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

Stefania M. Marotta, Filippo Giarratana, Alessio Parco, Domenico Neri, Grazziella Zino, Alessandro Giuffrida, Antonio Panebianco
Department of Veterinary Sciences, University of Messina, Messina, Italy

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 insensitive 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. The 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 EO's on a significant strains number of the same bacteria.

Materials and Methods

Essential oils collection

For this study, seven different samples of Citrus bergamia Riso essential oil (BEO) were tested. Among these, five were collected from local producers from Reggio Calabria district (BEOs-e), while, the remaining two from commercial products (BEOs: Mystic moments, Fordingbridge, UK; BEOg: 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 10⁵ colony forming unit/mL (SmartSpec Plus; Bio-Rad, Milan, Italy).

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 (NCCCLS/CLSI, 2015). Serial two-fold dilutions of each BEO were made in a concentration ranging from 5 to 0.31 µg/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 sterilized 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. Minimal 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).

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

Key words: Listeria monocytogenes; Bergamot; Essential oils; Antimicrobial activity.

Received for publication: 19 July 2016.
Revision received: 22 September 2016.
Accepted for publication: 22 September 2016.

This work is licensed under a Creative Commons Attribution-NonCommercial 4.0 International License (CC BY-NC 4.0).

Correspondence: Stefania Marotta, Department of Veterinary Sciences, University of Messina, Polo Universitario della Annunziata, 98168 Messina, Italy.
Tel. +39.090.3503768.
Email: smarotta@unime.it

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 (Frassineti 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 Riso, 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 bergaptenne) 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 BEO against several Listeria monocytogenes strains.

[Italian Journal of Food Safety 2016; 5:6176]
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 sensitivity against 4 oils and a strong reaction only for BEOd. Similar results are reported for 168me, except that for no reaction to BEOg. Strain 94me demonstrated weak inhibition zones for most of BEO, moderate for BEOc and a strong susceptibility for BEOd. Listeria 157me showed a weak reaction only for BEOa, BEOd and BEOe, 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 BEOe; anyway, among them, ATCC 19111 resulted, overall, the most sensitive, showing a strong inhibition for BEOd, moderate for BEOc 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) BEOd 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 BEOc, with a moderate action on 50% of bacteria and a weak (25%) to null (25%) inhibition in the other strains. BEOa resulted characterized by a mildly and weakly effective respectively on 37.5% and 50% of the strains studied. BEOb action was moderate and weak in both 37.5% of strains, while BEOc 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, BEOd and BEOg 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
BEOa, BEOb, BEOc, BEOd and BEOe, 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. BEOd had the lowest MIC (0.625 µL/mL), BEOa and BEOc a value of 1.25 µL/mL, while BEOb and BEOe the highest MIC (5 µL/mL).

Chemical composition
Seven compounds were detected in the most efficient Citrus bergamia Risso essential oil tested (BEOd). The compounds obtained and their abundance are in accordance with those reported by Navarra et al. (2015). The major compounds were hydrocarbons monoterprenes 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  | 12c      | Human |

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

| BEOs | Strains | ATCC19111 | ATCC13932 | ATCC7644 |
|------|---------|-----------|-----------|----------|
| a    | 11±1    | 14±1      | 10±1      | 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        | 11±1     | 9±1      | 8±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 sub-lethal 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) totally 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*’ 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.

**References**

Burt S, 2004. Essential oils: their antibacterial properties and potential applications in foods: a review. Int J Food Microb 94:223-53.

Cirmi S, Bisignano C, Mandalari G, Navarra M, 2016. Anti-infective potential of Citrus bergamia Risso et Poiteau (bergamot) derivatives: a systematic review. Phytother Res 30:1404-11.

Di Pasqua R, Betts G, Hoskins N, Edwards M, Ercolini D, Mauriello G, 2007. Membrane toxicity of antimicrobial compounds from essential oils. J Agr Food Chem 55:4863-70.

Fisher K, Phillips CA, 2006. The effect of lemon, orange and bergamot essential oils and their components on the survival of Campylobacter jejuni, Escherichia coli O157, *Listeria monocytogenes*, Bacillus cereus and Staphylococcus aureus in vitro and in food systems. J Appl Microbiol 101:1232-40.

Fister S, Fuchs S, Stessl B, Schoder D, Wagner M, Rossmanith P, 2016. Screening and characterisation of bacteriophage P100 insensitive *Listeria monocytogenes* isolates in Austrian dairy plants. Food Control 59:108-17.

Frassinetti S, Caltavuturo L, Cini M, Della Croce CM, Maserti BE, 2011 Antibacterial and Antioxidant activity of essential oils from Citrus spp. J Essent Oil Res 23:27-31.

Giarratana F, Muscolino D, Beninati C, Giuffrida A, Panebianco A, 2014. Activity of Thymus vulgaris essential oil against *Anisakis* larvae. Exp Parasitol 142:7-10.

Giarratana F, Muscolino D, Beninati C, Zilio G, Giuffrida A, Panebianco A, 2013. Effects of thyme and rosemary essential oils on the microbiology and shelf life of Italian Mortadella. Fleischwirtschaft 93:183-7.

Giarratana F, Muscolino D, Panebianco F, Patania A, Benianti C, Zilio G, Giuffrida A, 2015a. Activity of R (+) limonene against *Anisakis* larvae. Ital J Food Safety 4:4.

Giarratana F, Muscolino D, Ragonese C,
Beninati C, Sciarrone D, Ziino G, Mondello L, Giuffrida A, Panebianco A, 2016. Antimicrobial activity of combined thyme and rosemary essential oils against Listeria monocytogenes in Italian mortadella packaged in modified atmosphere. J Essent Oil Res 2016:1-8.

Giarratana F, Panebianco F, Muscolino D, Beninati C, Ziino G, Giuffrida A, 2015b. Effect of allyl isothiocyanate against Anisakis larvae during the anchovy marinating process. J Food Protect 78:767-71.

Hyldgaard M, Mygind T, Meyer RL, 2012. Essential oils in food preservation: mode of action, synergies and interactions with food matrix components. Front Microbiol 3:1-24.

ISO, 1985. Essential oils. Analysis by gas chromatography on capillary columns. General method. ISO Norm 7609:1985. International Standardization Organization ed., Geneva, Switzerland.

Klein G, Rüben C, Upmann M, 2013. Antimicrobial activity of essential oil components against potential food spoilage microorganisms. Curr Microbiol 67:200-8.

Rasooli I, Rezaei MB, Allameh A, 2006. Ultrastructural studies on antimicrobial efficacy of thyme essential oils on Listeria monocytogenes. Int J Infect Dis 10:236-241.

Rota MC, Herrera A, Martinez RM, Sotomayor JA, Jordan MJ, 2008. Antimicrobial activity and chemical composition of Thymus vulgaris, Thymus zygis and Thymus hyemalis essential oils. Food Control 19:681-7.

Salvo A, Bruno M, La Torre GL, Vadala R, Mottese AF, Saija E, Mangano V, Casale KE, Cicero N, Dugo G, 2016. Interdonato lemon from Nizza di Sicilia (Italy): chemical composition of hexane extract of lemon peel and histochemical investigation. Nat Prod Res 30:1517-25.

Sicari V, Loizzo MR, Branca V, Pellicanò TM, 2015. Bioactive and antioxidant activity from Citrus Bergamia Risso (Bergamot) juice collected in different areas of Reggio Calabria province, Italy. Int J Food Prop 1:10.

Sikkema J, De Bont JA, Poolman B, 1995. Mechanisms of membrane toxicity of hydrocarbons. Microbiol Rev 59:201-22.

Silva-Angulo AB, Zanini SF, Rosenthal A, Rodrigo D, Klein G, Martinez A, 2015. Comparative Study of the effects of citral on the growth and injury of Listeria innocua and Listeria monocytogenes cells. PloS One 10:e0114026.

Su X, Zhang J, Shi W, Yang X, Li Y, Pan H, Kuang D, Xu X, Shi X, Meng J, 2016. Molecular characterization and antimicrobial susceptibility of Listeria monocytogenes isolated from foods and humans. Food Control 70:96-102.

Trombetta D, Cimino F, Cristiani M, Mandalaris G, Saija A, Ginestra G, Speciale A, Chirafisi J, Bisignano G, Waldron K, Narbad A, Faulds CB, 2010. In vitro protective effects of two extracts from bergamot peels on human endothelial cells exposed to tumor necrosis factor-α (TNF-α). J Agr Food Chem 38:8430-6.