**ORIGINAL LABORATORY RESEARCH**

Evaluation of a bio-engineered honey and its synthetic equivalent as novel *Staphylococcus aureus* biofilm-targeted topical therapies in chronic rhinosinusitis

Dionyssia Papadopoulou MD1-3,**\***, Alicja Dabrowska PhD4-5,**\***, Philip G. Harries FRCS(ORL-HNS)3, Jeremy S. Webb PhD4-5, Raymond N. Allan PhD4-5, Rami J. Salib PhD1-3,5

1School of Clinical and Experimental Sciences, Faculty of Medicine, University of Southampton, Southampton, United Kingdom.

2Southampton NIHR Respiratory Biomedical Research Centre, University Hospital Southampton NHS Foundation Trust, Southampton, UK.

3University Hospital Southampton NHS Foundation Trust, Southampton, UK.

4School of Biological Sciences, Faculty of Environmental & Life Sciences, University of Southampton, Southampton, UK.

5National Biofilms Innovation Centre, University of Southampton, Southampton, UK.

**\*These authors contributed equally**.

**Corresponding Author**

Rami J. Salib. Associate Professor of Rhinology, Head of Upper Airway Research Group, Consultant ENT Surgeon, School of Clinical and Experimental Sciences, Mailpoint 810, Sir Henry Wellcome Laboratories, Faculty of Medicine, University Hospital Southampton NHS Foundation Trust, Tremona Road, Southampton SO16 6YD, UK. Phone: +44(0)2380 540276. Fax: +44(0)2380 825688. E-mail address: R.J.Salib@soton.ac.uk

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**ABSTRACT**

**Background** - Chronic rhinosinusitis (CRS) is a common condition which affects the quality of life of millions of patients worldwide and has a significant impact on healthcare resources. Whilst *Staphylococcus aureus* bacterial biofilms play an important role in this disease, antimicrobial therapy is rarely effective and may promote antibiotic resistance. Thus, development of novel biofilm-targeting and antibiotic-sparing therapies is highly desirable and urgently required.

**Objective** - This in vitro study evaluated the antimicrobial activity of a novel synthetic honey-equivalent product which was designed to have the same reactive oxygen release profile as the engineered honey SurgihoneyRO™.

**Methods** - Treatment efficacy was investigated by assessment of planktonic growth, biofilm viability, thickness and biomass, using 12 CRS-related *S. aureus* mucosal bacterial strains.

**Results** - Both SurgihoneyRO™ and the synthetic honey-equivalent product inhibited growth of planktonic methicillin-resistant and -sensitive *S. aureus* strains, with the synthetic honey-equivalent product exhibiting a lower minimum inhibitory concentration. Treatment of established *S. aureus* biofilms reduced biofilm viability with 24 hour treatment resulting in a 2-log reduction in viability of biofilms formed by methicillin-resistant strains, and a 1-log reduction in biofilms formed by methicillin-sensitive strains.

**Conclusions** - This preliminary study shows that the synthetic honey-equivalent product provides marked antimicrobial activity against *S. aureus* biofilms, with the potential for development in the clinical setting as an adjunctive biofilm-targeted therapy in CRS. The ultimate aim of such a product would be to reduce the need for antibiotics, steroids and invasive surgical procedures in CRS patients, as well as improving clinical outcomes following endoscopic sinus surgery.

**INTRODUCTION**

Chronic rhinosinusitis (CRS) with or without nasal polyps (CRSwNP and CRSsNP respectively), is a chronic inflammatory condition of the nose and paranasal sinuses1 presenting with symptoms such as nasal blockage, rhinorrhoea, facial pain or pressure, reduction or loss of smell. It is one of the most common conditions encountered in medicine1. Despite a wide range of medical therapies and surgical procedures undertaken to treat CRS, the condition remains recalcitrant in a large sub-set of patients2-3, affecting quality of life4 and increasing healthcare expenditure5. Thus, there is a large unmet need for the development of new therapeutic strategies for this condition aimed at reducing reliance on antibiotics, steroids and invasive surgical procedures, as well as improving clinical outcomes following endoscopic sinus surgery.

Biofilm is an association of micro-organisms in which microbial cells adhere to each other on a living or non-living surfaces within a self-produced matrix of extracellular polymeric substance. *Staphylococcus aureus (S. aureus)* biofilms are known mediators of the inflammatory reaction in CRS1,6, and are associated with more severe pre-operative disease, persistence of on-going mucosal inflammation and poor post-surgical outcomes7. *S. aureus*, a Gram positive, biofilm-forming bacterium, has previously been detected in sinus mucosal tissues of CRS patients8. A recent observation of *S. aureus* intracellular localization within mast cells of nasal polyps9 suggests possible presence of bacterial reservoirs within host tissues - a potential future therapeutic target. It is estimated that 20% of *S. aureus* clinical isolates are methicillin-resistant (MRSA) and exhibit substantial elevated levels of multidrug resistance as compared with non-MRSA strains10. It is likely that bacterial biofilms within the sinonasal mucosa mediate a continuing inflammatory reaction leading to early disease recurrence, development of chronicity and recalcitrance11. With similar mechanisms in other chronic respiratory infections12, conventional antimicrobial therapy is rarely effective in eradication of CRS bacterial biofilms and may lead to selection for antibiotic resistance13,14. As such the development of alternative biofilm-targeted therapies is both highly desirable and urgently required for a range of biofilm-mediated chronic respiratory conditions including CRS, otitis media, chronic obstructive pulmonary disease, primary ciliary dyskinesia, and cystic fibrosis15.

Honey is one of the oldest known antimicrobials, dating back to ancient times where the Egyptians used it as a natural antibiotic and to treat skin infections. Manuka honey has long been used in the treatment of chronic wounds and leg ulcers16. It is thought that its antibacterial properties are mediated by a combination of hydrogen peroxide accumulation through the inhibition of glucose oxidase, acidity, high osmolarity, and the presence of methylglyoxal17,18. The minimum inhibitory concentrations do vary depending on the bacterial strains and source of the honey. Thus far, two studies have evaluated the clinical use of topical manuka honey in the nose, one in allergic fungal sinusitis19, and the other in active chronic rhinosinusitis with previous sinus surgery20. Both studies showed some therapeutic benefit in those patients receiving the manuka honey.

SurgihoneyRO™ (Matoke Holdings Ltd, UK), a bio-engineered honey comprising gamma irradiated honey and glucose oxidase from *Aspergillus niger*21, has previously been shown to have anti-biofilm activity against a range of wound pathogens22 and the opportunistic pathogen non-typeable *Haemophilus influenzae*23. In the presence of water, glucose oxidase oxidizes glucose present in the natural honey. In the process, hydrogen peroxide, a reactive oxygen species, is released, leading to oxidation of DNA, proteins and membrane lipids in bacteria24. Although addition of glucose oxidase enhances the antimicrobial activity of honey and reduces the effect of natural variability between different batches of honey on the efficacy of the final product, there is a need for a fully standardized, artificial product. Apart from providing a reproducible antimicrobial treatment, which could be used externally and internally, a synthetic formulation would save natural resources and be suitable for patients unable or unwilling to use animal product-based treatments. A synthetic honey-equivalent antimicrobial product was therefore developed which contains glucose oxidase and a substrate for the enzyme to provide antimicrobial activity, but does not introduce substances such as proteins, minerals and traces of pollen present in natural honey. The product, Synthetic RO+, is sterile, has a pH of 7.04 and is composed of fructose (52% weight), glucose, (31%), citric acid/NaOH buffer (17%) and glucose oxidase (1000 ppm)25.

The aim of this study was to evaluate antibacterial activity of SurgihoneyRO™ and a novel synthetic honey-equivalent antimicrobial product against CRS-related mucosal MSSA and MRSA strains. The reported findings underpin the proposed clinical development of a synthetic honey-equivalent antimicrobial product as a novel topical biofilm-targeted adjunctive therapy in chronic respiratory infections, such as CRS.

**METHODS**

All methods were carried out in accordance with relevant guidelines and regulations. All experimental protocols were approved by the Southampton and South West Hampshire Research Ethics Committee (ref - 09/H0501/74). Informed consent was obtained from all participants.

***Bacterial strains and growth conditions***

*S. aureus* strains used in this study were isolated from twelve individual CRSwNP patients and validated by the UK Public Health England Microbiology Services laboratory, Southampton. Five strains displayed methicillin-sensitivity (MSSA1-5) and seven strains – methicillin-resistance (MRSA1-7). All strains were grown on Columbia blood agar plates (Oxoid, UK) for 18 hours at 37°C under a 5% CO2 atmosphere. Single, representative colonies were resuspended in Brain Heart Infusion broth (BHI; Oxoid) and used for further experiments upon reaching mid-exponential phase of growth.

***Planktonic growth***

Engineered honey (SurgihoneyRO™) and the synthetic honey-equivalent product with and without glucose oxidase (Synthetic RO+ and Synthetic RO- respectively) were prepared in BHI in clear flat-bottomed polystyrene 96-well plates (Corning Incorporated, USA) at a range of concentrations (0-383 g/L) previously used to investigate SurgihoneyRO treatment of planktonic non-typeable *Haemophilus influenzae* 23. Wells were inoculated with ~1.0 x 106 of either *S. aureus* MSSA1 or MRSA1 and cultures were incubated at 37°C under a 5% CO2 atmosphere for 18 hours. Growth was assessed by optical density measurements (absorbance at 595 nm; OD595) on EZ Read 400 microplate reader (Biochrom, UK), against a blank of sterile growth medium supplemented with the same concentration of a given product (n=4).

***Biofilm biomass and viability***

*S. aureus* MSSA1 and MRSA1 (~1.0 x 108 planktonic bacteria per well) were grown in BHI in clear polystyrene 6-well plates (Corning Incorporated, USA) at 37°C under static conditions with a 5% CO2 atmosphere. Spent media were replaced with fresh BHI at 24 hours. At 48 hours, spent media were removed and the biofilms washed twice with Hanks’ balanced salt solution (HBSS; Gibco, UK). A range of concentrations of Synthetic RO+ or Synthetic RO- in HBSS (0-142 g/L) were applied to the biofilms and the cultures incubated for a further 24 hours at 37°C under a 5% CO2 atmosphere. Treatments were removed and the cultures manually washed twice with HBSS to remove planktonic cells. The biofilms were disrupted by cell scraping in 1 mL of HBSS.

For assessment of cell viability, scraped cell suspensions were briefly vortexed then serial diluted for spot plating on Columbia blood agar plates. Plates were incubated for 18 hours at 37°C under a 5% CO2 atmosphere for colony forming unit (CFU) enumeration. To assess total biofilm biomass, aliquots of the same cell suspensions were diluted 10-fold in BHI. Total biomass was measured by OD595 on Jenway 6300 spectrophotometer (Cole-Parmer, UK) against a blank of sterile BHI.

Assessment of biofilm viability was also performed for a range of strains of *S. aureus*. Five methicillin-sensitive (MSSA1-5) and seven methicillin-resistant (MRSA1-7) strains were grown as described above, two wells per strain. At 48 hours, spent media were removed, biofilms were washed with HBSS and either HBSS or 71 g/L Synthetic RO+ in HBSS was applied. After 24 hours, biofilms were washed, resuspended, serially diluted and plated for CFU enumeration as described above. All assays were performed using 5 biological replicates.

***Confocal Laser Scanning Microscopy***

Mid-exponential planktonic cultures of *S. aureus* MSSA1 and MRSA1 grown in BHI 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 under static conditions at 37°C with a 5% CO2 atmosphere for 48 hours, replacing spent media with fresh BHI at 24 hours. At 48 hours, BHI was removed and biofilms were washed twice with HBSS. Cultures were treated with 71 g/L Synthetic RO+ in HBSS, 71 g/L Synthetic RO- in HBSS or HBSS (two treatment replicates per strain) and incubated under static conditions for 24 hours at 37°C with a 5% CO2 atmosphere. After 48 hours, treatments were removed and the biofilms were washed twice with HBSS before staining with LIVE/DEAD Baclight Bacterial Viability Kit (Life Technologies, UK) as per manufacturer’s instructions. Biofilms were examined using an inverted confocal microscope (Leica SP8; Leica Microsystems, UK) with 63x oil immersion lens and sequential scanning of 2 μm sections. Images of 5 fields of view per sample were taken to determine average maximum biofilm thickness after 48 hours of treatment.

***Hydrogen peroxide release***

Hydrogen peroxide released by the Synthetic RO+ and RO- products was measured over 24 hours using a Fluorimetric H2O2 Assay Kit (Sigma-Aldrich, UK) as per manufacturer’s instructions. The products were diluted in HBSS to a concentration of 71 g/L (three dilutions per product) and incubated at 37°C under a 5% CO2 atmosphere for 24 hours. Samples were taken every 15 minutes for the first hour, every hour for 7 hours and at 24 hours, flash frozen in liquid nitrogen and stored at -80°C until fluorimetric analysis (n=2).

***Statistical analyses***

Statistical analyses of planktonic and biofilm data were performed using one-way analysis of variance and Kruskal-Wallis tests. The level of significance was represented on graphs using the following star rating: \*p≤0.05; \*\*≤0.01; \*\*\*≤0.001. Error bars on graphs represent +/- 1 standard deviation (SD).

**RESULTS**

***Synthetic RO+ demonstrates antimicrobial activity against planktonic MRSA and MSSA***

Both SurgihoneyRO™ and the novel synthetic honey-equivalent product showed antimicrobial activity against planktonic *S. aureus* (Fig.1). Treatment of *S. aureus* MRSA1 and MSSA1 strains with a range of SurgihoneyRO™ concentrations resulted in an identical minimum inhibitory concentration (MIC) of 383 g/L for both strains. When treated with the same concentrations of the synthetic honey-equivalent product (Synthetic RO+), these strains were shown to have MICs lower than those of the original SurgihoneyRO™ product with MICs of the MRSA1 and MSSA1 strains being 192 g/L and 128 g/L respectively. In comparison, the synthetic honey-equivalent product lacking enzymatic activity (Synthetic RO-) did not inhibit planktonic growth of either strain, instead reducing growth only when treated with >64 g/L (MRSA1) or >6 g/L (MSSA1).

***Synthetic RO+ reduces MRSA and MSSA biofilm viability but increases biomass***

Treatment of established 48 hour *S. aureus* MRSA1 and MSSA1 biofilms revealed that treatment with the Synthetic RO+ product for 24 hours reduced biofilm viability in both instances (Fig.2 a-b). A log-fold reduction in viability was observed when *S. aureus* MRSA1 biofilms were treated with 53 and 71 g/L (p=0.0079), and a 2-log reduction when treated with 142 g/L (p=0.0159). In comparison, only a log-fold reduction was observed when *S. aureus* MSSA1 biofilms were treated with 36-142 g/L (p≤0.05). By comparison, treatment with Synthetic RO- had no effect on viability of biofilms formed by either strain (Fig.2 a-b). Treatment of *S. aureus* MRSA1 biofilms with either 36-71 g/L of Synthetic RO+ or Synthetic RO- for 24 hours resulted in a significant increase in overall biofilm biomass (p≤0.05), whilst all concentrations tested (7-142 g/L) significantly increased the biomass of *S. aureus* MSSA1 biofilms (p≤0.05; Fig.2 c-d). 71 g/L was chosen as the treatment concentration for all subsequent assays on the basis that it caused both a log-fold reduction in viability and an increase in biomass for both the MRSA1 and MSSA1 strain. By comparison, treatment of 48 hour MRSA1 biofilms with 71 g/L SurgihoneyRO™ for 24 hour resulted in a 2-log reduction in viability and an increase in biofilm biomass similar to the Synthetic RO+ product (p≤0.05; Fig. 2e). An increase in maximum biofilm thickness was also observed through confocal imaging when treating the same biofilms with 71 g/L RO+ or RO- for 24 hours (Fig.3 a-b).

***Synthetic RO+ reduces the viability of biofilms formed by different MRSA and MSSA clinical isolates***

Treatment of 48 hour established biofilms formed by several clinical MRSA (n=7) and MSSA (n=5) isolates revealed that the viability of every biofilm was reduced with an average log-fold reduction observed within each group (Fig.4 a-b). A hydrogen peroxide concentration of approximately 30 μM was reached within 15 minutes of activation of the Synthetic RO+ product and was maintained for 24 hours (Fig.4 c).

**DISCUSSION**

Both SurgihoneyRO™ and its synthetic RO+ equivalent showed antimicrobial activity against planktonic *S. aureus*. This, in addition to the inactivity of the RO- formulation lacking glucose oxidase, suggests that the reactive oxygen species produced by glucose oxidase results in oxidative damage and *S. aureus* cell death. The synthetic honey-equivalent product was more efficient at inhibiting growth of both strains than honey-based SurgihoneyRO™, which may be explained by a slight inhibition of the enzyme by other components present in natural honey.

At higher concentrations of the enzymatically inactive product (SyntheticRO-) for *S. aureus* MRSA1 strain and all concentrations for the MSSA1 strain, some inhibition of bacterial growth can be observed. *S. aureus* has been shown to have a range of carbohydrate transporters, including four glucose transporters, which enhance its ability to uptake glucose for growth, especially during infection of non-respiratory environments within a host26. This evolutionary advantage could become a disadvantage at unnaturally high levels of glucose and lead to toxicity and cell damage, which would explain reduced growth.

In established biofilms, all *S. aureus* strains showed reduced viability due to reactive oxygen activity after 24 hours of treatment with 71 g/L of the synthetic honey-equivalent product. This is a promising result, as bacterial biofilms, including *S. aureus*, have been linked to CRS recurrence, chronicity and recalcitrance11. If a topical application of the synthetic honey-equivalent product could disrupt existing biofilms or prevent biofilm formation, this could be used as an adjunctive treatment potentially delivered through a saline nasal rinse, thus reducing the need for systemic antibiotics and its attendant risk of antimicrobial resistance.

As part of the developmental process towards the final formulation, the increase in biomass associated with treatment of established *S. aureus* MRSA1 and MSSA1 biofilms with the synthetic honey-equivalent will require further investigation. This could be caused by the formulation characteristics, or by other factors such as osmotic stress levels. Future studies will also assess the response of human cells and evaluate the cytotoxicity profile of the synthetic honey-equivalent product using human primary tissues. This will enable us to assess the product in a more complex and biologically-relevant environment. The final phase will be validation of these results in a clinical setting using a proof of concept clinical trial.

With the increasing burden of infections caused by antibiotic-resistant bacteria on modern health care27, it is now more important than ever to develop new, efficient ways of preventing and treating bacterial infections. This study is the first report of antibacterial activity of SurgihoneyRO™ and a novel synthetic honey-equivalent antimicrobial product against CRS-related mucosal MSSA and MRSA strains. The results highlight the potential of the reactive oxygen platform for use in developing novel adjunctive therapies in CRS, particularly in recalcitrant biofilm-mediated disease. Apart from treatment of biofilm-related respiratory diseases, it has been suggested that formulations containing reactive oxygen species, such as the novel synthetic honey-equivalent product, could also be useful in treatment of skin and soft tissue infections, care of postoperative surgical sites, prophylaxis of prosthetic joints infections, and in chronic recurrent multidrug-resistant cystitis28.

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**FINANCIAL DISCLOSURES / CONFLICT OF INTEREST**

Matoke® Holdings Ltd, the developer of SurgihoneyRO™ and Reactive Oxygen®, has provided research funding contributions to the institutional departments involved in this study. Matoke® Holdings Ltd has provided the products free of charge for research and evaluation, but has had no decision-making involvement in design of the study, collection, analysis and interpretation of data, or writing of the manuscript. Matoke® Holdings Ltd has applied for patents for antimicrobial compositions with RJS listed as an inventor. DP, RNA, AD, PGH and JSW declare no conflict of interest or relevant financial disclosures.

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**FIGURE LEGENDS**

**Figure 1**. SurgihoneyRO™ and the synthetic honey-equivalent product with glucose oxidase (Synthetic RO+) inhibit *S. aureus* planktonic growth. Optical density (absorbance at 595 nm) of planktonic *S. aureus* MRSA1 (a) and MSSA1 (b) cultures was measured after 18 hours of growth in the presence of SurgihoneyRO™, Synthetic RO+ and Synthetic RO- (n=6 per strain). Error bars +/- 1 SD.

**Figure 2**. Treatment with synthetic honey-equivalent product with glucose oxidase (Synthetic RO+) reduces *S. aureus* biofilm viability, but increases biomass. Forty-eight hours *S. aureus* MRSA1 (a, c) and MSSA1 (b, d) biofilms were treated with Synthetic RO+ and Synthetic RO- for 24 hours (n=5 per strain). Viability was measured by CFU enumeration (a-b) and biomass was measured by optical density (OD595) (c-d). For comparison, forty-eight hour MRSA1 biofilms (n=4) were also treated with 71 g/L SurgihoneyRO™ for 24 hours with viability and biomass measured (e). \*p≤0.05; error bars +/- 1 SD.

**Figure 3.** Confocal imaging confirms that Synthetic RO+ reduces biofilm viability but increases biomass. Forty-eight hour MRSA1 and MSSA1 biofilms were treated with 71 g/L Synthetic RO+ and Synthetic RO- for 24 hours then assessed by confocal microscopy using live/dead staining to visualize live (green) and dead (red) cells (a). Maximum biofilm thickness was measured using the confocal z-stacks (b).

**Figure 4**. Synthetic honey-equivalent product with glucose oxidase (Synthetic RO+) releasing hydrogen peroxide is effective in reducing the viability of established biofilms formed by clinical *S. aureus* isolates. Forty-eight hours biofilms formed by 7 MRSA (a) and 5 MSSA (b) clinical isolates were treated with 71 g/L Synthetic RO+ for 24 hours. Their viability was measured by CFU enumeration. Hydrogen peroxide concentration (c) was measured using a fluorimetric H2O2 assay kit over 24 hours. \*\* p≤0.01; \*\*\*p≤0.001; error bars +/- 1 SD.