

Bovine Respiratory Disease Pathogen Inhibition by Tea Tree Oil (*Melaleuca alternifolia*) Vapours using a Novel Micro Vapour assay

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Abstract

Bovine respiratory disease (BRD) is a multifactorial disease primarily affecting beef cattle in high intensity feedlot environments and is a significant economic burden to the global industry. The disease is caused by a plethora of bacterial and viral pathogens, which can be treated by a variety of anti-microbials or vaccinations. The emergence of anti-microbial resistance is an increasing challenge to combatting BRD and natural product-based therapeutics may provide alternative or synergistic treatment strategies.

In this study, we demonstrate the effects of vapours generated from Australian Tea tree, *Maleluca alternafolia*, oil (TTO) and a nanoemulsion of TTO (nTTO) against the bacterium *Mannheimia haemolytica* and Bovine Herpesvirus 1 using a novel vapour-based assay. *Mannheimia haemolytica* was sensitive to TTO with a mean minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) of 0.078% and 0.115%, respectively. The mean MIC and MBC for nTTO were 0.104% and 0.118%, with those for oxytetracycline were 0.3125 µg/ml and 0.5 µg/ml. Vapours generated from both TTO and nTTO completely inactivated Bovine herpes virus 1 over 24-hours.

The micro vapour-based assay can be applied to measuring both bactericidal and virucidal effects allowing for rapid screening of essential oils and natural products against a range of respiratory pathogens.

Introduction

Bovine Respiratory Disease (BRD) is a multifactorial disease in cattle, predominantly in a feedlot environment and was previously known as 'Shipping' fever highlighting the contribution of the transportation period for young animals to transmission and stress (Ferraro et al. 2021; Poulsen and McGurik. 2009). Common bacterial pathogens associated with BRD include *Mannheimia haemolytica*, *Pasteurella multocida*, *Histophilus somni* and *Mycoplasma bovis*, whilst viral pathogens such as Bovine herpes type 1 (BHV1), Bovine Respiratory Syncytial Virus (BSRV), Parainfluenza 3 virus (PI3) and Bovine viral diarrhea virus (BVDV) can cause respiratory infections and immune suppression promoting bacterial infections (Guadino et al. 2022). A variety of clinical symptoms are associated with BRD including coughing and changes in breathing pattern, nasal and ocular discharge, fever and reduced appetite (Kamel et al. 2024). BRD has significant economic impact on the cattle industry accounting for up to 75% of mortality in feedlot cattle resulting in estimated losses of \$800 to \$900 million per year in North America (Peel. 2020), and in the Australian feedlot cattle industry losses of \$60 million per year (Blakebrough-Hall et al. 2020).

Common antibiotics used to treat BRD include tilmicosin, tetracyclines, oxytetracycline, and tulathromycin, and metaphylaxis of high-risk cattle has been shown to reduce mortality and improve herd health (Cameron and McAllister. 2016). The increasing prevalence of anti-microbial resistance (AMR) of common BRD pathogens contributes to increased morbidity and mortality in affected cattle, which in turn results in increased treatment costs. (Wilhelm et al. 2023).

The challenges of AMR have resulted in the development of alternative treatment approaches for BRD including screening natural products effective against common BRD bacterial pathogens.

Alternative treatment approaches may include the vapour phase of essential oils (EOs) derived from a range of plants which in one study have shown inhibitory activity against *M. hemolytica*, *P. multocida* and *H. somni* (Amat et al. 2017), where selected EOs derived from ajowan (*Trachyspermum ammi*), thyme (*Thymus vulgaris*) and cinnamon (*Cinnamomum cassia*) leaf showed varying levels of inhibitory activity. Subsequently, EOs from Ajowan, cinnamon leaf, citronella (*Cymbopogon nardus*), grapefruit (*Citrus paradisi*), fennel (*Foeniculum vulgare*) and thyme were investigated to assess their ability to modulate the bovine nasopharyngeal microbiota with ajowan, fennel and thyme having an effect (Amat et al. 2024). The antiviral effects of the selected EOs were tested against Bovine viral diarrhoea virus 1, with thyme showing the strongest effects.

A further study using a disc diffusion assay study demonstrated a number of EOs including cinnamon, lemongrass (*Cymbopogon* sp.) and thyme showed activity against *M. hemolytica* (Bismarck et al. 2022)

Nanoemulsion technology is a promising approach to enhance the properties of EOs. Antibacterial activity of a nanoemulsion of *Camphora longepaniculata* against *Escherichia coli* and *Staphylococcus aureus* was recently demonstrated (Yan et al 2025). A previous study described the formulation of a nanoemulsion of Australian tea tree (*Maleleuca alternifolia*) oil (TTO) with anti-inflammatory, antioxidant and diabetic activities (Sharma et al. 2023). Previously, TTO has demonstrated a range of potential anti-infective properties ranging from anti-bacterial (Carson et al. 2006), anti-parasitic (Lam et al. 2020) and anti-viral (Garozzo et al. 2009). Nanoemulsions of TTO (nTTO) have also been previously developed including enhanced delivery and of neomycin to skin and antibacterial effects (Elsewedy et al. 2022) and increased activity against AMR bacteria (Wei et al. 2022). Hybrid nanoemulsions have also been fabricated to enhance antibacterial effects against drug resistant strains, which have included combination of TTO with silver nanoparticles (Najafi-Taher et al. 2018) or Chitosan (Olivera et al. 2024).

In this study, we describe the formulation and characterisation of nTTO using ultrasonication and describe a novel micro vapour assay to determine antibacterial and virucidal effects. We tested the novel assay against common BRD causative pathogens, *M. haemolytica* and Bovine herpes virus 1.

Methods

2. MATERIALS AND METHODS

2.2. Preparation of bacteria Inoculum

Colonies of *M. haemolytica* were inoculated into 10 ml of Brain Heart Infusion (BHI) broth and incubated at 37°C with shaking overnight to obtain log phase growth. This culture broth was used to prepare a bacterial suspension with turbidity equivalent to a 0.5 McFarland standard, which contains

approximately 1 to 2 x 10⁸ colony forming units (CFU)/ml. Prior to experimental use, this was further diluted 1:150 to produce a working suspension of approximately 1 x 10⁶ CFU/ml.

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

The standard broth dilution method was used for measuring the MIC and MBC of TTO and nTTO. Briefly, two-fold serial dilutions of the test liquid were prepared with final test concentrations of TTO (10.4%, 5.2%, 2.6%, 1.3%, 0.65%, 0.325%, 0.16%, 0.081%, 0.041%, 0.02%), nTTO (4%, 2%, 1%, 0.5%, 0.25%, 0.125%, 0.0625%, 0.0313%, 0.0156%, 0.0078%) and mixed with bacterial suspensions at 0.5 McFarland standard to produce a final bacterial inoculum of approximately 5 x 10⁵ CFU/ml. Oxytetracycline (512, 256, 128, 64, 32, 16, 8, 4, 2, 1, 0.5, 0.25, 0.125, 0.0625) µg/ml was also included. Medium without antimicrobial agents was used as a negative control, while medium without antimicrobial agents inoculated with bacteria served as a positive control. To ensure no separation of oil from media and adequate aeration, test vials were incubated at 37°C for 24 hours on a rotating mixer. The MIC was determined by the absence of visible microbial growth. Bacterial suspensions with test agent concentrations no less than the MIC were plated onto blood agar and incubated at 37°C on a further 24 hours. The MBC was determined by the concentration of test agent that produced no visible colonies after subculture (Tan et al. 2019; Qi et al. 2021). The MIC and MBC were obtained from at least three independent experiments performed in duplicates.

2.4. Preparation of tea tree oil nanoemulsion

Tea tree oil extracted from *Maleluca alternafolia* was kindly donated from AgriFutures Australia. A 12.5% Nanoemulsion of TTO was prepared by combining the TTO with 5.9% (w/v) soy lecithin granules (macro wholefoods) and 2% (v/v) Poloxamer 188 solution (Sigma Aldrich) made up with distilled water and stirred at room temperature overnight. Once dissolved, the mixture was further subjected to probe-type ultrasonication on ice with the following cycle for a 25 ml sample (amplitude: time (mins)) 20:2.5; 40:2.5; 50:2, 60: 3.

2.5. Characterisation of nanoemulsion by droplet diameter, zeta-potential and cryo-electron microscopy.

The droplet diameter of nanoemulsions and non-sonicated control emulsions were measured using laser diffraction (Mastersizer 2000 Hydro, Malvern Instruments Ltd) at 25°C. The droplet diameter was represented as the surface-weighted mean diameter.

For characterisation of TTO Nanoemulsion by cryo-electron microscopy, the nTTO samples were diluted 1 in 10 in pure water and 4 µl aliquots were added to holey carbon coated 3 mm copper grids and hand blotted for 6 secs before vitrification in liquid ethane using a Leica GP2 freeze plunger. Micrographs were collected on a JEOL F200 CR Transmission Electron Microscope at 200kV with a K3 camera using a Gatan ELSA side entry holder.

2.6. Bovine herpes virus 1

Bovine herpes virus 1 (BHV1) was maintained in Madin-Darby bovine kidney cells (MDBK) cultured at 37°C with 5% CO₂ in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% foetal bovine serum and penicillin/strep [1mg/ml] with viral stocks kept at -80°C. The standard 50% tissue culture infectious dose (TCID₅₀) assay was used to measure infectious viral titre. Upon recovery of viral particles after vapour treatment by the Micro-Vapour assay, the virus-media solutions were serially diluted 10-fold in a 96-well plate. The virus dilutions were then added to MDBK pre-seeded overnight. The cells were assessed for cytopathic effects (CPE) by microscopy.

2.7. Micro-Vapour Assay

The newly developed Micro-Vapour assay is a scalable in vitro method for measuring the antibacterial and anti-viral properties of vapours from volatile organic compounds such as EOs. For fastidious bacteria, such as *M. haemolytica* blood agar is used, or a solid version of BHI can be prepared by addition of agar powder.

For virus assays, sterile paper discs were cut for a tight fit in the microtube lids in place of a solid media plug. For application to bovine herpes virus 1, we added 15 µl of BHV1 stock to each filter paper disc. This volume was selected based on the TCID₅₀ methodology for determining viral titre. Briefly, after 24 hours of TTO vapour or PBS control exposure using the Micro-Vapour Assay, the control and viral-loaded discs were placed in a microcentrifuge tube containing 270ul of cell culture media and vortexed for 30 seconds. Serial dilutions of the recovered virus were made and 15ul of each dilution was added to MDBK cells cultured in a 96 well format. Five replicates were performed for each dilution and the TCID₅₀ was calculated using the Reed Muench method

To test the effect of TTO and nTTO vapour on the agar substrate, plugs were exposed to vapour at undiluted, 10%, 1%, 0.1% and 0.01% solutions and PBS as a control for 24 hours at 37°C. The lids containing the vapour-exposed plugs were removed and transferred to a petri dish and seeded with 5 µl of 0.5 McFarland standard bacteria suspension and incubated for 48 hours. Visible growth was recorded after incubation. Plugs showing no growth as well as the PBS control were soaked and vortexed in 1ml of BHI broth, removed and the broth further incubated for 24 hours with shaking and examined for turbidity. The workflow of the Micro-Vapour assay is detailed in Fig. 2

2.8. Statistical analysis

Mann-Whitney U-test was used to identify any statistical differences between the MIC of TTO and nTTO and the MBC of these. This method was selected as visual inspection of the histogram of the data showed that parametric assumptions of normality and homoscedasticity (variances are not equal in each group) were not met. Statistical significance level was set at $p = 0.05$. Analysis was conducted using GraphPad Prism 10.2.0.

RESULTS

3.1. Particle Sizing of Tea Tree Oil Nanoemulsion

Particle size analysis of the non-sonicated control emulsion (Fig. 1A) revealed a broad peak up to 100 μm , which upon ultrasonication was resolved to an average particle size of 200 nm typical of a nanoemulsion (Fig. 1B) and with an associated zeta potential of 72.5 mV (Fig. 1C). Cryo-electron microscopy revealed the morphology of nTTO as spherical droplets with a unique rosette structure and an average size range of up to 200nm (Fig. 1D, E).

3.2. Development of a Micro-Vapour Assay Workflow

Briefly, microtubes (1.5 ml) were filled with 1.3 ml of test liquid (Fig. 2A). A blood agar plate was lawn inoculated with 100 μl of *M. haemolytica* suspension at 0.5 McFarland standard and incubated at 37°C for 1 hour. After incubation (at this stage no visible colonies would be seen), circular plugs of inoculated blood agar were cut using a sterile microtube lid as a standardised cutter. Using sterile forceps, the agar plug was transferred to another cap without disturbing the surface, ensuring the inoculated side faced outwards (Fig. 2A). This was closed onto a microtube containing the test liquid, forming a standardised distance between the liquid and agar surface for vapour exposure for 24 hours at 37°C. After incubation, the lids were visually examined for growth. For enumeration of bacterial growth post-vapour exposure, each plug was aseptically removed using a sterile small tip and transferred to into 1 ml of BHI (Fig. 2B). After soaking for 10 minutes and vortexed for 30 seconds. Plugs noted with visible growth were further serially diluted 10^2 , 10^3 , 10^4 for enumeration of colony forming units per millilitre (CFU/ml). With a calibrated loop, 10 μL of the BHI broth was lawn inoculated onto blood agar and incubated for 24 hours at 37°C. On day 3, colonies were counted for quantification of growth.

3.3. Effects of Tea Tree Oil and Nanoemulsion Tea Tree Oil Vapour on *Mannheimia haemolytica*

Volatile vapour from undiluted, 10%, 1% TTO and nTTO inhibited the growth of *M. haemolytica* after overnight incubation. Visible colonies were observed after exposure to vapour from 0.1% and 0.01% solutions as well as the PBS control (Fig. 3).

After harvesting bacterial cells from the agar plugs post-vapour exposure, further testing was performed to determine whether the vapour effects were inhibitory or bactericidal.

Figure 3 should appear here in the text

3.4. Determination of Minimum Inhibitory Concentration (MIC) and Minimum bactericidal concentration (MBC)

In this study, *M. haemolytica* was sensitive to TTO with a mean MIC and MBC of 0.078% and 0.115%, respectively. The mean MIC and MBC for nTTO were 0.104% and 0.118%, with those for oxytetracycline

were 0.3125 µg/ml and 0.5 µg/ml. No statistically significant difference was found between the MIC of TTO and nTTO with a difference of medians of 0.044 ($p = 0.14$). Likewise, no statistically significance difference was found between the median MBC of TTO and nTTO with a difference of medians of 0.022 ($p = 0.95$).

3.5. Virus inactivating effects of TTO and nTTO

In duplicate experiments, using the microvapour assay, vapours from both TTO and nTTO completely inactivated bovine herpes virus 1 at a titre of when exposed for 24hrs from a titre of approximately 5.37×10^5 TCID₅₀ units.

3.6. Effects of Vapour from TTO and nTTO on Growth Substrate

Blood agar exposed to high concentrations (10% and undiluted) of TTO and nTTO did not support microbial growth. Medium exposed to vapour from 1% solutions did not produce visible growth as observed in PBS control, 0.1% and 0.01% solutions for both TTO and nTTO. Although, upon subculture into fresh medium, bacteria growth was visible from the agar plugs exposed to the 1% solution after 24hrs, but not at 10% and undiluted. Similar observations were noted for BHI solid agar.

DISCUSSION

Nanoemulsions are colloidal dispersions composed of water, oil and surfactant formed by mechanical forces with particle sizes at the nano scale commonly between 20–500 nm but may be as high as 1000 nm (Kale et al. 2016).

Pratap-Singh et al (2021), investigated the effects of sonication amplitude, treatment time and sample volumes and reported that the optimal ultrasonication process time remains constant for hemp oil and olive oil and tween 80, where a 10 min ultrasound treatment produced the same nano-particle size as a 60 min ultrasonication.

Our study used a sonication volume of 50 ml of tea tree oil emulsion and found that 9–10 mins of ultrasonication produced consistent uniform nanodroplets. The zeta potential of -72.5 mV indicates a stable Nanoemulsion as (Che Marzuki et al. 2019).

A variety of methods have been used to characterise nanoemulsions including measurement of size and charge. Electron microscopy approaches including TEM and cryo-EM have also been applied to essential oil nanoemulsion characterisation including *Lavandula agustifolia* (Miastkowska et al. 2023) and Thymol (Kumari et al.2023). Tea tree oil nano emulsions have been developed which enhanced the action of encapsulated antibiotics against multi drug resistant antibiotics (Wei et al. 2022) and formulation of a nTTO using high speed shearing (Han et al. 2023) has shown antibacterial activity against *S. aureus* and *E. coli*.

We noted a discolouration of the blood agar medium from opaque red to opaque brown (i.e. appearance similar to chocolate agar) after exposure to vapour liberated from undiluted tea tree oil and signs of haemolysis from opaque red to transparent red for concentrations of TTO and nTTO above 1%. Such discolouration is likely attributed to the denaturation of haemoglobin in the erythrocytes. To discern whether this impacted the medium's capacity to support microbial growth, a comparison study was performed for subjecting blood agar to vapours from various concentrations of TTO and nTTO to control, prior to adding bacteria inoculum. Our observation showed that substrate exposed to vapour from TTO and nTTO at > 10% concentrations failed to support microbial growth in blood agar and solid BHI. This may be due to an adsorption effect of the volatile organic compounds from TTO into the matrix of the substrate, as evident by the detection of TTO scent when the plugs were removed from the lids after vapour exposure. This potential alteration of agar properties has not been examined in previous studies that investigated the antimicrobial properties of TTO against bacteria (Amat et al. 2017).

In Conclusion the presented micro-vapour assay is a potential in vitro screening method for the antimicrobial effects of vapour liberated from volatile substances such as EOs, against bacteria and viruses. The method is relatively cheap and utilises readily accessible and standardised plasticware

Declarations

Data Availability Statement

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

1: Animal Ethics

Not Applicable, No Animals were used in this study

2: Funding

Agrifutures Australia

3: Data availability: All data supporting the findings of this study are available within the paper and can also be provided by the authors upon request.

4: Consent to Participate: Not Applicable

5: Consent for publication: All authors have provided consent for publication

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Figures

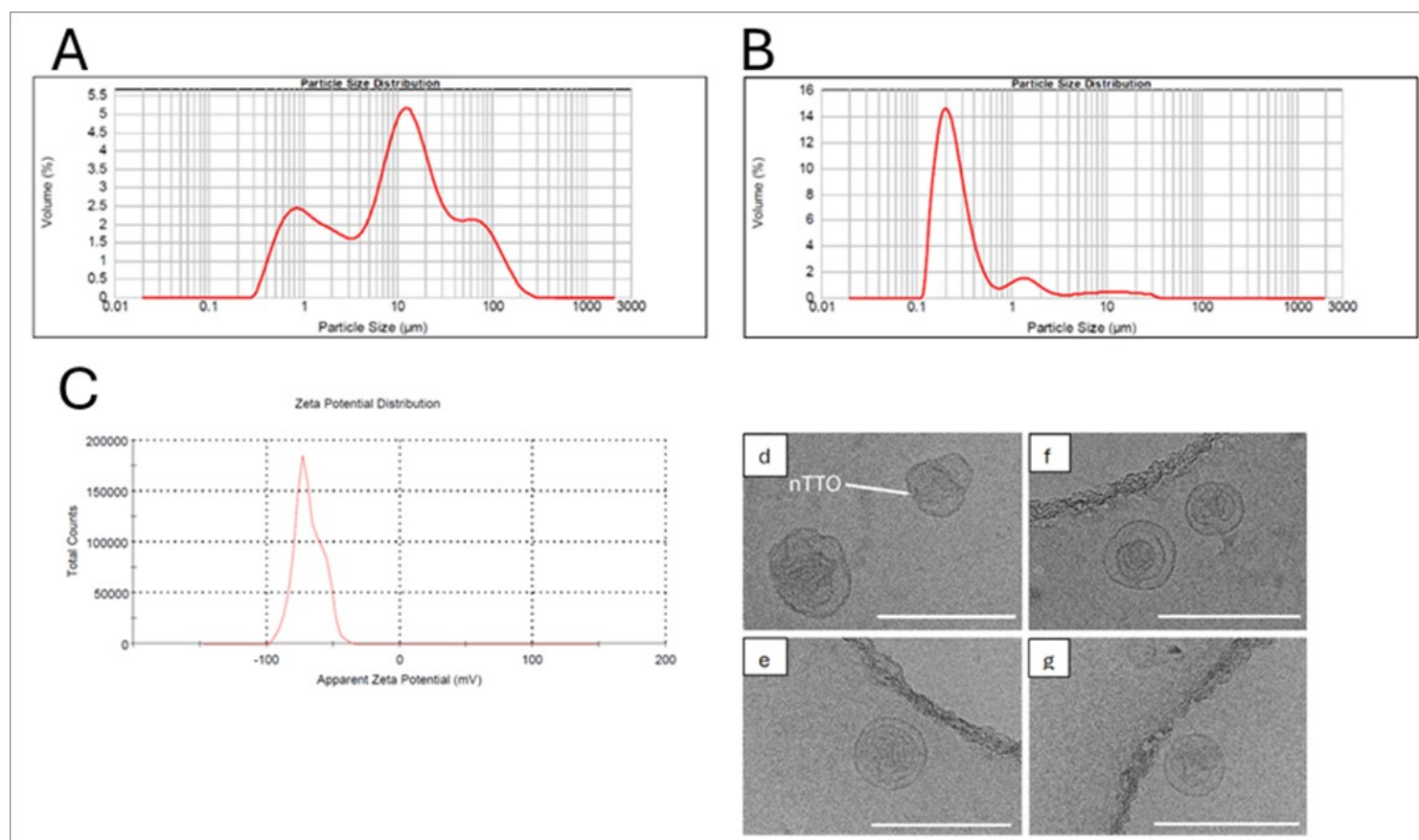


Figure 1

(A-G): Characterization of nano-emulsion tea tree oil solution before(a) and after (b) ultrasonication by particle size distribution and measurement of zeta (c) potential distribution and analysis by cryo-electron

microscopy of the ultrasonicated nano-emulsion (d-g).

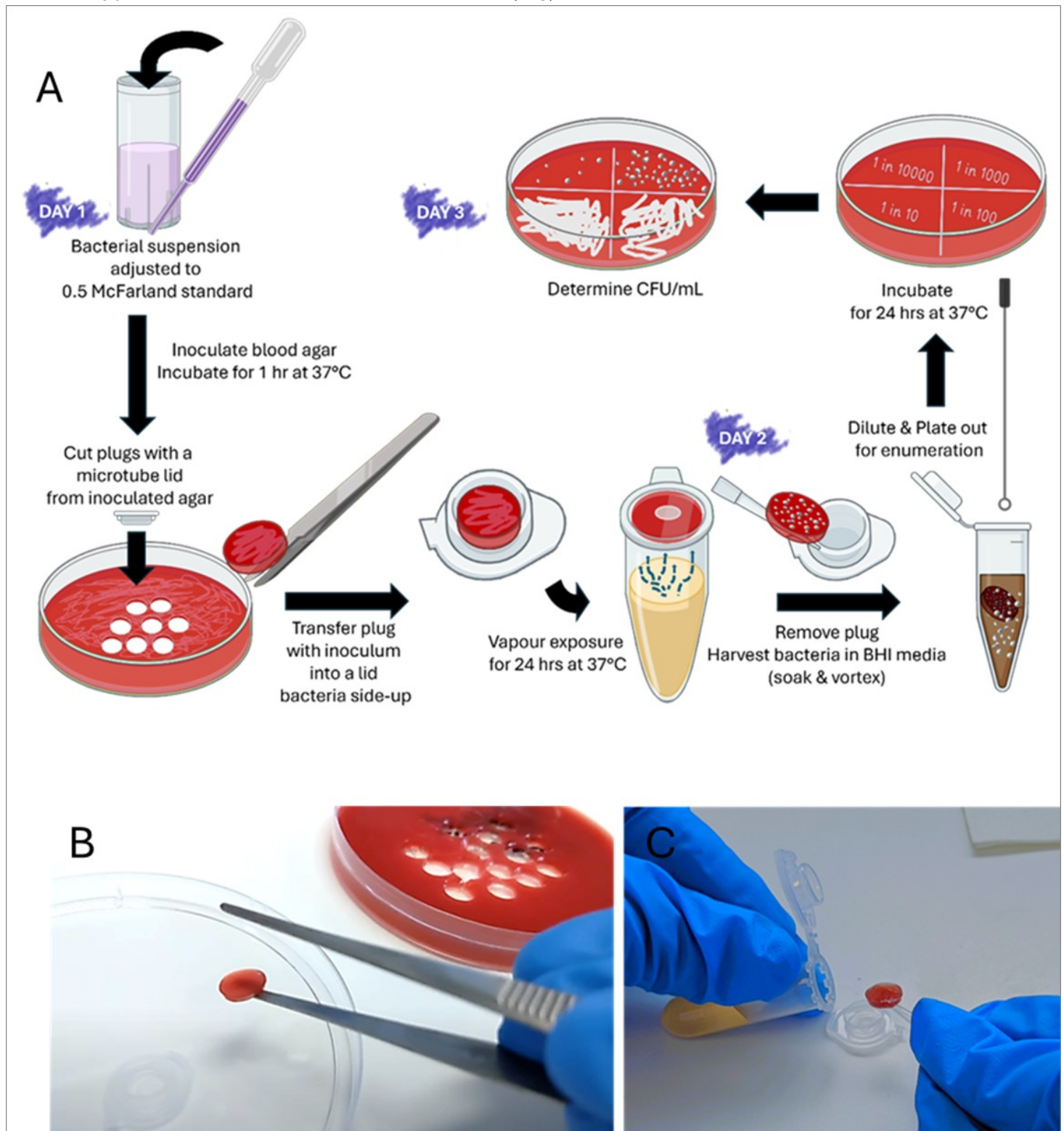


Figure 2

(A-B): Workflow of the Plug-Vapour assay. BHI = Brain heart infusion; CFU = colony forming units.

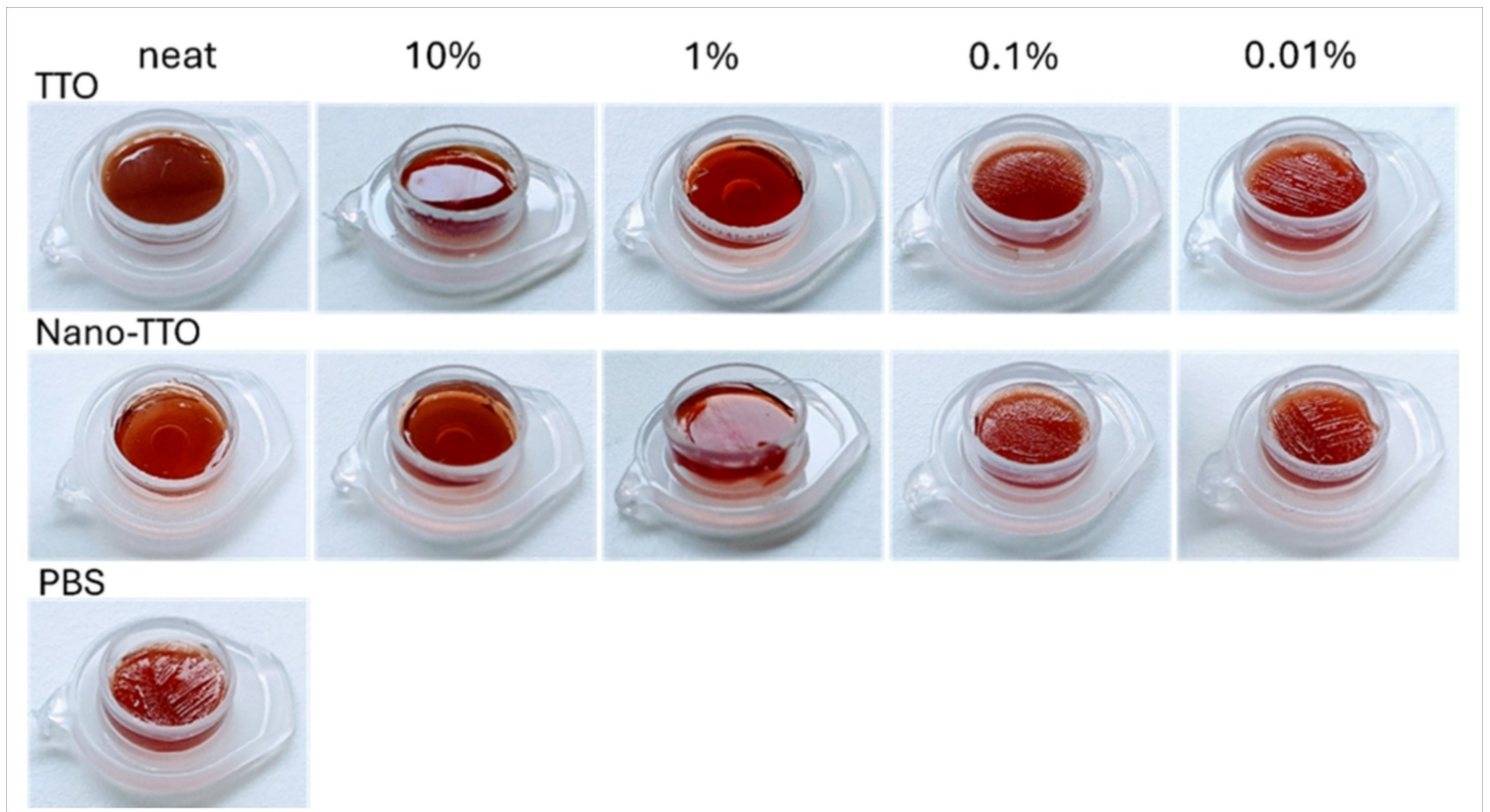


Figure 3

Growth of *Mannheimia haemolytica* after 24-hour vapour exposure from tea tree oil (TTO) and nano-emulsion (nTTO) at various concentrations. Control included vapour from phosphate buffered saline (PBS).