**Antimicrobial activity of a novel bioengineered honey against non-typeable *Haemophilus influenzae* biofilms: an *in vitro* study**

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**ABSTRACT**

The opportunistic pathogen non-typeable *Haemophilus influenzae* (NTHi) plays an important role in many chronic respiratory diseases including otitis media, chronic rhinosinusitis, cystic fibrosis, and chronic obstructive pulmonary disease. Biofilm formation has been implicated in NTHi colonisation, persistence of infection, and recalcitrance towards antimicrobials. There is therefore a pressing need for the development of novel treatment strategies that are effective against NTHi biofilm-associated diseases. SurgihoneyROTM is a honey-based product that has been bioengineered to enable the slow release of H2O2, a reactive oxygen species to which *H. influenzae* is susceptible. Treatment of established NTHi biofilms with SurgihoneyROTM significantly reduced biofilm viability through enhanced H2O2 production, and was shown to be more effective than the conventional antibiotic co-amoxiclav.

**INTRODUCTION**

Non-typeable *Haemophilus influenzae* (NTHi) is an opportunistic pathogen that plays an important role in a number of chronic diseases including otitis media, chronic rhinosinusitis, chronic obstructive pulmonary disorder, and cystic fibrosis. The persistence of NTHi infections is often associated with biofilm formation, where individual bacterial cells form surface-associated aggregates surrounded by a self-produced extracellular polymeric substance. These heterogeneous bacterial populations play an important role in long-term NTHi colonisation of the human respiratory tract and are of significant clinical importance, responsible for causing infections after long periods of quiescence.[1] Bacteria residing within biofilms are protected from host immune responses, are less susceptible to antibiotic treatment, and also have an increased propensity for development of antimicrobial resistance (AMR). Treatment of biofilm-associated infections is therefore challenging, and with the increasing prevalence of AMR there is a pressing need for the development of new treatment strategies.[2]

A diverse range of factors can influence the growth and survival of bacteria within the human host. Understanding the interactions between the various members of the microbiota, their adaptive responses towards the unique environmental niches they occupy, and the role of biofilms in colonisation can provide an avenue for the development of novel treatments. NTHi co-exists and competes with several other bacterial species in the human nasopharynx, however, its interaction with another opportunistic pathogen *Streptococcus pneumoniae*, has attracted the most interest. Co-culture of *S. pneumoniae* with NTHi has been shown to stimulate up-regulation of the pneumococcal virulence factor pyruvate oxidase (SpxB), an enzyme responsible for catalysing the production of hydrogen peroxide (H2O2),[3] whilst increased expression of SpxB has also been demonstrated in monospecies pneumococcal biofilms.[4,5] The production of H2O2 confers *S. pneumoniae* with a competitive advantage over other bacterial species occupying the nasopharynx,[6] and has been shown to reduce *H. influenzae* viability in co-culture.[7]

SurgihoneyROTM is a licensed, CE marked sterile honey that has been bioengineered to enable the controlled release of H2O2 over a prolonged period.[8] This product has been shown to be effective in preventing biofilm formation and treating established biofilms formed by clinically relevant pathogens including *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Klebsiella pneumoniae,* and *Escherichia coli*.[9-11] Based on the susceptibility of NTHi towards exogenous H2O2, we hypothesised that this bioengineered product would be effective in the treatment of biofilms formed by this species.

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions**

Bacterial strains used in this study were isolated from nasopharyngeal swabs of children aged 4 years and under following written informed consent (Southampton and South West Hampshire Research Ethics 06/Q1704/105). NTHi was sub-cultured from frozen stocks onto Colombia agar with chocolated horse blood (Oxoid, UK) and incubated for 18 h at 37oC and 5% CO2, following which colonies were resuspended in Brain Heart Infusion broth supplemented with 10 μg/ml Hemin and 2 μg/ml NAD (sBHI), and grown to mid-exponential phase for experiments. The *Pseudomonas aeruginosa* reference strain PAO1-UW[12] and a clinical methicillin-resistant *Staphylococcus aureus* isolate were subcultured onto Colombia blood agar plates (Oxoid, UK) and grown in BHI.

**Planktonic assays**

Flat-bottomed 96-well plates (Fisher Scientific, UK) were inoculated with ~1.0 x 106 planktonic bacteria per well (grown in supplemented BHI). SurgihoneyROTM and the non-engineered base honey (Acacia) were both prepared in sBHI and added to wells at final concentrations of 6 g/L to 319 g/L. Acacia was sterilised using a 0.22 μm syringe filter. Supplemented BHI alone was added in place of treatments for untreated controls. Cultures were incubated at 37oC and 5% CO2 for 18 hours then turbidity measured by absorbance (OD595) using an EZRead 400 spectrophotometer (Biochrom; n=6).

**Biofilm assays**

Mid-exponential planktonic cultures were used to inoculate individual wells of untreated 6-well polystyrene plates (~1.0 x 108 planktonic bacteria per well; Corning Incorporated, USA). Cultures were incubated at 37oC and 5% CO2 for 48 h, replacing spent media with fresh sBHI (NTHi) or BHI (MRSA, *P. aeruginosa*) at 24 h. Prior to treatment spent media was removed and biofilms washed twice with Hanks’ balanced salt solution (HBSS; Gibco, UK). Biofilms were treated with SurgihoneyROTM or Acacia (both prepared in HBSS) at final concentrations of 7 to 213 g/L. To assess the effect of pH biofilms were treated with HBSS adjusted to pH6.3 (the pH of 71 g/L SurgihoneyROTM in HBSS). For adjuvant assays biofilms were treated with 300 μg/mL amoxicillin and 60 μg/mL clavulanic acid (Co-amoxiclav). HBSS alone was added in place of treatments for untreated controls. Biofilms were incubated at 37oC and 5% CO2 for 2 h, following which the treatments were removed and biofilms washed twice with HBSS to remove unattached cells. Biofilms were resuspended in 1 mL HBSS by cell scraping, briefly vortexed, then serially diluted onto Colombia agar with chocolated horse blood (NTHi) or Columbia blood agar (MRSA, *P. aeruginosa*). Plates were incubated at 37oC and 5% CO2 and colony forming units (c.f.u.) enumerated (n=4).

**Confocal Microscopy**

Mid-exponential planktonic cultures were used to inoculate 35 mm untreated glass-bottom CellView cell culture dishes (~1.0 x 108 planktonic bacteria per well; Greiner Bio One, UK). Cultures were incubated at 37oC and 5% CO2 for 48 h, replacing spent media with fresh sBHI at 24 h. Media was removed, biofilms washed twice with HBSS, then treated with 71 g/L SurgihoneyROTM, 300/60 μg.ml co-amoxiclav or HBSS alone (untreated control) for 2 h at 37oC and 5% CO2. Treatments were removed and biofilms washed twice with HBSS before staining with a LIVE/DEAD Baclight Bacterial Viability Kit (Life Technologies, UK) as per manufacturer’s instructions. Biofilms were examined using an inverted Leica SP8 confocal microscope using a 63x oil immersion lens with sequential scanning of 2 μm sections (Leica Microsystems, UK).

**Hydrogen peroxide measurements**

SurgihoneyROTM and Acacia were prepared at a range of concentrations between 7 to 213 g/L in HBSS and incubated at 37oC and 5% CO2 for 2 h. H2O2 production was then measured using a Fluorimetric H2O2 Assay Kit (Sigma-Aldrich, UK) as per manufacturer’s instructions.

**Statistical analyses**

Statistical analyses were performed using one-way analysis of variance (ANOVA) and Kruskal-Wallis multiple comparisons tests. Comparative data with a P value of ≤0.05 were considered as statistically significant.

**RESULTS**

**SurgihoneyROTM and the non-engineered base honey (Acacia) are equally efficacious against planktonic NTHi**

As *H. influenzae* has previously been shown to be sensitive towards H2O2 produced by *S. pneumoniae* we investigated whether SurgihoneyROTM, on account of its bioengineered H2O2-generating properties, would be effective against planktonic NTHi. SurgihoneyROTM treatment resulted in a dose-dependent response, with a significant reduction in growth at concentrations ≥178 g/L (P≤0.05) and a minimum inhibitory concentration (MIC) of 192 g/L (Fig. 1a). To determine if the bioengineering process increased the antibacterial properties of SurgihoneyROTM, the response towards the non-engineered base honey (Acacia) was also investigated. Acacia demonstrated an antibacterial profile similar to that of SurgihoneyROTM, also resulting in a significant reduction in growth at concentrations ≥178 g/L (P≤0.05) whilst possessing a marginally increased MIC of 255 g/L (Fig. 1a).

**SurgihoneyROTM treatment reduces NTHi biofilm viability through increased H2O2 generation**

Having established that both SurgihoneyROTM and Acacia were both equally as effective in the treatment of planktonic NTHi, the response of the biofilm phenotype was then assessed. Treatment of established 48 h *in vitro* biofilms with 71 and 142 g/L SurgihoneyROTM for 2 h resulted in a 4-log and 3-log reduction in viability respectively (P≤0.05), whilst treatment with 213 g/L reduced viability 5-log (P≤0.01; Fig. 1b). In comparison, treatment with the equivalent concentrations of Acacia had no effect on biofilm viability (P=0.75; Fig. 1b). A dose-dependent increase in H2O2 levels in the surrounding media was also observed when treating with both Acacia and SurgihoneyROTM (Fig. 1c). SurgihoneyROTM, however, produced significantly higher levels of H2O2 at all concentrations tested, ranging from 10.7 - 71.2 μM in comparison with 0.24 - 6.5 μM generated when treating with the equivalent concentrations of Acacia. Furthermore, these data indicate that the minimum concentration of H2O2 effective in reducing NTHi biofilm viability, as evidenced with 71 g/L SurgihoneyROTM, is approximately 25.7 μM (Fig. 1c). To account for a pH-mediated response NTHi biofilms were also treated with HBSS adjusted to pH6.3 (the pH of 71 g/L SurgihoneyROTM), revealing no effect on biofilm viability (Fig. 1d). To confirm that the response was H2O2-mediated biofilms were treated with 71 g/L SurgihoneyROTM in the presence of 2 mg/mL catalase, revealing a reduction in SurgihoneyROTM activity (Fig. 1e).

**SurgihoneyROTM is more effective than co-amoxiclav in the treatment of NTHi biofilms**

The activity of SurgihoneyROTM was compared to the conventional antibiotic co-amoxiclav, and also whether it could improve antibiotic efficacy when used as an adjuvant. Treatment of established 48 h *in vitro* biofilms with 71 g/L SurgihoneyROTM for 2 hours resulted in a 5-log reduction in viability (P=0.029) whereas treatment with 300/60 μg.ml co-amoxiclav had no effect on viability (P=0.343; Fig. 2a). Combined treatment did not improve co-amoxiclav efficacy (Fig. 2a). Confocal laser scanning microscopy confirmed the reduction in biofilm viability of 48h NTHi biofilms following treatment with 71 g/L SurgihoneyROTM for 2 hours, and also the ineffectiveness of co-amoxiclav (Fig. 2b-d). The confocal micrographs also demonstrated that SurgihoneyROTM treatment had no obvious effect on overall biofilm biomass or ultrastructure, with all biofilms 70-80 μm in maximum height (Fig. 2b-e).

**SurgihoneyROTM treatment is ineffective against *in vitro* MRSA and *P. aeruginosa* biofilms**

The same treatment conditions successful in reducing NTHi biofilm viability (71 g/L SurgihoneyROTM for 2 h) were tested on established 48 h *in vitro* biofilms formed by a clinical MRSA isolate and *P. aeruginosa* PAO1-UW to determine whether a similar reduction in viability would be observed (Fig. 3). Treatment of *P. aeruginosa* biofilms with either SurgihoneyROTM or Acacia had no effect on viability (p=0.2539) whereas MRSA biofilms demonstrated a small but significant increase in viability when treating with both formulations (p=0.0286).

**DISCUSSION**

NTHi biofilm-associated diseases manifesting in a number of chronic infective respiratory conditions including otitis media, chronic rhinosinusitis, cystic fibrosis, bronchitis, chronic obstructive pulmonary disease, are inherently resistant to antimicrobials and thus represent a significant challenge in clinical settings. The susceptibility of *H. influenzae* to exogenous H2O2 offers a potential avenue for the development of new treatment strategies. SurgihoneyROTM, through its bioengineered H2O2-releasing properties, represents one such treatment. Interestingly, planktonic NTHi demonstrated similar susceptibility towards both SurgihoneyROTM and the non-engineered base honey Acacia, indicating that the antibacterial properties of Acacia are sufficient against this phenotype in the absence of enhanced H2O2 production. Acacia was, however, ineffective against established NTHi biofilms with viability remaining unaffected when treating with 213 g/L, evidencing the well documented tolerance of the biofilm phenotype. In comparison, SurgihoneyROTM reduced the viability of NTHi biofilms 4-log when treating with 71-142 g/L and 5-log when treating with 213 g/L, confirming that the bioengineering process enhanced the antibacterial efficacy, with 25.7 μM being the minimum effective concentration of H2O2. To overcome the recalcitrant nature of biofilms, it is becoming widely recognised that combination treatments targeting various aspects of the biofilm are required.[2] SurgihoneyROTM was therefore tested as an adjuvant therapy with the conventional antibiotic co-amoxiclav. Whereas treatment with co-amoxiclav alone at a concentration 300 times in excess of the planktonic MIC (1 μg/mL) had no effect on biofilm viability 71 g/L, SurgihoneyROTM produced a 5-log reduction demonstrating its potential as an antibiotic-sparing therapy. When used in combination there was no increase in antibiotic efficacy. This could be attributed to the fact that SurgihoneyROTM had no impact on overall biofilm biomass, thus restricting antibiotic diffusion into the biofilm, or that the surviving cells were tolerant towards both treatments. Interestingly, treatment of *P. aeruginosa* and MRSA biofilms using the same conditions (71 g/L for 2 h) was ineffective, having no effect on *P. aeruginosa* viability, and even increasing MRSA biofilm viability. This is in contrast to a previous study that demonstrated reduced seeding and disruption of biofilms formed by both species.[10] It is worth noting, however, that these biofilms were exposed to SurgihoneyROTM for 24 h as opposed to 2 h in this study. Whilst further validation of these data using biologically relevant models will be required, this study demonstrates a potential role for SurgihoneyROTM as a novel adjuvant treatment in NTHi biofilm-associated infections.

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**Figure 1: SurgihoneyROTM treatment of *in vitro* non-typeable *H. influenzae* biofilms reduces viability in the presence of increasing H2O2 concentrations. (a)** NTHi *in vitro* planktonic cultures were grown in the presence of SurghioneyROTM or Acacia for 18 h then growth assessed by measurement of absorbance (OD595). **(b)** Forty-eight hour *in vitro* NTHi biofilms were treated with SurghioneyROTM or Acacia for 2 h and biofilm viability measured by c.f.u. enumeration. **(c)** Fluorimetric measurements of H2O2 production by different SurgihoneyROTM and Acacia concentrations at 2 h. **(d)** Forty-eight hour *in vitro* NTHi biofilms treated with HBSS adjusted to pH6.3 (equivalent pH to 71 g/L SurgihoneyROTM) for 2 h and biofilm viability measured by c.f.u. enumeration. **(e)** Forty-eight hour *in vitro* NTHi biofilms treated for 2 h with SurgihoneyROTM in the presence of catalase and biofilm viability measured by c.f.u. enumeration. \*P≤0.05; \*\*P≤0.01.

**Figure 2: SurgihoneyROTM is more effective than co-amoxiclav** **in the** **treatment of *in vitro* non-typeable *H. influenzae* biofilms. (a)** Forty-eight hour *in vitro* NTHi biofilms were treated with 71 g/L SurgihoneyROTM and 300/60 μg.ml co-amoxiclav alone, and in combination for 2 hours with viability measured by c.f.u. enumeration. Confocal microscopy was performed on **(b)** untreated 48 h NTHi biofilms, biofilms treated for 2 hours with **(c)** 300/60 μg.ml co-amoxiclav, and **(d)** 71 g/L SurgihoneyROTM. Biofilms were imaged using Live/Dead staining to visualize live cells (green fluorescence) and dead cells (red fluorescence). **(e)** Maximum biofilm height measured using Leica confocal software. \*P≤0.05.

**Figure 3: SurgihoneyROTM was ineffective in the treatment of established methicillin-resistant *S. aureus* and *P. aeruginosa* biofilms.** Forty-eight hour MRSA and *P. aeruginosa* biofilms were treated for 2 h with 71 g/L SurgihoneyROTM or Acacia and biofilm viability measured by c.f.u. enumeration. \*P≤0.05.