Antibacterial and anti-virulence activity of manuka honey against genetically diverse Staphylococcus pseudintermedius

Running title: Manuka honey activity against S. pseudintermedius

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Abstract

Staphylococcus pseudintermedius causes opportunistic infections in dogs. It also has significant zoonotic potential, with the emergence of multidrug-resistance leading to difficulty treating both animal and human infections. Manuka honey has previously been reported to inhibit many bacterial pathogens including methicillin resistant Staphylococcus aureus and is successfully utilised in both clinical and veterinary practice. Here we evaluated the ability of manuka honey to inhibit strains of S. pseudintermedius growth alone and in combination with antibiotics, and its capacity to modulate virulence within multiple S. pseudintermedius. All 18 of the genetically diverse S. pseudintermedius strains sequenced and tested were inhibited by ≤ 12% (w/v) medical grade manuka honey, although tolerance to five clinically relevant antibiotics was observed. The susceptibility of the isolates to four of these antibiotics was significantly increased (p ≤ 0.05) when combined with sub lethal concentrations of honey, although sensitivity to oxacillin was decreased. Virulence (DNase, protease and haemolysin) activity was also significantly reduced (p ≤ 0.05) in over half of isolates when cultured with sub lethal concentrations of honey (13, 9 and 10 isolates respectively). These findings highlight the potential for manuka honey to be utilised against S. pseudintermedius infections.

Importance

Staphylococcus pseudintermedius is an important member of the skin microbial community in animals and can cause opportunistic infections in both pets and their owners. The high incidence of antimicrobial resistance in S. pseudintermedius highlights that this opportunistic zoonotic pathogen can cause infections which require prolonged and intensive treatment to resolve. Manuka honey has proven efficacy against many bacterial pathogens and is an
accepted topical treatment for infections in both veterinary and clinical practice so is a particularly appropriate antimicrobial for use with zoonotic pathogens such as *S. pseudintermedius*. Here we demonstrate that manuka honey is not only highly potent against novel multi-drug resistant *S. pseudintermedius* isolates, but also acts synergistically with clinically relevant antibiotics. In addition, manuka honey modulates *S. pseudintermedius* virulence activity, even at subinhibitory concentrations. In a clinical setting these attributes may assist in controlling infection, allowing a more rapid resolution and reducing antibiotic use.

Keywords: manuka honey, antibiotic resistance, synergy, haemolysis, proteolysis, aggregation

**Introduction**

*Staphylococcus pseudintermedius* is a commensal bacterium of the skin and mucous membranes of up to 80% of dog populations [1] and is frequently associated with opportunistic veterinary infections such as post-surgical infections and pyoderma [2, 3]. Increasingly, methicillin resistant *S. pseudintermedius* (MRSP) is being isolated from these infections, and reports now indicate that *S. pseudintermedius* isolates display resistance to a range of antibiotics including: erythromycin, clindamycin, ciprofloxacin and gentamicin [4]. This resistance to multiple classes of antibiotics has made *S. pseudintermedius* a global clinical challenge in veterinary medicine as treatment of infections is increasingly problematic. The prevalence of *S. pseudintermedius* has implications that reach beyond the veterinary field as studies have shown that *S. pseudintermedius* can cause zoonotic infections in humans, making it a significant one health issue [5, 6].
The zoonotic potential of this organism has only recently been recognised as *S. pseudintermedius* infections in humans have previously been misidentified as *Staphylococcus aureus* due to their phenotypic similarities and coagulase positivity [7]. They share traits such as acquired methicillin resistance, ability to form biofilms, modulation of the host immune system and production of proteolytic enzymes and toxins [8]. Recent advances in diagnostics have allowed clearer separation of the two species and have shown that, as well as a zoonotic agent, *S. pseudintermedius* is able to colonise the nasal passageways of humans. This colonisation provides a reservoir for reinfection of both animals and humans, and a potential reservoir for antimicrobial resistance gene transfer [9]. As *S. pseudintermedius* exhibits a decreasing level of antibiotic susceptibility and can cause severe infection in both animals and humans there are grounds for evaluating whether new strategies or novel antimicrobial agents could be used to enhance treatment options [10].

Manuka honey is used as a topical antimicrobial agent for infections in both humans and animals, exhibiting activity against a wide range of pathogens [11]. Antimicrobial activity is due to the presence of multiple antimicrobial compounds within the honey, the best studied of which is methylglyoxal, alongside its high osmotic potential (for a review of the activity of manuka honey see [12]). Manuka honey has been used to successfully eradicate *S. aureus* infections in the clinic and can inhibit the growth of methicillin resistant *S. aureus* (MRSA) and *Pseudomonas aeruginosa* at a low concentrations *in vitro* [13, 14]. The mode of action of manuka honey against MRSA has been partially elucidated; and due to the phenotypic similarity between *S. aureus* and *S. pseudintermedius* is highly likely to be similarly effective in inhibiting *S. pseudintermedius* and MRSP [15]. More recently studies have also highlighted the ability of manuka honey to increase efficacy of some clinically relevant antibiotics against MRSA which could also be useful in the case of difficult to treat antibiotic resistant *S. pseudintermedius* infections [15]. In parallel, manuka honey has been utilised by
the veterinary community, in particular for treatment of equine wounds [16]. Previous work has demonstrated *in vitro* activity against a wide range of equine bacterial isolates [17] with *in vivo* activity also recently reported [18].

Here we aimed to establish whether manuka honey could inhibit the growth of a range of genetically diverse *S. pseudintermedius*, increase the sensitivity of clinical *S. pseudintermedius* isolates to a range of antibiotics and reduce expression of key virulence factors. Activity in these areas would indicate potential for manuka honey in the treatment of difficult to treat infections caused by *S. pseudintermedius*.

**Results**

*Genome sequencing highlighted that isolates were genetically diverse and contained novel MLST sequences*

As these isolates have not previously been reported they were sequenced in order to interrogate their multilocus sequence typing (MLST) and antimicrobial resistance (AMR) profiles and detect how closely related the isolates were genetically. Sequencing reads and genome assemblies from this study are available from NCBI via the BioProject record PRJNA561036. MLST allele sequences were derived from assemblies and submitted to the *Staphylococcus pseudintermedius* PubMLST database. New allele sequences were detected for the *pta* loci of isolate C and the *ack* loci of isolate G. A total of 10 novel sequence types (ST) were identified within this dataset with a further 8 STs identified, 4 of which belonged to ST71 (Assembly and MLST information found in Supplementary Table 1). A summary table showing the different antibiotic resistance genes present in each isolate can be seen in table 1.
A core genome SNP phylogeny accounting for the removal of detected recombination and consisting of the isolates documented within this study along with *Staphylococcus pseudintermedius* genome assemblies displayed very little obvious clustering of isolates based on geographical location or the health status of the individual from which they were isolated (Figure 1).

Minimum inhibitory/bactericidal concentration testing of honey and antibiotics showed universal activity of honey against the *S. pseudintermedius* isolates tested.

Previous work by our group indicates that manuka honey has antibacterial activity against *Staphylococcus* sp., including, antibiotic resistant (AMR) isolates [11, 13]. In this study all 18 isolates of *S. pseudintermedius* demonstrated susceptibility to low concentrations of manuka honey ≤ 12% (w/v), with 9/18 inhibited at 12% w/v and 9/18 inhibited at 10% w/v manuka honey. No antimicrobial activity was detected at concentrations of less than 8 % w/v manuka honey. The minimum bactericidal concentration was ≤ 12% (w/v) manuka honey for all isolates.

The sensitivity of the *S. pseudintermedius* isolates to tetracycline, penicillin, chloramphenicol, gentamicin and oxacillin was also determined. For tetracycline, penicillin and gentamicin more than half of the isolates tested (9, 16 and 11 isolates respectively) had breakpoints that indicated resistance according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines. Only chloramphenicol and oxacillin remained
effective against a majority of isolates with 10 and 14 isolates remaining susceptible respectively.

Assessment of antibiotic interactions showed that honey was able to increase the activity of the majority of antibiotics tested

There has been significant recent interest in combination therapy, combinations of antibiotics with other antibiotics, peptides and plant extracts, to increase antibiotic efficacy [19, 20]. Since the isolates within the collection tested here display a high level of resistance to commonly used antibiotic treatments, it was speculated that antibiotic efficacy might be improved if used in combination with sub lethal concentrations of manuka honey.

Manuka honey increased the sensitivity of a number of isolates to various antibiotics, with small but statistically significant increases in zone size observed for the majority of isolates (Figure 2 and Supplementary figure 1). The exact increase in susceptibility was both strain and antibiotic specific, full details of the inhibition of each isolate to the five antibiotics with and without honey is shown in Supplementary figure 1. The numbers of isolates which displayed significantly larger zones of inhibition ($p \leq 0.05$) when treated with sub lethal concentration of honey (5% w/v) combined with antibiotic compared to antibiotic alone were: tetracycline 89% (16/18), penicillin 56% (10/18) chloramphenicol 83% (15/18) gentamicin 67% (12/18). In some instances (isolates C, D, E, K, M and X for tetracycline and isolates O and X for Chloramphenicol) the isolates showed complete resistance to the antibiotic in standard medium, however in the presence of subinhibitory honey a zone of inhibition was present. When manuka honey was used in combination with oxacillin none of the isolates displayed increased sensitivity and 33% (6/18) isolates showed significantly decreased sensitivity (Figure 2) data for individual isolates in supplementary figure 1.

(Figure 2)
Generally, there was a good correlation between the AMR phenotype observed in the disc diffusion assays and the genotype of each isolate. There were several instances where discrepancies between genotype and phenotype were detected but only two are of note. Firstly, no beta lactam inhibiting genes were detected in isolate G despite a clear phenotypic resistance to penicillin but not oxacillin. There was a similar observation for isolate X, which was phenotypically resistant to chloramphenicol, despite no identifiable resistance gene being identified encoding chloramphenicol inactivation enzymes (acquired genes) (Supplementary table 1). Thus, in this case, 50S ribosomal subunit mutational effects are the likely resistance source.

**Characterisation of virulence factor activity indicated that manuka honey was able to reduce activity of several key virulence factors**

Alongside its antimicrobial activity, manuka honey has previously been shown to affect protein and gene expression in the closely related *S. aureus* [21]. It was therefore of interest to determine if manuka honey could elicit phenotypic changes in the virulence profile of *S. pseudintermedius* isolates. Initially the haemolytic, protease, lipase and DNase activity of the isolates was assessed without manuka honey, alongside the isolate’s ability to aggregate (a property linked to biofilm formation).

All 18 isolates showed haemolytic, proteolytic, DNase, and aggregation activity. None of the isolates demonstrated lipase or lecithinase capabilities (data not shown). The activity was then reassessed using a sub lethal concentration (5% w/v) of manuka honey. As with the antibiotic activity (Figure 2), the presence of subinhibitory concentrations of honey were able to elicit alterations in the virulence activity of the *S. pseudintermedius* isolates. As with the alterations in antibiotic susceptibility, alterations in virulence activity appeared to be highly
isolate specific. In most of the isolates the observed effects were subtle, although statistically significant. However, four of the strains showed a complete reduction of haemolysis (strains G and H) or proteolysis (Strains B and C) activity. Two further strains (D and F) showed complete reduction of both haemolysis and proteolysis. The number of isolates which had significantly changed haemolysis, proteolysis and DNase activity $p = \leq 0.05$ respectively when grown in sub lethal concentration of honey (5% w/v) compared to control cells were: haemolytic 56% (10/18), protease 50% (9/18) and DNase 72% (13/18) (Figure 3 and Supplementary figure 2).

(Figure 3)

Sub inhibitory concentrations of manuka honey inhibited aggregation and reduced mature biofilm biomass of *S. pseudintermedius* suspensions

All 18 isolates displayed the ability to agglutinate. After 24 hours incubation control isolates had a mean optical density of 25% of the 0 hours value, indicating that bacterial cells had agglutinated, leading to a decreased OD$_{600}$ (Figure 4A). In contrast, 15 of the isolates displayed a significantly reduced ability to agglutinate ($p = \leq 0.001$) after incubation with a sub lethal (5% w/v) concentration honey, with a mean optical density of 65% of the 0-hour value (Figure 4A). Total viable cells counts after 24 hours of incubation with and without manuka honey highlighted that the increased optical density was not linked to an increase in cell numbers, with total viable counts indicating that $\sim 4 \times 10^7$ CFU/ml viable cells remained in each vessel following incubation. With the exception of isolates B and H which were moderate biofilms formers and isolate O which was a weak biofilm former, all the isolates were strong biofilm formers as defined by Stepanovic *et al* [22].
As expected, the concentrations of manuka honey needed to disrupt established (24 h) biofilm was higher than the MIC (Figure 4B). There was a reduction in biomass at concentrations of \( \geq 20\% \) w/v manuka honey, with reductions becoming significant \((p = \leq 0.01)\), at concentrations of \( \geq 30\% \).

**Discussion**

The increase in *S. pseudintermedius* antibiotic resistance is of concern to both clinicians and veterinarians [23, 24]. New strategies for infection control are required and a one health approach must be utilised during discovery to ensure that therapeutics can be used harmoniously across medical disciplines. Clinicians and veterinarians have previously been criticised for excessive use of antibiotics [25], and following the publication of the review on antimicrobial resistance [26] there has been a drive to improve collaboration between medical, veterinary and agricultural disciplines [27] in addressing the issue of AMR. The report by O'Neill [26] also highlighted that the development of novel therapeutics to enhance the activity of, or replace antibiotic treatments, was an essential strategy to preserve our ability to effectively treat infectious diseases.

Zoonotic pathogens are of concern, since inappropriate treatment and resolution of animal infections has the potential to increase disease severity and recalcitrance to treatment of any subsequent human infection. Other members of the staphylococcus genus, such as *S. aureus* and *S. epidermidis*, are persistent colonisers of the human body, causing significant morbidity and mortality, and display high levels of AMR. *S. pseudintermedius* has also been shown to
colonise healthy humans [28] and appears to have similar disease progression and resistance
designs to other members of the genus [23, 24, 29].

The *S. pseudintermedius* in this study showed a wide range of genetic diversity (Figure 1),
including the presence of several novel MLST combinations. The genetic diversity of these
samples allowed an estimation of how effective manuka honey might be for treatment of *S.
pseudintermedius* infections as multiple strains of *S. pseudintermedius* with varied antibiotic
resistance profiles can co-colonise at an infection site [30]. The majority of isolates also
contained genes known to confer antibiotic resistance, with many isolates also showing
phenotypic resistance to one or more of the antibiotics tested. Only in a couple of instances
was there a discrepancy between the phenotypic resistance patterns and the presence of
acquired AMR genes. However, since the genomes presented within this paper are not closed
it is possible that genes are present in regions of the genome currently unassembled. Further,
presence of point mutations giving rise to resistance were not screened for and thus could
offer alternative resistance strategies.

The results presented here provide evidence that low concentrations of manuka honey can
inhibit the growth of clinical isolates of *S. pseudintermedius*, with a bactericidal mode of
action. All isolates displayed sensitivity to manuka honey and had a range of sensitivities to
the conventional antibiotics tested. This is in line with previous studies which have
demonstrated that manuka honey can inhibit *S. aureus*, MRSA and vancomycin-intermediate
*S. aureus* (VISA) at low concentrations [31-33]. The only study looking at the ability of
manuka honey to inhibit *S. pseudintermedius* was by Uri *et al* [34] which showed a low level
of antibacterial activity. However, this was likely to be due to issues with the homogeneity of
the honey solution used in the study as highlighted by the authors of that paper and not a true
reflection of poor manuka honey efficacy, particularly given the many *in vitro* and *in vivo
studies showing efficacy against other bacterial species [11, 13, 21].
The results of the antibiotic susceptibility testing presented here reflect the general trend of increased antimicrobial resistance seen globally, with 88% of the isolates tested displaying resistance to two or more of the antibiotics (Figure 2). The levels of resistance for gentamicin and chloramphenicol presented here are broadly similar to findings from earlier studies where *S. pseudintermedius* resistance to chloramphenicol and gentamicin was ~75 and 55%, respectively, whereas the levels of resistance for tetracycline were lower in this study at ~50% than those previously reported (94.2%) [35, 36]. There were only four MRSP isolates within our collection, a lower incidence of resistance than previously reported [35, 36].

In addition to obtaining the sensitivity profiles of these bacteria and in view of the growing interest in the use of topical and combination therapies for staphylococcal infections [37] the ability of manuka honey to enhance antibiotic activity was tested. Our results highlight that sub lethal concentrations of honey are capable of significantly improving (p < 0.05) the activity of antibiotics from a variety of classes, for most isolates tested (Figure 2). The exception to this was oxacillin, in which combination with honey showed no improvement in activity against any of the MRSP isolates. Importantly, in six of the methicillin susceptible *S. pseudintermedius* (MSSP) isolates manuka honey was associated with a significant decrease in the efficacy of oxacillin, although the isolates did remain sensitive to oxacillin according to the breakpoints. This was an unexpected finding as previous studies have identified synergy between honey and oxacillin when tested against MRSA, as well as reporting a possible mechanism of action for this synergy through the MecR1 pathway [32, 38]. It is interesting to note that synergy has been observed for MRSA with *mecA* mechanism of resistance previously, and the four MRSPs here all have resistance mediated by *mecA* (supplementary table 1) the same as seen in the MRSA, so a similar response would have been expected, this difference in response could be investigated further in future work. The fact that the isolates with the altered response are phenotypically methicillin sensitive, and therefore unlikely to
contain a functioning mecR1 gene, highlights why the response is different to that previously observed. The mecR1 gene has not shown to be common within *Staphylococcus pseudintermedius* isolates, with a recent study only detecting it in 7 of 17 isolates tested [39]. It has also been observed that some MRSP do also not contain mecR1 [40], again showing that even where isolates do have phenotypic resistance to penicillin’s, honey may not be able to act on them via the previously described MecR1 pathway. This reduction in sensitivity to oxacillin when exposed to sub lethal concentrations of manuka honey is an important observation for medical practice, where oxacillin is a commonly used treatment, as it suggests that using manuka honey in combination with the other antibiotics tested here would be more effective than oxacillin as a potential combination therapy. Our observation also highlights the need, previously stressed within the O’Neill Report [26], for novel bedside diagnostic tools in order to better determine suitable treatments for patients presenting with infection. If clinicians are able to rapidly distinguishing between opportunistic infections caused by *S. aureus* and *S. pseudintermedius* then suitable combination therapies can be selected, improving clinical outcome and reducing the opportunity for microbial persistence. Within veterinary practise, where *S. pseudintermedius* infection is more commonly encountered, oxacillin is not considered for first line treatment of skin infections [41] and so our observation is of less significance. It should also be noted that our data did not show decreased susceptibility to penicillin, indicating that manuka honey/oxacillin combination therapies may not be suitable for treatment of *S. pseudintermedius*, this is unlikely to be the case for all penicillin’s.

Investigation into the mechanism by which honey is inhibiting *S. pseudintermedius* might shed light onto why there is this discrepancy between *S. pseudintermedius* and *S. aureus* when treated with oxacillin and honey; potentially genetic variation in the two species might account for the reduced efficacy of the combination in this case. Despite this and given the high levels of antibiotic resistance increasingly being reported, the results presented here...
show that there is real potential for honey to be used alone or as a topical adjuvant to certain antibiotic treatments to help improve antibiotic efficacy against *S. pseudintermedius* infections.

Alongside investigation of synergy, antivirulence compounds are of great interest as they reduce pathogenicity while leaving bacterial growth unaffected and desirable host microbes unharmed [42]. As *S. pseudintermedius* are known to produce a range of virulence factors which enhance their ability to cause severe disease in both animal and humans [23, 43] the effect of sub lethal concentrations of manuka honey on the activity of virulence factors in *S. pseudintermedius* was tested. Investigating the effect of sub lethal concentrations of honey on virulence provided novel evidence that honey can significantly reduce virulence factor activity in some *S. pseudintermedius* isolates (Figure 3). In infection the ability to produce an extensive array of virulence factors allows bacteria to break down host tissue, evade the immune system and acquire host nutrients [5, 44]. The isolates in this study all displayed varying levels of β haemolytic, protease, DNase activity as well as the ability to autoagglutinate. They were all negative for lipase and lecinthinase activity, correlating well with previous findings charting the virulence profiles of *S. pseudintermedius* isolated from humans and dogs [45]. The data presented here establishes that sub lethal concentrations of honey significantly reduced haemolysin activity in half the *S. pseudintermedius* isolates tested, indicating a strain specific mode of action. As there was a significant increase in haemolytic activity of one isolate further work would be needed before this could be considered of interest in a clinical setting. To date there is little evidence of the role of β-haemolysin in *S. pseudintermedius* pathogenicity. In *S. aureus* β haemolysin production promotes efficient skin colonisation [46]. It is also thought to cause host cell cytotoxicity, act as a biofilm ligase and conferring a selective advantage to those strains which produce it [47]. It is logical to suggest that ability of honey to reduce β haemolytic activity would thereby reduce the
capability of S. pseudintermedius to colonise host cells and cause cytotoxicity and reduce zoonotic transmission.

Similarly, the results presented here show that sub lethal manuka honey significantly reduces the ability of half the S. pseudintermedius strains to produce protease and did not increase protease activity in any of the other strains. As the ability of staphylococci to produce proteases has been linked to their ability to evade host immunity as well as cleaving host proteins and contributing to bacterial dissemination within the host [48, 49] reduction in this activity could be clinically relevant. Previously published work showed that protease null mutants of S. aureus have decreased virulence and reduced dissemination and invasion in vivo [50]. S. pseudintermedius produces a similar range of proteases and, if they perform an analogous role to those seen in S. aureus, then honey could have an important function in decreasing the severity of localised infection and reducing the spread of S. pseudintermedius within the host.

Linked to infection duration and antibiotic tolerance is DNase activity, which has been reported to aid in the formation of bacteria aggregates, maintenance of biofilm [51] and degradation of neutrophil extracellular traps [52]. These actions all assist the bacteria in evading the immune system. The reduction of DNase activity seen in over half the S. pseudintermedius tested (13/18) again suggests a role for manuka honey in reducing bacterial virulence and supporting host function in infection. It is possible that the significant decrease in DNase activity after treatment with sub MIC honey in in this study could be related to the significant reduction in aggregation seen here. The ability to agglutinate has been linked to the ability of isolates to form biofilms, an important mechanism of persistence [53]. Aggregation is known to be partially depended on components displayed on the cell surface [54] but the role of DNase in staphylococcus sp. aggregation is less well understood. Some studies observe increased expression of DNase from planktonic cells [55] and others
describing increased expression of DNase from sessile cells [56]. It has been shown that the presence of extracellular DNA (eDNA) improves the ability of bacteria to aggregate and adhere to surfaces [57], therefore a reduction in DNase expression would be expected to lead to increased aggregation. As exposure to sub lethal concentrations of manuka honey led to a reduction in both DNase activity and bacterial aggregation, there could well be additional factors at work here. Further work to elucidate the mechanisms which cause these effects need to be undertaken.

The ability to form and persist within, biofilms is a known Staphylococcus sp. virulence trait which decreases the susceptibility of in vivo populations to antimicrobial treatment and provides a reservoir for system infection [58]. Significant effort has been dedicated to removing and inactivating the biofilms of S. aureus and S. epidermidis, with manuka honey showing significant promise in both species [11, 59, 60]. The data presented here suggests that S. pseudintermedius isolates are likely no exception from this trend, with all the isolates within our collection showing a statistically significant reduction in mature biofilm biomass at manuka honey concentrations of ≥ 30 % (w/v). This suggests that manuka honey is able to disperse preformed biofilms, and individual bacterium which are shed form the biofilms will rapidly succumb to the antimicrobial effects which manuka honey clearly showed against planktonic populations.

The data presented shows that in vitro manuka honey is able to reduce the activity of the virulence factors tested, however caution should be used when interpreting the data as the changes seen in vitro might not be replicated when tested in vivo. Although the activity assays used within this study allow investigators to measure differences in activity of the supernatant this technique has limitations as it does not distinguish between an alteration in activity due to changes in protein efficiency or affects produced by changes in gene expression and/or protein production. In order to provide more information about the exact
mechanism of virulence factor modulation by manuka honey the technique used here must be
combined with an analysis of gene presence (WGS), gene expression (qPCR and RNAseq)
and protein expression (2D gel electrophoresis and mass spectrometry). This additional
analysis is outside the scope of the current work, however, would be of significant interest to
help elucidate the honeys mechanism of activity against *S. pseudintermedius*. Further *in vivo*
testing will be required to ensure that significant reductions in activity highlighted here
translate to a biological difference in the virulence of the bacteria within animals and their
owners.

It is clear from the results presented here that manuka honey alone can effectively inhibit
antibiotic sensitive and resistant *S. pseudintermedius* at low concentrations, and when
combined with selected antibiotics can increase their efficacy. It is also apparent that the
addition of sub lethal manuka honey to *S. pseudintermedius* has multiple effects on the
virulence activity of those isolates with over half seeing a significant reduction in virulence
activity *in vitro*. *S. pseudintermedius* is recognised as a major cause of post-operative
infections in small animals, and more recently as a coloniser and opportunistic pathogen of
humans [6]. Our data indicates that use of manuka honey in clinical treatment against *S.*
*pseudintermedius* infections could be possible and that manuka honey could potentially be
used as an antibiotic adjuvant for difficult to treat multi-antibiotic resistant strains. Further
investigation via microbroth dilution on the effect of manuka honey on antibiotic sensitivity
would help determine which interactions are antagonistic, indifferent or synergistic. In
addition to this there is strong evidence that manuka honey could function as a topical
antivirulence treatment to help reduce the severity of *S. pseudintermedius* infections and limit
further colonisation. Investigations into the mechanisms by which manuka honey is eliciting
these effects needs to be undertaken to determine the mechanistic processes and strain
specific effects seen.
Methods

Strains and Culture conditions

Eighteen *S. pseudintermedius* isolated from dogs were used throughout this study. All were provided by Nottingham Veterinary School, UK (18 isolates). These cultures were stored at -80°C on cryobeads and revived on to Muller Hinton Agar (MHA) (Oxoid, UK) at 37°C before testing. Throughout the experiment Mueller Hinton Broth (MHB) was used where liquid medium was required.

Antimicrobial agents

Sterile medical grade manuka honey (Derma Sciences, Medihoney) was used throughout and was a gift from Derma Sciences, Europe. Antimicrobial susceptibility testing discs of; penicillin (1 unit), tetracycline (30 µg), chloramphenicol (30 µg), gentamicin (10 µg) and oxacillin (1 µg) were purchased from Oxoid (Hampshire, UK). These concentrations were chosen to match those with break points in the EUCAST breakpoint tables.

DNA extraction/sequencing

DNA was extracted from cultures using the Qiagen DNAeasy kit according to the manufacturer’s guidelines prior to quantifying yield and quality using spectrophotometric and fluorometric methods. DNA libraries for sequencing were constructed using the Illumina Nextera XT kit and sequenced on an Illumina MiSeq platform using a V3 reagent kit and 2x300 bp reads.

Genome Assembly and Analysis

Raw sequence reads were passed through cutadapt (v2.0) [61] for the removal of nextera sequence adapters only prior to genome assembly using SPAdes (v3.12.0) in careful mode with read error correction and auto k-mer detection. [62]. Quast (v5.0.0) [63] was used to assess the quality of the genome assemblies. MLST allele sequences, IDs and isolate ST were
queried against the genome assemblies using the MLST tool (v2.16) (currently unpublished, Seemann T, mlst Github https://github.com/tseemann/mlst). A core SNP phylogeny was reconstructed using the isolates from this study along with 137 S. pseudintermedius genome assemblies obtained from NCBI and using variant sites called by SNIPPY (v4.3.2) (currently unpublished, Seemann T, SNIPPY Github https://github.com/tseemann/SNIPPY).

Recombination was accounted for with the use of Gubbins (v 2.3.1) [64] prior to inference of a maximum-likelihood phylogenetic tree using FastTree (v 2.1.10), applying the generalized time-reversible model (gtr) [65]. A final tree was annotated with the use of iTOL [66]. The presence of antimicrobial resistance genes was determined using ABRICATE (v0.8.11) (Seemann T, Abricate, Github https://github.com/tseemann/abricate). A threshold of > 90% sequence identity was used to determine a good match between the database genes and hits within the genome sequences.

**Minimum Inhibitory/Bactericidal Concentration Testing**

Minimum Inhibitory Concentration (MIC) of manuka honey antimicrobial susceptibility testing was completed using an adapted European Committee on Antimicrobial Susceptibility Testing methodology. Briefly, mid-logarithmic growth phase cells were diluted to 0.5 McFarland standard diluted 1 in 100 before it was mixed 1:1 with MHB to a final volume of 200 µl (creating a cell density of ~5x10⁶ CFU/ml) with the manuka honey set out as 2% w/v increments, diluted in MHB. The isolates were then grown for a period of 16-20 hours at 37°C before measurement of optical density at a wavelength of 600nm (OD₆₀₀). The MIC was considered to be the lowest concentration which inhibited cell growth (defined as blank corrected OD₆₀₀ measurements equaling ≤0). The minimum bactericidal concentration (MBC) was determined by plating 10 µl of cells onto MHA from all wells showing no growth at 18 hrs and incubating for a further 18 hours at 37°C. The MBC was defined as the lowest concentration that reduced the viability of the initial bacterial inoculum ≥ 99.9%.
Positive control wells contained growth media and bacterial cells with no antibiotic or honey, negative control (used for blank correction) contained growth media and no cells.

**Antibiotic Disc Susceptibility**

Susceptibility of isolates to antibiotics alone and in combination with sub lethal concentrations of manuka honey was determined using disc diffusion on MHA. Briefly the cells were diluted to 0.5 McFarland standard then using a sterile cotton swab a lawn plate was created and antibiotic discs were immediately applied using sterile forceps. The plates were incubated for 16-20 hours at 37 °C before the diameter of the zones of inhibition was measured. Zone sizes were interpreted using EUCAST Clinical Breakpoint Tables v. 9.0, where *S. pseudintermedius* breakpoints were unavailable those for *S. aureus* were used. To screen for potential synergy between antibiotics and honey the disc diffusion assay was repeated with MHA supplemented post-sterilisation with a sub lethal dose of 5% (w/v) manuka honey.

**Virulence testing**

All isolates were screened for their ability to produce the virulence factors; haemolysin, protease, lipase, lecithinase and DNase. Agar plates were prepared by supplementing MHA with either: 5% (v/v) sheep blood, 5% (v/v) skim milk powder or 5% (v/v) egg yolk (Sigma, UK). Supplements were added after the media had been autoclaved and were mixed before pouring into plates. DNase agar was purchased directly from Sigma and prepared following manufactures guidelines. Prior to use the plates were equilibrated to room temperature and 5 mm diameter wells were cut in the agar surface using a sterile cork borer. Overnight cultures were diluted to a density of OD$_{600}$ 0.8 – 1.0 (equivalent to a cell density of ~1 x $10^8$ CFU/ml) and pelleted before resuspension in 0.4 % MHA, with or without 5% w/v honey. A volume of 100 µl of this adjusted cell suspension was added to the plate wells and the plates cooled quickly to allow the soft agar to rapidly set and form a plug within the well. The plates were
then incubated for 24 hours at 37°C and a zone of activity around bacterial growth was recorded as a positive result for each virulence factor.

**Auto aggregation**

To assess aggregation overnight cultures were adjusted to OD$_{600}$ 0.9, 1 ml was transferred to 1.5ml Eppendorf tube, centrifuged at 14,000 x g for 5 minutes and the pellet re-suspended in 1 ml of phosphate buffered saline (Oxoid, UK; PBS) or PBS + honey (5 % (w/v). The inoculum was then transferred to microcuvettes and OD$_{600}$ measured at 0 and 24 hours with static incubation at room temperature between the measurements. Bacterial cells that strongly agglutinate do not remain in the aqueous phase leading to a decrease in the OD$_{600}$, if prevented from agglutinating the OD$_{600}$ remains relatively stable. The percentage change of OD$_{600}$ was calculated after 24 hours. Viable counts were taken using the Miles Misra method before and after incubation to confirm that any differences in auto aggregation were not due to cell death or proliferation.

**Biofilm assessment**

To determine the effect of manuka honey on mature biofilm *S. pseudintermedius* strains were diluted as described for MIC testing and diluted 1:1 with MHB to a final volume of 200 µl before incubating for 24 hours at 37°C to allow biofilm formation. Following incubation the supernatant was removed carefully from the wells, so as not to disturb the biofilm, and washed once with sterile PBS. Manuka honey was diluted to concentrations of 10 to 50 % w/v increments in MHB. A volume of 200 µl of these solutions was added to the biofilms before incubating for a further 24 hours at 37°C. Following incubation, supernatant was removed and wells gently rinsed twice with PBS before fixing remaining biofilms with methanol for 15 minutes and staining with crystal violet (1% v/v diluted in distilled water) for 15 minutes. Excess stain was rinsed until water ran clear and wells were dried before adding
7% acetic acid (diluted in distilled water) for 15 minutes to dissolve the bound crystal violet.

Absorbance was measured at a wavelength of 590 nm.

The ability of the *S. pseudintermedius* strains to attach to surfaces and form biofilm was performed following the method and classification proposed by Stepanovic *et al* [22].

Biofilm cultures were established, fixed and stained as described above and then classified as weakly, moderately, or strongly adherent, based upon the following formula: 

$$\text{OD}_c < \text{OD} \leq 2 \times \text{OD}_c = \text{weak adherence},$$  
$$2 \times \text{OD}_c < \text{OD} \leq 4 \times \text{OD}_c = \text{moderate adherence and } 4 \times \text{OD}_c < \text{OD} = \text{strong adherence}.$$

ODc was defined as three standard deviations above the mean OD of the negative (medium only) control.

**Statistical analysis**

All tests were performed in triplicate and as data was nonparametric (determined using a Shapiro-Wilk test) was analysed using Mann Whitney (auto aggregation) and Kruskal Wallis method for all other experiments (GraphPad Prism 8). Unless otherwise stated all bars in figures show median values and error bars represent 95% confidence limit.

**Acknowledgements**

We would like to thank Dr. Anette Loeffler of the Royal Veterinary College for providing the original bacterial strains to Nottingham University. Bioinformatic analyses were carried out using MRC CLIMB Infrastructure (Grant – MR/L015080/1). Funding was provided for JC by the Microbiology Society (VS15-20).

RJ, HLB, GM, JC, HD, MH, LS performed experiments. MH carried out bioinformatics analysis. RA, TW and RJ conceived the study. Manuscript was drafted by HLB, MH, TSW, RA and RJ.

The authors declare no competing interests.

**Data availability**
Sequencing reads and genome assemblies from this study are available from NCBI via the BioProject record PRJNA561036. Accession numbers: Isolate A (SAMN12347716,SRR9998529), Isolate B (SAMN12347717,SRR9998521,SRR9998530), Isolate C (SAMN12347718,SRR9998528,SRR9998519), Isolate D (SAMN12347719,SRR9998518), Isolate E (SAMN12347720,SRR9998522), Isolate F (SAMN12347721,SRR9998525), Isolate G (SAMN12347722,SRR9998517), Isolate H (SAMN12347723,SRR9998524), Isolate I (SAMN12347724,SRR9998520), Isolate J (SAMN12347725,SRR9998523), Isolate K (SAMN12347726,SRR9998528), Isolate M (SAMN12347728,SRR9998534), Isolate N (SAMN12347729,SRR9998536), Isolate P (SAMN12347730,SRR9998526,SRR9998527), Isolate Q (SAMN12347731,SRR9998531), Isolate T (SAMN12347732,SRR9998516), Isolate X (SAMN12347733,SRR9998532).

Figure legends

Supplementary Table 1: Antibiotic resistance genes and products present in S. pseudintermedius isolates identified using ABRICATE. A threshold of >90% sequence identity was used to determine a good match between the database genes and hits within the genome sequences (see attached excel file).

References:


27. Torjesen, I., (2018), Doctors and vets working together for antibiotic stewardship. BMJ. 362: p. k3014. 10.1136/bmj.k3014


**Figures and Tables**

**Table 1:** A summary of the antibiotic resistance genes found by sequencing for each isolate

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<th>Isolate Identity</th>
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<th>Beta lactam</th>
<th>Chloramphenicol</th>
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<th>Streptothricin</th>
<th>Tetracycline</th>
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Figure 1: A maximum likelihood phylogeny of 156 *Staphylococcus pseudintermedius* isolates reconstructed from core SNPs, with removal of detected recombination sites. Samples sequenced in this study are distributed across the tree as noted by the outer black triangle. Coloured bars indicate geographical location of isolation source. Isolates with metadata related to host health have terminal nodes coloured green for healthy or red for confirmed infection.
**Figure 2:** Graph A-E shows the zone diameter in mm of tetracycline, penicillin, chloramphenicol, gentamicin, and oxacillin respectively against 18 isolates of *S. pseudintermedius* in MHB (Control) and with a sub lethal concentration of manuka honey, 5% (w/v) (Treated). Those isolates showing a significant changes in sensitivity are marked with asterisk (** p ≤ 0.01, *** p ≤ 0.001, **** p ≤ 0.0001). Data points show median values and error bars represent 95% confidence limit.
**Figure 3:** Manuka honey is able to inhibit virulence factor production by *S. pseudintermedius*. DNase (A), Haemolytic (B) proteolytic (C) and activity of the 18 *S. pseudintermedius* isolates in the absence (Control) or presence (Treated) of a sub lethal (5% w/v) concentration of manuka honey. Those isolates showing a significant changes in sensitivity are marked with asterisk (** p ≤ 0.01, **** p ≤ 0.0001). Data points show median values and error bars represent 95% confidence limit.
Figure 4: The ability of *S. pseudintermedius* isolates to aggregate is reduced in the presence of sub-inhibitory 5% (w/v) manuka honey. A shows the effect of sub-lethal (5% w/v) manuka honey on 18 *S. pseudintermedius* isolates. Isolates showed a significant decrease (*p = ≤ 0.001*) in their ability to aggregate when treated with 5% w/v manuka honey. B shows the effect of increasing concentrations of manuka honey on preformed 24 h biofilms with a significant (**) *p = ≤ 0.01* and (***) *p = 0.001*) reduction in biofilm seen at concentrations ≥ 30% w/v. Data points indicate median values and error bars represent the 95% confidence limit.