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Short communication: Interaction of the isomers carvacrol and thymol with the antibiotics doxycycline and tilmicosin: In vitro effects against pathogenic bacteria commonly found in the respiratory tract from calves

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ABSTRACT

Bovine respiratory disease is the major problem faced by cattle, specially calves, leading to reduced animal performance and increased mortality, consequently causing important economic losses. Hence, calves must be submitted to antibiotic therapy to counteract this infection usually initiated by the combination of environmental stress factors and viral infection, altering the animal's defense mechanism, and thus allowing lung colonization by the opportunistic bacteria *Mannheimia haemolytica* and *Pasteurella multocida.* Essential oils appear to be candidates to replace antibiotics or to act as antibiotic adjuvants due to their antimicrobial properties. In the present study, we aimed to evaluate the 4 essential oil components carvacrol, thymol, trans-anethole, and 1,8 cineole as antibacterial agents or as adjuvants for the antibiotics doxycycline and tilmicosin against *M. haemolytica* and *P. multocida.* Bacteria were cultured according to standard protocols, followed by the determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration. A checkerboard assay was applied to detect possible interactions between components, between antibiotics, and between components and antibiotics. Doxycycline at 0.25 and 0.125 μg/mL inhibited the growth of *P. multocida* and *M. haemolytica,* respectively, whereas tilmicosin MIC values were 1.0 and 4.0 μg/mL for *P. multocida* and *M. haemolytica*, respectively. Carvacrol MIC values were 2.5 and 1.25 m*M* for *P. multocida* and *M. haemolytica*, respectively, whereas thymol MIC values were 1.25 and 0.625 m*M* for *P. multocida* and *M. haemolytica*, respectively. Trans-anethole and 1,8 cineole did not present any antibacterial effect even at 40 m*M* against the investigated pathogens. All minimum bactericidal concentration values were the same as MIC, except when thymol was tested against *M. haemolytica*, being twice the MIC data (i.e., 1.25 m*M* of thymol). Based on fractional inhibitory concentration checkerboard assay, no interaction was observed between doxycycline and tilmicosin. Carvacrol and thymol presented an additive effect when one of them was combined with tilmicosin. Additive effect was also observed when doxycycline was combined with thymol. Synergism was observed when carvacrol was combined with doxycycline or with thymol. Although the antibacterial effects of the tested essential oil components were observed at high concentrations for in vitro conditions, the additive and synergic effects of carvacrol and thymol with antibiotics suggest the option to apply them as antibiotic adjuvants.
Key words: bovine respiratory disease, essential oils, macrolide, tetracycline

Short Communication

Bovine respiratory disease (**BRD**) remains one of the major factors playing a role in economic losses in dairy cattle industry, and is commonly associated with environmental stress and viral infections, combined with severe pneumonia caused by the opportunistic gram-negative bacteria *Pasteurella multocida* and *Mannheimia haemolytica* (Crouch et al., 2012). Both bacteria are members of the *Pasteurellaceae* family, and are naturally found in bovine upper respiratory tract. Stress conditions and viral infections predispose to an inefficient immunodefense in calves, and as consequence, *P. multocida* and *M. haemolytica* proliferate, colonizing the upper and the lower respiratory tract, resulting in lung infections (Roier et al., 2013). Although vaccines are commercially available, not all of them show potential results on BRD prevention (Aubry et al., 2001; Purtle et al., 2016). This is probably due to the fact that a very limited number of bacteria serotypes are targeted by vaccines, leading to their failure. Hence, therapeutic intervention is often chosen to

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counteract BRD. Two widely used antibiotics are the macrolide tilmicosin (Crepieux et al., 2016) and the tetracycline doxycycline, which is lipophilic with a high oral bioavailability (Khamesipour et al., 2014). Both antibiotics have a good penetration and accumulation in lung tissue (Cunha et al., 1982; Modric et al., 1999) and are commonly used by practitioners (De Briyne et al., 2014). After tetracyclines, macrolides are the most used antibiotics and are the first-line treatment especially against *M. haemolytica* (Zaheer et al., 2013), but it is important to note that BRD is not limited to this bacterium. *Mannheimia haemolytica* resistance to doxycycline can reach 18% of bacterial clinical isolates from cattle suffering BRD (Katsuda et al., 2009), but no resistant *P. multocida* isolates were found (Khamesipour et al., 2014). Although *M. haemolytica* and *P. multocida* resistance to tilmicosin has been considered uncommon (McClary et al., 2011), recent indications were found of extra pathways leading to macrolide resistance by these bacteria (Olsen et al., 2015). To counteract antibiotic resistance, the antibacterial properties of essential oils are commonly claimed (Burt, 2004), especially by their ability to act as antibiotic adjuvants and optimize the antibiotic effect (Langeveld et al., 2014; Yap et al., 2014). In the present study, we selected 4 essential oil components (carvacrol, thymol, trans-anethole, and 1,8 cineole) with claimed antibacterial effect, at concentrations ranging of 0.0001 to 33 m*M*, based on a scientific literature survey (see Supplemental Table S1; https://doi.org/10.3168/jds.2016-11536). Carvacrol and thymol appear as the most efficient terpenes against bacteria (Andrade-Ochoa et al., 2015). Besides being found in the oil of thyme, carvacrol is also encountered in oregano oil together with trans-anethole and 1,8 cineole, both also with antibacterial activity (Dadalioglu and Evrendilek, 2004). As no information was found on *P. multocida* and *M. haemolytica*, we checked for the essential oils activity against different bacteria to select the concentration range. The in vitro antibacterial activity of these essential oils was evaluated at different concentrations (0.078–40 m*M*) to determine if they could be useful as alternative or as adjuvants for the antibiotics doxycycline and tilmicosin against *M. haemolytica* and *P. multocida.*

All bacterial strains were purchased from the American Type Culture Collection (**ATCC**; Mercatorstr. 51, Wesel, Germany). *Pasteurella multocida* (ATCC 51689) and *M. haemolytica* (ATCC 33398) were plated on tryptone soya agar slants at 4°C (tryptone soya broth + 1% bacteriological agar, Oxoid, Waltham, MA). Bacteria were grown in the nutrient-rich medium brain-heart infusion medium (**BHI**) at 37°C with 150 rpm shaking for 20 h under aerobic conditions, before starting the experiment.

The compounds used for the assays were carvacrol (98%), trans-anethole (99%), 1,8 cineole (99%), thymol (>99.5%), doxycycline (99%), and tilmicosin (98%). All of these compounds were obtained from Sigma-Aldrich, Zwijndrecht, the Netherlands.

Essential oil components were tested at concentrations of 0.078, 0.156, 0.3125, 0.625, 1.25, 2.5, 5, 10, 20, or 40 m*M* in BHI medium. Doxycycline was tested at concentrations of 0.0039, 0.0078, 0.0156, 0.03125, 0.0625, 0.125, 0.25, 0.5, or 1 μg/mL in BHI medium. Tilmicosin was tested at concentrations of 0.0625, 0.125, 0.25, 0.5, 1, 2, 4, 8, or 16 μg/mL also in BHI medium. All these concentrations were selected based on scientific literature, considering essential oils with antibacterial activity (Dadalioglu and Evrendilek, 2004; Li et al., 2014; Du et al., 2015) and antibiotics used for therapy in calves (McClary et al., 2011; Goldstein et al., 2012). Both antibiotic groups are indicated in Europe to treat respiratory diseases (De Briyne et al., 2014). Vials containing oil preparations were kept sealed and refrigerated when not in use. All oil-containing vials were vortexed for 15 s before starting each trial. For each bacterial species, 4 replicates of every treatment were tested at every concentration with positive (BHI medium + bacteria) and negative (only BHI medium) controls. Replicates were randomized by date, bacterium, and treatment to minimize experimental bias.

Portions of 100 μL increasing concentrations of the chosen test compounds (i.e., carvacrol, trans-anethole, 1,8 cineole, thymol, doxycycline, or tilmicosin in BHI) were placed in 96-well microplates. Aliquots (100 μL) of each bacterial suspension (*P. multocida* or *M. haemolytica*) were added to achieve a bacterial density of 10^5 cfu per well (200 μL). The plates were incubated at 37°C with shaking. At the end of culture, the bacterial optical density was measured at 655 nm wavelength. The MIC was determined as the lowest concentration at which no bacterial growth was measured. For determination of the minimum bactericidal concentration (**MBC**), 10-μL portions from microplate wells showing no bacterial growth were plated out onto BHI agar (BHI broth + 1% wt/vol bacteriological agar, Oxoid) and incubated for 24 h at 37°C. The MBC was the lowest concentration at which no viable bacteria could be cultured. Each experiment was carried out 4 times in quadruplicates.

To detect any synergic, additive, or antagonistic effect between the test compounds and the selected antibiotics, checkerboard assays were carried out whereby increasing concentrations of one compound were placed in the rows and increasing concentrations of the other in the columns of a microplate (Fassi Fehri et al., 2007). In brief, a culture of each strain was incubated with 2-fold dilutions of antibiotic-antibiotic, antibiotic-