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 of calves

W. Kissels,*¹ X. Wu,† and R. R. Santos‡

*ECOSTyle B.V., 8431 SM Oosterwolde, the Netherlands

†Hubert Department of Global Health, Rollins School of Public Health, Emory University, Atlanta, GA 30322

‡Institute for Risk Assessment Sciences, Division Veterinary Pharmacology, Pharmacotherapy and Toxicology, Faculty of Veterinary Medicine, Utrecht University, 3584 CM Utrecht, the Netherlands

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 mM for *P. multocida* and *M. haemolytica*, respectively, whereas thymol MIC values were 1.25 and 0.625 mM for *P. multocida* and *M. haemolytica*, respectively. Trans-anethole and 1,8 cineole did not present any antibacterial effect even at 40 mM 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 mM 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 it 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

Received May 30, 2016.

Accepted October 25, 2016.

¹Corresponding author: w.kissels@ecostyle.nl

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 from 0.0001 to 33 mM, 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 mM) 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 soy agar slants at 4°C (tryptone soy 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 mM 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-

compound, or compound-compound associations from $4 \times \text{MIC}$ to $\text{MIC}/16$. After adding bacteria, plates were incubated for 24 h at 37°C with shaking, and the inhibition of growth was monitored by measuring optical density values and comparing with control (only BHI medium). The fractional inhibitory concentrations (FIC) were calculated from these results using the following formulae:

$$\text{FIC (A)} = \frac{\text{MIC (A in the presence of B)}}{\text{MIC (A)}}, \text{ and}$$

$$\text{FIC (B)} = \frac{\text{MIC (B in the presence of A)}}{\text{MIC (B)}},$$

where A represents each tested compound and B represents each antibiotic.

The FIC index for each combination was then calculated as the sum of the FIC from both test compounds. The results were interpreted as synergistic when the FIC index for the combination was less than or equal to 0.5, as additive when the index was between 0.5 and 1.0, as indifferent when the index was between 1.0 and 2.0, and as antagonistic when the index was greater than 2.0 (EUCAST, 2000). An additive effect means that the combined effect will result from the sum of each individual effect, synergy means that the combined effect is greater than the sum of each individual effect, and antagonism occurs when the sum of each individual effect results in a lower effect (Yap et al., 2014).

The MIC and MBC values for carvacrol, thymol, 1,8 cineole, and trans-anethole, as well as for the antibiotics doxycycline and tilimicosin, are shown in Table 1. Doxycycline at 0.25 and 0.125 µg/mL and tilimicosin at 1.0 and 4.0 µg/mL inhibited the growth of *P. multocida* and *M. haemolytica*, respectively. Carvacrol MIC values were 2.5 and 1.25 mM for *P. multocida* and *M. haemolytica*, respectively, whereas thymol MIC values were 1.25 and 0.625 mM for *P. multocida* and *M. haemolytica*, respectively. The 1,8 cineole and trans-anethole did not present any antibacterial effect even at 40 mM. All MBC values were the same of MIC, except when thymol was tested against *M. haemolytica*, being twice the MIC data (i.e., 1.25 mM of thymol).

The MIC values for the antibiotics against both bacteria were within the range as previously reported in the literature [i.e., 0.06–0.5 µg/mL for doxycycline (Goldstein et al., 2012) and ≤16 µg/mL for tilimicosin (McClary et al., 2011)]. Essential oils are often indicated as alternatives to synthetic antibiotics, due to their antimicrobial activity, as well as low risks of side effects or bacterial resistance (Lindeman et al., 2014). However, such application only becomes true if these compounds

are used at high concentrations as feed (Melo et al., 2015) or food additives (Wu et al., 2015), or topically administered (Lindeman et al., 2014). Their effective concentrations as antibiotics are not realistic for other routes of administration, as confirmed in the present study. However, this cannot exclude their potential application as antibiotic adjuvants, which appear as promising new therapeutic application. For instance, recently it was demonstrated that although 0.5 mM carvacrol cannot directly affect viability of *Salmonella typhimurium*, it inhibits bacterial invasion in intestinal porcine cells (Burt et al., 2016).

The FIC of carvacrol, thymol, as well as of the antibiotics doxycycline and tilimicosin are shown in Table 2. Based on the obtained data, no interaction was observed between doxycycline and tilimicosin as previously reported in the literature (Womble et al., 2006). No antibacterial effects of trans-anethole and 1,8-cineole against both bacterial strains was observed, different from previous reports (Kubo and Fujita, 2001; Hendry et al., 2009). It could be argued that these components may present other effects than direct antibacterial activity, for instance acting as immunomodulators (Sadlon and Lamson, 2010). Therefore, only carvacrol and thymol were selected for the checkerboard assay together with the antibiotics. Carvacrol and thymol presented an additive effect when one of them was combined with tilimicosin. Additive effect was also observed when doxycycline was combined with thymol. Synergism was observed when carvacrol was combined with doxycycline or with thymol. Synergic effects of carvacrol and thymol was observed when these compounds were combined with organic acids against *Salmonella typhimurium* (Zhou et al., 2007). Recently, synergic interactions were shown between doxycycline and terpenic components such as carvacrol (Valcourt et al., 2016). Considering that thymol is also a terpene, a synergic interaction of thymol–doxycycline could be expected. However, differences in chemical structure may

Table 1. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values for the 4 tested compounds and the 2 antibiotics against *Pasteurella multocida* and *Mannheimia haemolytica*

Tested compound	<i>P. multocida</i>		<i>M. haemolytica</i>	
	MIC	MBC	MIC	MBC
Doxycycline (µg/mL)	0.25		0.125	
Tilimicosin (µg/mL)	1.00		4.00	
Carvacrol (mM)	2.50	2.50	1.25	1.25
Thymol (mM)	1.25	1.25	0.625	1.25
1,8 Cineole (mM)	>40	NA ¹	>40	NA
Trans-anethole (mM)	>40	NA	>40	NA

¹NA: not applicable.

Table 2. Fractional inhibitory concentration (FIC) values of the components against *Pasteurella multocida* and *Mannheimia haemolytica*

Combination ¹	Bacterial strains	FIC	FIC index	Remark
Dox + Til	<i>P. multocida</i>	0.25/1.0	1.25	Indifferent
	<i>M. haemolytica</i>	0.5/0.5	1.0	Indifferent
Dox + Cvc	<i>P. multocida</i>	0.125/0.125	0.25	Synergy
	<i>M. haemolytica</i>	0.0625/0.0625	0.125	Synergy
Dox + Thy	<i>P. multocida</i>	0.5/0.0625	0.5625	Additive
	<i>M. haemolytica</i>	0.5/0.0625	0.5625	Additive
Til + Cvc	<i>P. multocida</i>	0.5/0.0625	0.5625	Additive
	<i>M. haemolytica</i>	0.0625/0.5	0.5625	Additive
Til + Thy	<i>P. multocida</i>	0.0625/0.5	0.5625	Additive
	<i>M. haemolytica</i>	0.5/0.25	0.75	Additive
Cvc + Thy	<i>P. multocida</i>	0.25/0.125	0.375	Synergy
	<i>M. haemolytica</i>	0.25/0.125	0.375	Synergy

¹Dox = doxycycline; Til = tilmicosin; Cvc = carvacrol; and Thy = thymol.

explain the fact that thymol has an additive effect with doxycycline. It was reported that both carvacrol and thymol enhance tetracycline effects against *Staphylococcus aureus* by inhibiting the activity of efflux pumps, thus modulating drug resistance (Cirino et al., 2014). No information was obtained regarding interaction of tilmicosin with essential oils against pathogenic bacteria. The mechanisms involved in these additive and synergistic effects remain unknown.

The new concept of combining essential oils with conventional drugs leads to a reduced effective dose of antibiotics when treating infections, which means decrease in the adverse effects of antibiotics. More importantly, antibiotics associated with essential oils may present a different and more potent mechanism of action, and thus avoiding bacterial resistance (Yap et al., 2014). The combination of essential oils with antibiotics that present synergistic effect provides a basis for the development of novel therapy against multi-drug-resistant bacteria. Drug combinations was already claimed as a potential strategy for controlling bacterial resistance (Bollenbach, 2015), which means that the combination proposed herein is promising. As essential oils alone do not have a specific target against bacteria, resistance risk is negligible (Langeveld et al., 2014).

In practice, excretion of essential oils via the lungs can occur, although no specific information is given for bovine. For instance, it is claimed that pulmonary excretion of thymol induces an increase in mucus secretion and ciliary movement of the bronchi, resulting in local antiseptic and antibacterial effects (Brinckmann and Lindemann, 2004). However, excretion of thymol as well as other essential oils such as 1,8-cineole is considered negligible without clinical consequences (Grisk and Fischer, 1969).

In conclusion, we show in the present study the additive and synergistic effect of the isomers carvacrol and thymol when combined with each other, or with

doxycycline or tilmicosin against *P. multocida* and *M. haemolytica*. These findings indicate the use of both components not as antibacterial substances as such, but as potential adjuvants to minimize or avoid bacterial resistance during antibiotic therapy, especially those related to BRD. The topic about the possible combinations of essential oils and antibiotics is important in the veterinary field, because animals receive them with feed additives. However, this study does not intend to give any economic justification to the selection of such essential oil components, which can be studied further.

ACKNOWLEDGMENTS

This project was funded by ECOstyle B.V. The authors thank Lilian J. L. de Nijs-Tjon (IRAS-Toxicology, Utrecht University), Gertie C. A. M. Bokken (IRAS-Veterinary Public Health, Utrecht University), and Angel D. J. Timman (IRAS-Veterinary Public Health, Utrecht University) for their technical support. None of the authors have a conflict of interest.

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