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Inhibitory activity of yarrow essential oil on *Listeria* planktonic cells and biofilms

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ABSTRACT

The inhibitory effects of the essential oil obtained from yarrow (*Achillea millefolium*) were tested against planktonic cells and biofilms of *Listeria monocytogenes* and *Listeria innocua* isolates obtained from food processing environments. The plant essential oil was found to have strong bactericidal activity against planktonic cells which was found to be equivalent to that of tea tree oil (*Melaleuca alternifolia*). Inhibition of biofilm formation and growth after incubation with different concentrations of yarrow essential oil was assessed by the crystal violet and 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide reduction assay. Yarrow essential oil significantly inhibited the initial cell attachment of the *Listeria* cells on polystyrene, stainless steel and high density polyethylene surfaces but was less inhibitory towards 6 h preformed biofilms formed on polystyrene surface. Metabolic activity of the biofilms decreased considerably after incubation with the oil. Yarrow essential oil, or its constituents, may be useful additives for the development of new disinfectant and sanitizer formulations for application in the food industry.

1. Introduction

The genus *Listeria* is comprised of seven species of gram positive bacteria (Graves et al., 2010). Amongst these, the species *Listeria ivanovii* and *Listeria monocytogenes* are pathogenic to humans and can cause listeriosis, a predominately foodborne disease which has a higher mortality rate compared to other foodborne illnesses. Listeriosis mostly affects the elderly, neonates, pregnant women and immunocompromised populations. It can result in meningitis, septicaemia, encephalitis or can also be responsible for still births and abortions in pregnant women. Some common foods associated with the presence of *L. monocytogenes* include uncooked meat products, vegetables and soft cheeses (Jadhav, Bhave, & Palombo, 2012). The presence of this pathogen in a food product leads to a recall in most countries. Hence, it is obligatory for the food industry to carry out hazard analysis and critical control point (HACCP) approach to periodically check the contamination levels of this bacterium in the processing facilities (Blatter, Giezendanner, Stephan, & Zweifel, 2010).

This industrially important foodborne pathogen can survive refrigeration temperatures, prevalent in food processing environments (Hansen & Vogel, 2011). It forms biofilms in such environments and can hence lead to recurrent contamination of food products (Leonard, Virijevic, Regnier, & Combrinck, 2010). In case of bacteria, biofilm formation takes place in multiple stages. The initial stage involves the reversible attachment of the cells to an inert surface, followed by irreversible adhesion of the cells (second stage). In the third stage, the bacteria grow on the surface to form microcolonies. In this maturation stage, a well-organised complex three dimensional biofilm is formed and finally, in the fourth stage, the cells detach from the mature biofilm (Rieu, Weidmann, Garmyn, Piveteau, & Guzzo, 2007; Xu, Zou, Lee, & Ahn, 2010). Cells in a biofilm have been observed to be more resistant to heat, drying, acidic environments, salinity, antimicrobials and food preservatives, compared to their planktonic counterparts (Xu, Lee, & Ahn, 2011). Hence, better sanitizing and disinfecting strategies are required to deal with this issue.

Recently, the emergence of antibiotic resistant strains and the reluctance of consumers towards consumption of chemically-treated goods have encouraged the development of natural antimicrobial agents. Substances obtained from plants are preferred over synthetic biocides as they may have been used in traditional medicine for a long time. Overall, they are generally considered to be safe by consumers and are not known to cause harm to the environment (Leonard et al., 2010).

In the current study, the inhibitory effects of yarrow (*Achillea millefolium*) essential oil (YEO) on planktonic cells and biofilms were studied using *L. monocytogenes* and *Listeria innocua* isolates obtained from food processing environments. *L. innocua* is a non-pathogenic species which is often used as a surrogate to

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L. monocytogenes, as it is taxonomically closely related (Oulahal, Brice, Martial, & Degraeve, 2008). Native to Europe and western Asia, *A. millefolium* (family Asteraceae) can now be found in southern Australia, New Zealand and parts of North America (Rahimmalek, Sayed Tabatabaei, Arzani, & Etemadi, 2009). YEO is conventionally obtained from different structural parts such as flowers, leaves and stems by steam distillation or hydrodistillation (Bocevska & Sovová, 2007). The annual worldwide production of the oil is 800 tonnes and it has been used traditionally as an anti-inflammatory medicine, to treat headaches and to control bleeding of wounds and haemorrhoids (Tajik, Jalali, Sobhani, Shahbazi, & Zadeh, 2008). Hydroalcoholic extracts of this herb have also exhibited anti-nociceptive activity (Lakshmi, Geetha, Roy, & Kumar, 2011). The antimicrobial activity of the alcoholic extract of this plant has been screened against a range of other bacteria and the extract was found to be most active against *Staphylococcus aureus* (Tajik et al., 2008).

The effect of various essential oils and their individual constituents on biofilms formed by *L. monocytogenes* has been investigated previously (Leonard et al., 2010; Oliveira, Brugnera, Alves, & Piccoli, 2010; Sandasi, Leonard, & Viljoen, 2008, 2010). However, to our knowledge, this is the first report of the anti-listerial and antibiofilm effects of YEO.

2. Materials and methods

2.1. Preparation of bacterial cultures

Several isolates of *L. monocytogenes* and *L. innocua* were obtained from food processing environments and subcultured on Brain Heart Infusion (BHI, Oxoid) agar and incubated at 37 °C for 24 h. Prior to each experiment, a loopful of the BHI culture was inoculated in Tryptone Soya broth (TSB, Oxoid) and incubated at 37 °C for 24 h. Following incubation, a hundred-fold dilution of the overnight culture matched to the 0.5 McFarland turbidity standard (approximately 10^8 cfu/ml) was used for experimental procedures.

2.2. Essential oils

Yarrow essential oil (YEO, 100% pure) was obtained from New Directions (Sydney, Australia) and essential oil of *Melaleuca alternifolia*, commonly known as tea tree oil (TTO, 100% pure) was obtained from Thursday Plantations (Queensland, Australia). All other chemicals of analytical grade were obtained from Sigma–Aldrich Co. Ltd. (Dorset, UK) unless otherwise specified.

2.3. Agar disk diffusion assay

The anti-listerial activity of YEO was evaluated using the agar disk diffusion assay. The assay was carried out as per Fisher and Phillips (2006), with some modifications. Initially, 100 μl of 24 h bacterial cultures (in TSB) adjusted to a 0.5 McFarland standard was uniformly spread on the surface of BHI agar plates. Three sterile discs impregnated with 20 μl of YEO were then placed on the surface of the plates. This experiment was carried out on two independent occasions. The solvent used for subsequent preparation of dilutions of YEO (methanol) was used as negative control while chloramphenicol discs (30 μg) were used as positive control. The plates were incubated at 37 °C for 24 h before measuring the zone of inhibition (diameter in mm).

2.4. Determination of minimal inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The MIC of YEO was determined in BHI broth using a broth microdilution method in the 96-well round-bottomed polystyrene microtitre plates (Corning, NY), as conducted by Mihajilov-Krstev, Radnović, Kitić, Stojanović-Radić, and Zlatković (2010), with some modifications. One hundred microlitres of YEO (50% v/v) were added to an equal volume of BHI broth and a series of two-fold serial dilution was performed with BHI broth in the microtitre plate to achieve a concentration gradient from 25% v/v to 0.781% v/v. Inhibition of bacterial growth in the wells containing YEO was assessed by comparison with growth in blank control wells and the inhibitory activity was compared to that of TTO by simultaneous determination of the MIC of TTO against both the isolates.

Bacterial cultures in TSB were adjusted to 0.5 McFarland turbidity equivalents. Ten microlitres of the inoculum were added to each well and incubated for 24 h at 37 °C. Positive growth controls (inoculum + BHI) and vehicle growth controls (inoculum + BHI + methanol) were also included in the plate. The MIC values were defined as the lowest concentrations of the oil which inhibited visible growth of the bacteria. For determination of MBC of YEO and TTO, 50 μl of solution from all wells having no turbidity were spread onto the surface of BHI agar plates and incubated for 24 h at 37 °C. The sample with the lowest concentration of the oil that showed no growth on BHI agar was recorded as the MBC.

2.5. Determination of biofilm inhibitory activity of YEO

2.5.1. Inhibition of initial cell attachment

The effect of YEO on biofilm formation was evaluated as described by Sandasi et al. (2010). Solutions of YEO (equivalent to 0.5× MIC, 1× MIC and 2× MIC) were prepared. One hundred microlitres of each solution were added to individual wells of a sterile flat-bottomed 96-well polystyrene microtitre plates (Corning, NY). Equal volumes of methanol or water were added as negative controls while tetracycline (0.00125 mg ml^{-1}) was added as a positive control. One hundred microlitres of the bacterial cultures (prepared as described above in Section 2.1) were then added to the wells to yield a final volume of 200 μL in each well. The cultures were added into the wells in quadruplicate and sterile TSB was also added as an additional control to confirm the sterility of the medium. The plates were sealed and incubated for 24 h at 37 °C under sterile conditions to allow cell attachment. Biofilm formation was assessed using the crystal violet assay and the metabolic activity of the cells incubated with YEO was investigated using the MTT assay (assays described below).

2.5.2. Inhibition of preformed biofilm

The effect of essential oil on biofilm growth and development was evaluated as described by Sandasi et al. (2010), with some modifications. Biofilms were allowed to be formed for 6 h prior to addition of YEO. Biofilm formation was achieved by transferring 100 μl of bacterial culture (prepared as described above in Section 2.1) into the wells of sterile flat-bottomed 96-well polystyrene microtitre plates in quadruplicates. The microtitre plates were covered and incubated for 6 h at 37 °C to allow cell attachment and biofilm formation. Following incubation, 100 μl of each stock solution of the oil was added to each well to yield a final volume of 200 μl. Equal volumes of methanol or water were added as negative controls while tetracycline (0.00125 mg ml^{-1}) was added as a positive control. After the treatment of preformed biofilms with YEO, the plates were incubated for 1 h, 5 h, and 20 h. Following incubation, the biofilms were assessed for biomass attachment