% yeast extract. At this solution 5% (v/v) Tween-20 (Biolife) was incorporated into the

broth medium to enhance oil solubility. The inoculums were prepared from overnight broth cultures of sensitive strains (logarithmic growth phase cells). A 400-µL suspension of tested microorganisms was added to each tube. For positive and negative control we used two broth tubes containing respectively 50 µg/mL of Streptomycin and only microorganism inoculums. MIC was assumed as the concentration in the lowest serial dilution of the BEOs that resulted in the lack of visible microorganism growth in tubes after 24 h incubation.

Gas chromatography

Analysis of most efficient BEO was carried out by as gas chromatography with flame ionization detection according to ISO 7609:1985 (ISO, 1985).

Statistical analysis

Each experiment was carried out in triplicate on two separate occasions. Results are expressed as mean values±standard deviation. One-way ANOVA test was performed to determine the mean significant differences among different BEOs treatment and strains tested, significance was assumed as $P<0.01$ (XLSTAT, Microsoft Excel; Addinsoft, New York, NY, USA).

Results

Antimicrobial activity: *Listeria monocytogenes*'s variability

Results are showed in Table 2. *L. monocytogenes* strains expressed a various range of susceptibility to BEOs action (Figure 1). In particular, among the 5 wild type strains, 115me resulted the most sensitive, revealing weak inhibition zones for 2 BEO, moderate sensibility against 4 oils and a strong reaction only for BEO*d*. Similar results are reported for 168me, except that for no reaction to BEO*g*. Strain 94me demonstrated weak inhibition zones for most of BEO, moderate for BEO*c* and a strong susceptibility for BEO*d*. Listeria 157me showed a weak reaction only for BEO*a*, BEO*d* and BEO*e*, no inhibition zones were observed for others oils. Finally, 163me showed a potential resistance to BEOs antibacterial activity with no inhibition zones for all the oils employed. All the ATCC strains resulted completely insensitive to BEO*e*; anyway, among them, ATCC 19111 resulted, overall, the most sensitive, showing a strong inhibition for BEO*d*, moderate for BEO*c* and weak for the other samples. Finally, the remaining ATCC 13932 and ATCC 7644 strains demonstrated a weak reaction to all the BEOs tested.

Antimicrobial activity: bergamot essential oils' variability

Among the seven BEOs employed in our study (Figure 2) BEO*d* was the most efficient in restricting *L. monocytogenes* growth. It was the only BEO characterized by a strong activity, with an inhibition diameter of 20 mm in 50% of the strains and weak inhibition zones in the remaining sensitive strains. Follows BEO*c*, with a moderate action on 50% of bacteria and a weak (25%) to null (25%) inhibition in the other strains. BEO*a* resulted characterized by a mildly and weakly effective respectively on 37.5% and 50% of the strains studied. BEO*b* action was moderate and weak in both 37.5% of strains, while BEO*e* showed an activity from weak to moderate in 50% of *L. monocytogenes* strains and no reaction in the left 50% including 163me and all the ATCC strains. Finally, BEO*f* and BEO*g* resulted the less effective showing only a weak activity respectively in the 75 and 62.5% of the strains and no inhibition in the remaining others.

Determination of minimal inhibitory concentration

BEO*a*, BEO*b*, BEO*c*, BEO*d* and BEO*e,* on the bases of antimicrobial activity, were selected for MIC determination. MIC values confirmed the results obtained by the agar disk diffusion method, as well as, their variable levels of inhibition. BEO*d* had the lowest MIC (0.625 µL/mL), BEO*a* and BEO*c* a value of 1.25 µL/mL, while BEO*b* and BEO*e* the highest MIC (5 µL/mL).

Chemical composition

Seven compounds were detected in the most efficient *Citrus bergamia Risso* essential oil tested (BEO*d*). The compounds obtained and their abundance are in accordance with those reported by Navarra *et al*. (2015). The major compounds were hydrocarbons monoterpenes like Limonene (35.5%) and Linalyl acetate (33.1%); followed by Linalolo (9.9%), γ-Terpinene (6.9%), β-Pinene (5.9%), β-Bisabolene (0.6%) and Geranile (0.2%).

Table 1. *Listeria monocytogenes* strains.

ID strains	Serotype	Origin
94me	-	Wild type - smoked salmon fillets
115me	-	Wild type - smoked salmon fillets
157me	-	Wild type - smoked salmon fillets
163me	-	Wild type - fresh salmon fillets
168me	-	Wild type - fresh salmon fillets
ATCC 13932	4b	Human
ATCC 19111	1/2	Poultry
ATCC 7644	1/2c	Human

ATCC, American Type Culture Collection.

Table 2. Bergamot essential oils' antimicrobial activity against the tested microorganisms.

BEOs	Strains							
	94me	115me	157me	163me	168me	ATCC19111	ATCC13932	ATCC7644
a	11±1	14±1	10±1	0	14±0	12±1	11±1	10±1
b	12±0	14±1	0	0	16±1	11±0	8±1	7±0
c	15±1	14±0	0	0	16±1	15±1	10±0	11±1
d	20±0	20±1	10±1	0	20±0	20±1	11±1	11±1
e	12±1	14±1	8±1	0	12±1	0	0	0
f	12±1	12±0	0	0	12±0	10±1	8±1	8±0
g	8±0	8±1	0	0	0	11±1	9±1	8±1

BEOs, bergamot essential oils; ATCC, American Type Culture Collection. Results are expressed as mean±standard deviation from the experiments in triplicate. The diameter of the disks (Ø=6 mm) was included.

Discussion

Terpenes antibacterial activity is well recognized. They pass through the cell wall and permeabilize cytoplasmic membrane, by destroying the multi-layers structure of polysaccharides, fatty acids and phospholipids. In bacteria, these events are associated with loss of ions and reduction of membrane potential, which lead to the proton pump collapse and depletion of ATP pool and lysis (Burt, 2004; Di Pasqua *et al.,* 2007; Oussalah *et al.,* 2007; Sikkema *et al.,* 1995). It was reported that *Listeria monocytogenes* strains exposed to EOs activity react with the thickening and rupture of cellular membrane, and the progressive lack of cytoplasm material (Rasooli *et al.,* 2006). Despite that, ANOVA test reported that our extremely variable results are significantly connected ($P<0.01$) to the strains tested; thus, to different cellular answers, when occurring injuring factors. It is well known that sublethal stressors can induce *L. monocytogenes* adaptation and develop of specific resistance against several substances such as antibiotics, bacteriophages and disinfectants (Fister *et al.,* 2016; Kovacevic *et al.,* 2016; Su *et al.,* 2016). *L. monocytogenes* population is characterized by different stress robustness parameters that may represent an advantage in unfavorable condition. This pathogen, indeed, exhibits a mutable response upon stress exposure, which can be partially attributed to the presence of stable stress resistant variants (Metselaar *et al.,* 2016). Some authors, also, suppose a multi-factorial genomic regulation to explain this various resistant patterns (Kovacevic *et al.,* 2016). Indeed, we found a wild type strain (163me) total insensitive to oils action, probably due to an acquired resistance against lytic BEOs substances, whose mechanism of action has to be further investigated. Furthermore, we did not found any significant relation ($P>0.01$) between our results and the different types of BEO tested. Thus, different susceptibilities are, probably, imputable to the strains tested, rather than to oils compounds. Our best MIC result (0.625 µL/mL – 0.625 % v/v) is higher than value reported by Cirmi *et al*. (2016) (0.125 % v/v) on a single *L. monocytogenes* strain.

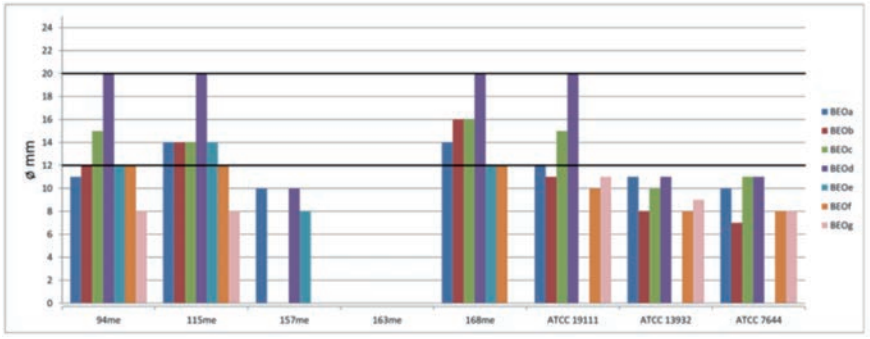

Figure 1. ***Listeria monocytogenes*** **variable response to the activity of bergamot essential oils. Black lines represent critical limit fixed to assess oils' activity as weak, moderate or strong.**

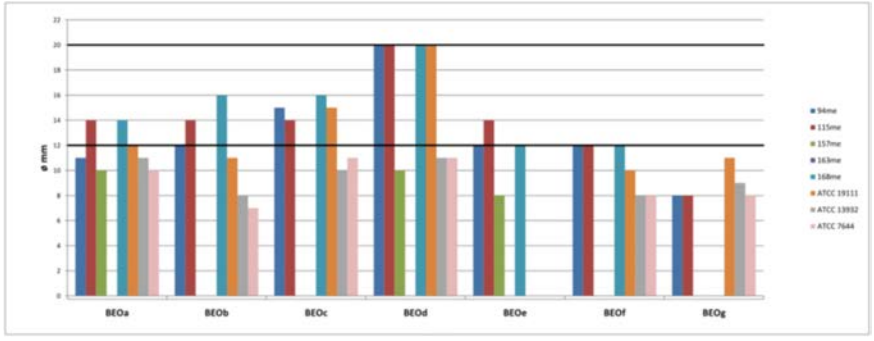

Figure 2. Bergamot essential oils' inhibitory activity on ***Listeria monocytogenes*** **strains. Black lines represent critical limit fixed to assess oils' activity as weak, moderate or strong.**

Conclusions

In conclusion, on the basis of our results, the different BEOs activities registered are mostly related to individual susceptibility of bacteria. Considering the extreme variability of *Listeria monocytogenes*'s response to BEOs action, it is recommended to estimate their efficacy on a significative number of pathogenic strains in order to prospect a concrete employ in food industries as a valid natural alternative for the bio-control of the pathogen.

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