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Antimicrobial activity of several essential oils on pathogenic and beneficial bacteria

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ABSTRACT

The possibility to transfer antibiotic resistance from animal gut bacteria to human pathogens by animal source foods has stimulated the search for alternatives to replace the antibiotic use on animal production. Essential oils (EOs) and beneficial bacteria have been considered as alternatives. This study evaluated, *in vitro*, the antibacterial activity of EOs, individually and in binary blends, on pathogenic and beneficial bacteria that can occur in the swine and poultry gut. An initial screening was made with 28 EOs to verify their antibacterial activity on a model pathogenic bacterium *Salmonella* Enteritidis and a model probiotic bacteria *Lactobacillus plantarum*. The EOs from leaves of *Eucalyptus globulus*, *E. exserta*, *Pimenta pseudocaryophylllus*, and also two EOs, named Orange Oil Phase Essence, and Citrus Terpens, which are by-products of orange juice production, presented the greatest activity on pathogenic bacterium (*S.* Enteritidis) and the lowest activity on beneficial bacterium (*L. plantarum*). These five EOs were tested additionally, alone and in binary blends, against other four pathogenic bacteria: *Staphylococcus aureus, Escherichia coli, Enterococcus faecalis* and *Listeria innocua*, and against other two beneficial bacteria: *Lactobacillus rhamnosus* and *Bacillus subtilis*. Orange Oil Phase Essence and Citrus Terpens were oils that showed the greatest activity on pathogenic bacteria and the lowest activity on beneficial bacteria ($p < 0.05$), therefore presenting the best selective antibacterial activity between both groups of bacteria. The possibility of having a new usage for citrus EOs, which are by-products of the food industry, represents important information.

1. Introduction

Pig and poultry farmers have long used, for health maintenance and improvement of animal weight gain, antibiotics in sub therapeutic doses that are called Antimicrobial Growth Promoters (AGP). However, since the beginning of the use of AGP, there have been reports on the emergence of resistance to some antibiotics in bacteria isolated from livestock. Consequently, the concern about the possibility of transferring that resistance to human pathogens through food chain arose (Dibner and Richards, 2005) as a public health problem, since it is estimated that at least 61% of all human pathogens are of animal origin (WHO, 2016). As a consequence of this concern, some countries, such as Sweden in 1986 (Aarestrup, 2003) and Denmark in 2000 (WHO, 2000) banned the use of antibiotics as AGP, and in 2006 the European Community, through the Regulation (EC) N° 1831/2003 of the European Parliament and The Council (EU, 2003) extended this ban to all member states.

Therefore, due to the increase of bacterial resistance and the legal restriction of AGP use, there has been an intense search for alternatives to substitute AGP and so maintain efficiency in animal production. Among the alternatives studied, essential oils (EOs), according to their antimicrobial characteristics, have received significant attention (Franz et al., 2010). For instance, some studies have already evaluated the antibacterial activity of EOs, and they have shown their potential to fight pathogenic bacteria (Elaissi et al., 2011; Zhang et al., 2016). Moreover, synergic effect improving antibacterial activity has also been reported for blends of EOs (Hyldgaard et al., 2012). EOs individually (Kirkpinar et al., 2014) or in blends (Franz et al., 2010; Khattak et al., 2014), have been evaluated as possible antimicrobials in pig and poultry diets, with the

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aim of obtaining better inhibition of pathogenic bacteria and the improvement of animal performance. The use of EOs as an antimicrobial agent presents the additional advantage of decreasing the possibility of antimicrobial resistance appearance, since EOs are made up of different chemical compounds which may have different antimicrobial modes of action (Burt, 2004).

The control of pathogenic bacteria by antimicrobials is important, but cannot be considered as the only way to obtain good animal production performance. It is known that an optimal composition of the gastrointestinal microbiota is necessary for animal intestinal health, which allows for achieving an immune status against harmful agents such as pathogenic bacteria (Choct, 2009). Among microorganisms that compose the animal gastrointestinal microbiota are the so called beneficial bacteria or probiotic, such as *Lactobacillus* and *Bifidobacterium*, whose benefic action is the inhibition of pathogenic bacteria by competitive exclusion (Sugiharto, 2014). Several strains of beneficial microorganism are already used in animal feed (Gaggìa et al., 2010). Because of this, it is desirable that EOs are selective, exerting an antibacterial action against pathogenic, and not against benefic bacteria from animal gastrointestinal tract (Ouwehand et al., 2010). Thus, both EOs and beneficial bacteria can together contribute to the inhibition of pathogenic bacteria. In this context, the present study aimed to evaluate, *in vitro*, the antibacterial activity of different EOs, individually and in binary blends, on pathogenic and beneficial bacteria that can occur in the gastrointestinal tract of pigs and poultry. Twenty- eight EOs from different sources were evaluated for their antimicrobial activity. Twenty of them were obtained from different vegetal species and eight were obtained from different steps of a commercial orange juice production line.

2. Materials and methods

2.1. Source of materials

Twenty-eight EOs were studied, from which twenty were obtained directly from leaves or rhizomes of different vegetal species, as shown in Table 1. The remaining eight EOs were comprised by-products from orange juice production and were supplied by a factory in São Paulo State, Brazil. These oils were named by the factory as follows: Orange Peel Oil, Oil Five Fold, Orange Oil Phase Essence, Orange Terpens (Brazilian orange terpens), Thaiti Lime Water, Thaiti Lime Oil Phase, Thaiti Lime Peel Oil, CitrusTerpens.

2.2. Essential oil extraction

The 20 EOs from leaves or rhizomes were obtained by 4-h hydrodistillation using the standard Clevenger apparatus. The ratio of vegetal material and distilled water was 1:5 (volume/weight). The volume of EOs obtained were measured, dehydrated by passing through anhydrous sodium sulfate, and then stored in an amber vial kept under refrigeration (4 °C) and protected from light, until their use.

2.3. Bacterial strains

All bacterial strains evaluated were standard cultures from the American Type Culture Collection (ATCC). For this study, bacteria were classified in potentially pathogenic or beneficial, according to their importance to pigs and poultry production. The potentially pathogenic bacteria evaluated were *Salmonella* Enteritidis ATCC 13076, *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Listeria innocua* ATCC 33090 and *Enterococcus faecalis* ATCC 29212. The beneficial bacteria evaluated were *Bacillus subtilis* ATCC 6623, *Lactobacillus plantarum* ATCC 8014 and *Lactobacillus rhamnosus* ATCC 7469.

Table 1
Plant species used in the experiment for EOs extraction.

Plant species	Plant material	Origin
Baccharis dracunculifolia *Cordia verbenácea.*	Leaves	Campinas – São Paulo/Brasil 22°53'36.3"S 47°03'55.6"W
Cymbopogon citratus, Lippia alba, Thymus vulgaris, Lippia sidoides.	Leaves	Campinas – São Paulo/Brasil 22°47'42.5"S 47°06'40.4"W
Pimenta pseudocaryophyllus	Leaves	Registro – São Paulo/Brasil 24° 29′ 16″ S, 47° 50′ 38″ W
Eucalyptus camaldulensis, Eucalyptus crebra, Eucalyptus exserta, Eucalyptus glóbulos, Eucalyptus grandis, Eucalyptus staigeriana, Eucalyptus urograndis, Eucalyptus urophylla, Corymbia citriodora	Leaves	Itatinga – São Paulo/Brazil 22°59'34.8"S 48°41'14.4"W
Zingiber officinale	Rizhomes	Piracicaba- São Paulo/Brazil 22°43′31″S 47°38′57″O
Melaleuca alternifolia, Melaleuca leucadendron	Leaves	Piracicaba- São Paulo/Brazil 22°43′31″S 47°38′57″O
Mintostachys mollis	Leaves	Cochas-El Tambo/Huancayo-Junín/Peru 12°00'31.0"S 75°11'49.2"W

2.4. Antibacterial activity

2.4.1. Initial screening

All EOs were initially screened to measure their antibacterial activity by disk diffusion method, following the standard protocol M02-A11 from the Clinical and Laboratory Standards Institute (CLSI, 2012). This screening was carried out using, as a potential pathogenic bacterium, *Salmonella* Enteritidis ATCC 13076 and, as a beneficial bacterium, *Lactobacillus plantarum* ATCC 8014.

EOs solutions were prepared at 90% (v/v), using acetone as an emulsifier to improve the EOs dispersion. *S.* Enteritidis and *L. plantarum* were grown on TSA agar for 18–24 h (Tryptic Soy Agar-Difco) and MRS agar for 48 h, respectively. Isolated colonies of each bacteria were transferred to tubes containing sterile saline (0.85%) until it reached an optical density within 0.08–0.1 abs, at 625 nm, which corresponds to 0.5 McFarland standard, therefore containing $\sim 10^8$ CFU/mL (CLSI, 2012). After this, Mueller Hinton Agar plates (*S.* Enteritidis) or MRS Agar plates (*L. plantarum*) were inoculated with the respective bacterial inoculum. Seven microliters of each EO solution (90% v/v) were placed on a 6-mm diameter sterile paper disc (Whatman N° 3), which was transferred to the inoculated agar plate. Three discs with the oil solution were placed in each plate; one disc of streptomycin (10 μg/disk) was used as a positive control, and one disk of acetone (10 μL) was used as a negative control after its non-antimicrobial activity had been proved. The agar plates were incubated at 37 °C for 24 h for *S.* Enteritidis and at 30 °C for 48 h for *L. plantarum*. Inhibition zone diameters (IZD) were measured