A vapour phase assay for evaluating the antimicrobial activities of essential oils against bovine respiratory bacterial pathogens

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Significance and Impact of the Study: In this study, we present a new vapour phase assay for evaluating the antimicrobial activities of essential oils (EO) against bovine respiratory pathogens (BRPs). Using this assay, we identified EOs, such as ajowan, thyme and cinnamon leaf, that can effectively inhibit growth of the BRPs Mannheimia haemolytica serotype 1, Pasteurella multocida and Histophilus somni. This is the first study to demonstrate the vapour phase antimicrobial activity of EOs against BRPs.

Keywords
antimicrobial activity, bovine respiratory bacterial pathogens, essential oils, in vitro, vapour phase assay.

Abstract
The objectives of this study were to develop a new assay for the evaluation of the antimicrobial activities of essential oils (EOs) in vapour phase and to demonstrate the antimicrobial activities of commercial EOs against BRPs. To achieve the first objective, a microtube cap containing 100 μl of EO was embedded in an agar plate. An agar plug (diameter 13 mm) inoculated with a bacterial suspension containing 108 CFU per ml was then placed over the cap and incubated at 37°C for 24 h. Subsequently, bacteria were recovered from the agar plug by immersion in 5 ml of broth for 10 min, followed by vortexing for 30 s, and the broths were then plated for enumeration. To demonstrate the usefulness of the assay, nine commercial EOs derived from the following specific plants: ajowan, carrot seed, cinnamon leaf, citronella, fennel, ginger grass, lavender, rosemary and thyme were first evaluated for their vapour phase antimicrobial activities against Mannheimia haemolytica serotype 1. Selected EOs were further tested against Pasteurella multocida and Histophilus somni. The EOs of ajowan, thyme and cinnamon leaf completely or partially inhibited BRPs growth. This new assay provided reproducible results on the vapour phase antimicrobial activities of EOs against BRPs. These results support further study of EOs as a potential mitigation strategy against BRPs.

Introduction
Bovine respiratory disease is a leading cause of morbidity and mortality in feedlot cattle, contributing $1 billion in annual losses to the North American beef industry (Miles 2009). The main bacterial respiratory pathogens (BRPs) associated with the disease include Mannheimia haemolytica, Pasteurella multocida, Histophilus somni and Mycoplasma bovis. As part of control strategies, antibiotics are commonly used to prevent (metaphylaxis) and treat bovine respiratory disease (Checkley et al. 2010; USDA, 2013). However, despite the use of antibiotics and vaccines, the prevalence of bovine respiratory disease has increased (Hilton 2014). Recent studies have also indicated that multidrug resistance in BRPs has expanded to encompass the main antibiotics used to treat bronchopneumonia in North American feedlots (Klima et al. 2014). Thus, there is an impetus to develop novel antimicrobial agents to mitigate BRPs. EOs extracted from aromatic and medicinal plants have been demonstrated to inhibit the growth of human respiratory pathogens in vitro (Fabio et al. 2007; Cermelli...
Essential oils inhibit bovine respiratory bacteria

et al. 2008) and have a broad range of antimicrobial activities against both Gram-negative and Gram-positive bacterial pathogens. For example, EOs including cinnamon bark, thyme and spotted beebalm inhibited the growth of Streptococcus pyogenes and S. pneumoniae in vitro (Inouye et al. 2001; Li et al. 2014). Interestingly, EOs exhibit minimal effects on lactic acid bacteria including Lactobacilli and Bifidobacteria, and therefore may have limited negative effects on beneficial bacteria within the host microbiota, when they are administered to target pathogens (Hawrelak et al. 2009).

EOs exhibit their antimicrobial activities in both liquid and vapour phases. Agar diffusion and dilution assays are most commonly used for testing the liquid phase antimicrobial activities of EOs. However, the accuracy of these assays is often compromised by active volatile compounds, and poorly diffusive insoluble compounds of an EO in the agar medium, as well as by the addition of solvents (Griffin et al. 2010). Increasingly, the evaluation of the vapour phase antimicrobial activity of EOs has been recognized as a more reliable method for determining their antimicrobial activity (Goni et al. 2009; Nedorostova et al. 2009). Disc volatilization, which is a simple modification of disc diffusion assay used for antibiotic susceptibility testing (Lopez et al. 2005), and agar vapour (Inouye et al. 2006) assays are often used to determine the vapour phase antimicrobial activity of EOs. However, limitations such as providing only a qualitative measurement, testing a single EO in one concentration with one agar plate and requiring sealing of the agar plate, hinder their application in screening larger quantities of EO samples. To date, there are no standardized vapour phase assays available for evaluating the antimicrobial activities of EOs (Al-Yousef 2014; Bueno 2015). The objective of this study was to develop a new assay for the evaluation of the antimicrobial activities of EOs in vapour phase and to demonstrate the antimicrobial activities of commercial EOs against BRPs.

Results and discussion

Visual assessment of the vapour phase assay

In the present study, we developed a new agar plug vapour phase assay for evaluating the antimicrobial activities of EOs (Fig. 1). This assay was tested to evaluate commercial EOs for their antimicrobial activities against M. haemolytica serotype 1 (S1), P. multocida and H. somni. This new assay provided both qualitative and quantitative measurements on the vapour phase antimicrobial activities of EOs. Following 24 h of exposure to an EO, the agar plugs seeded with pathogens were examined visually for the growth of bacterial cells. If the pathogen was not inhibited or was only partially inhibited by an EO, a bacterial lawn or individual colonies were observed on the surface of the agar plug respectively (Fig. 2). If the pathogen was inhibited completely, the surface of the agar plug was clear. No viable colonies were detected from these clear agar plugs. If the screening purpose is to identify qualitatively those EOs that inhibit the growth of target pathogen, then visual observation on agar plug after a 24-h incubation period is sufficient and no further steps are required. However, for EOs that only partially inhibit pathogenic bacteria, enumeration of bacterial cells recovered from the agar plug can be used to measure the extent of inhibition. This was performed on M. haemolytica S1, P. multocida, and H. somni using several EOs.
Quantitative assessment of vapour phase antimicrobial activities of EOs against \textit{M. haemolytica} S1, \textit{P. multocida} and \textit{H. somni}

Among the nine commercial EOs tested, the ajowan and thyme EOs completely inhibited the growth of \textit{M. haemolytica} S1, with no colonies being observable for enumeration (Fig. 3). A moderate inhibition of \textit{M. haemolytica} S1 was observed with ginger grass (reduction of 2.2 log\textsubscript{10} CFU) and cinnamon leaf (reduction of 3.7 log\textsubscript{10} CFU per agar plug) compared to the control (P < 0.05). In contrast, the growth of \textit{M. haemolytica} S1 was not affected by any of the other EOs tested including fennel, rosemary, carrot seed, lavender and citronella (P > 0.05).

The four EOs (ajowan, thyme, cinnamon leaf and ginger grass) that inhibited the growth of \textit{M. haemolytica} S1 were also tested against \textit{P. multocida} and \textit{H. somni}. Similar to \textit{M. haemolytica} S1, \textit{P. multocida} was completely inhibited by ajowan and thyme EOs (Fig. 4a). Cinnamon leaf and ginger grass EOs reduced the growth of \textit{P. multocida} by 6.8 and 2.4 log\textsubscript{10} CFU per agar plug respectively. Ajowan, thyme and cinnamon leaf EOs completely inhibited the growth of \textit{H. somni} (Fig. 4b). Moderate inhibition of \textit{H. somni} growth was also observed with ginger grass EO.

Ajowan and thyme EOs completely inhibited the growth of all BRPs tested. Ajowan EO has been reported to strongly inhibit the growth of Gram-negative bacteria such as \textit{Escherichia coli} and \textit{Klebsiella}, and the Gram-positive bacterium \textit{Staphylococcus aureus} in liquid phase (Sharifi-Mood et al. 2014). Although there is limited information on its vapour phase antibacterial properties, the vapour phase of ajowan has recently been reported to display antifungal activity (Kim et al. 2016). Similar to our observation, Dobre et al. (2011) observed vapour phase antimicrobial activities of thyme EO against several Gram-positive and Gram-negative bacterial species. The liquid phase antimicrobial activities of thyme EO against a wide range of bacterial pathogens have also been
documented (Melo et al. 2015). The major volatile compound of ajowan and thyme EOs used in the present study was thymol (46.5 and 58.5% respectively) (Amat et al. unpubl. data). The vapour of this compound has previously been shown to inhibit the growth of foodborne bacterial pathogens (Zhou et al. 2013). Hence, it is possible that inhibition of BRPs by ajowan and thyme EOs observed in the present study were in part attributed to the presence of the thymol.

Cinnamon leaf EO has been reported to inhibit Bacillus cereus in its vapour phase (Dobre et al. 2011), in addition to moderate inhibition of several Gram-negative and Gram-positive bacteria (Lopez et al. 2007). In our study, Cinnamon leaf EO displayed moderate (M. haemolytica S1, P. multocida) to complete (H. somni) growth inhibition, indicating that susceptibility to this EO is variable in BRPs. The different susceptibility of the BRPs towards cinnamon leaf EO might be due to the different cell membrane structure of these pathogens. Both M. haemolytica and P. multocida are encapsulated, whereas H. somni is nonencapsulated (Sandal et al. 2011). The lack of cell capsule in H. somni might attribute the higher susceptibility of H. somni towards cinnamon leaf EO compared to M. haemolytica and P. multocida.

Ginger grass EO showed a weaker inhibition against all three BRPs compared to ajowan, thyme and cinnamon leaf EOs. The antimicrobial activity of ginger grass EO has been less well characterized. Overall, ajowan, thyme and cinnamon leaf EOs all inhibited the growth of M. haemolytica, P. multocida and H. somni in their vapour phase, suggesting the potential of use of these EOs as a nasal spray to mitigate the colonization and proliferation of BRPs in the respiratory tract of cattle.
Currently, disc volatilization and agar vapour assays are used for evaluating vapour phase antimicrobial activities of EOs (Bueno 2015). For disc volatilization, an agar plate seeded with bacteria is inserted upside down on top of a container, and a paper disc containing an EO is placed at the bottom of the container before sealing with parafilm. Current agar vapour assays use a paper disc containing an EO placed on an agar plate seeded with bacterial culture. The paper disc is then surrounded by a plastic ring and covered by a piece of glass, followed by sealing with vinyl tape and inversion. In both assays, the agar plates are incubated at 30°C for 24 h prior to measurement of the inhibition zones and minimum inhibition dose for each EO. Our assay has an advantage over these two assays in that it provides a quantitative measurement. Compared to zone inhibition measurements, the viable cell counting method provides data on reduced growth potential and whether the EO antimicrobial effect is biostatic or biocidal. In addition, because the bacterial cells exposed to EO vapour are easily recovered from the agar plug, this assay can also be used for the assessment of morphological damage of bacterial cells by using microscopy. The enumeration aspect of the proposed new assay may also be applicable for testing EOs against bacterial pathogens that are not easily visible. For example, M. bovis colonies are relatively small and translucent, and as such, the measurement of the inhibition zones after exposure to an EO is challenging. Finally, our assay enhances throughput for evaluating the antimicrobial vapour effects of EOs. We were able to analyse four caps on a single agar plate. This enabled us to test several replicate samples of each EO against a pathogenic bacterium, or to test one EO sample against several different bacterial pathogens at once.

In conclusion, the new agar plug-based vapour phase assay presented in this study was found to be simple, provided low variation, and adaptable for the primary screening of EOs for their antimicrobial activity against bacterial pathogens. Ajowan, thyme and cinnamon EOs all showed vapour phase antimicrobial activity against M. haemolytica S1, H. somni and P. multocida. This is the first study to demonstrate the vapour phase antimicrobial activities of commercial EOs against BRPs. Future studies are needed to determine whether the application of these EOs as a nasal spray can mitigate BRPs, as alternatives to antibiotics.

Materials and methods

Essential oils

Nine commercially available EOs were evaluated for their antimicrobial activity in vapour phase against BRPs (Table 1). The EOs were purchased from Essential Hydrosol (Brampton, ON, Canada) and stored at 4°C until use. All EOs were first evaluated for antimicrobial activity against M. haemolytica S1. Four of the EOs that inhibited growth of M. haemolytica S1 (ajowan, cinnamon leaf, ginger grass and thyme) were then further tested against P. multocida and H. somni.

Bacterial strains and culture conditions

Bovine respiratory bacterial pathogens used in this study were M. haemolytica S1 (LO24A), P. multocida (Y2680-011NA) and H. somni (G1999-027NA). M. haemolytica S1 strain was previously isolated from a feedlot beef steer that died of pneumonia in Alberta, Canada (Klima et al. 2011). The P. multocida and H. somni strains were previously isolated from the nasopharynx of morbid feedlot

Table 1 List of essential oils and their properties

| EO name (abbreviation) | Botanical name         | Country of origin | Plant part extracted | Main constituents†            |
|------------------------|------------------------|-------------------|----------------------|-----------------------------|
| Ajowan (AJO)           | Carum copticum         | India             | Seed                 | Thymol, γ-Terpene and Cymene|
| Carrot Seed (CAR)      | Daucus carota          | China             | Seed                 | β-Bisabolene and Asarone    |
| Cinnamon Leaf (CIN)    | Cinnamomum verum       | Sri Lanka         | Leaf                 | Eugenol                     |
| Citronella (Java) (CITR)| Cymbopogon winterianus| Indonesia         | Leaf                 | Citronellal and Geranole    |
| Fennel (sweet) (FEN)   | Foeniculum vulgare dulce| Hungary          | Seed                 | Anethole and Fenchone       |
| Ginger Grass (GEN)     | Cymbopogon martini var sophia | India | Grass                | Geraniol and Limonene       |
| Lavender (LAV)         | Lavandula angustifolia | France            | Flower               | Linool and Linyl acetate    |
| Rosemary (ROS)         | Rosmarinus officinalis | Spain             | Leaf                 | Camphor, α-Pine and Eucalyptol|
| Thyme (THY)            | Thymus zygis           | Spain             | Leaf                 | Thymol and Cymene           |

*The information listed in this table (except for main constituents of essential oils) was obtained from the manufacturer (http://www.essentialoilsSCANADA.ca/).
†Main constituents of essential oils were obtained from Gas Chromatography-Mass Spectrometry (GC-MS) analysis. The GC-MS analysis was performed according to Yap et al. (2014).
cattle diagnosed with bronchopneumonia in Alberta, Canada. Bacterial cultures were grown on tryptic soy agar (TSA) with 5% sheep blood (Dalynn Biologicals, Calgary, AB, Canada) and incubated overnight (M. haemolytica and P. multocida) or 36 h (H. somni) at 37°C. A single colony from each plate was inoculated into 5 ml of brain heart infusion broth (Oxoid, Nepean, ON, Canada) and incubated for 18 h at 37°C with shaking at 200 rev min⁻¹. A 100-µl aliquot of each culture adjusted to 1 x 10⁸ CFU per-ml was plated on TSA blood agar and incubated at 37°C for 1 h. These plates were then used for the vapour phase assays.

Agar plug-based vapour phase assay

A schematic representation of the vapour phase assay is shown in Fig. 1. A maximum of four holes (10 mm in diameter) were pressed through a TSA blood agar plate using a sterile hollow punch (Tekton - 12 PC Hollow Punch Set – 6588). A sterile cap from a 1.5-ml disposable/conical freestanding microtube (VWR, Mississauga, ON, Canada) was tightly embedded into each hole in the agar. The cap consisted of an inner and outer compartment and 100 µl of an EO was placed in the inner compartment of the cap. For the control, 100 µl of BHI broth was used instead of an EO. Agar plugs (from the plated bacteria described above) were prepared using a second sterile hollow punch (13 mm in diameter). These plugs were removed using forceps, placed on the top of an embedded cap containing EO with the surface covered in bacteria facing outwards, and incubated at 37°C for 24 h. The space between the EO and agar plug, allowed for the EO to evaporate. Following incubation, the agar plugs exposed to the EO vapour were examined visually for growth. The agar plug was then removed with sterile forceps and immersed in 5 ml of BHI broth for 10 min, followed by vortexing for 30 s to recover the bacterial cells from the agar plug. Tenfold dilutions of the bacterial cell suspensions were plated on TSA blood agar to test cell viability and enumerate the bacteria. The inhibition activity of EO in vapour phase was determined by comparing bacterial counts from the agar plug exposed to EO to the BHI control, with a limit of detection set at five colonies per plug. Each test was performed in triplicate. To avoid the interference of EO vapour, the enumeration results for the controls were obtained from control agar plugs that had been placed in a separate agar plate.

Statistical analysis

Data were analysed as a one-way analysis of variance (ANOVA) using Proc Glimmix in SAS (ver. 9.4, SAS Institute Inc, Cary, NC). The LSMEANS statement was used to compare means and significance was declared at P < 0.05.

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Conflict of Interest

No conflict of interest declared.

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