Manuka honey reduces the motility of *Pseudomonas aeruginosa* by suppression of flagella-associated genes

Aled E. L. Roberts, Sarah E. Maddocks* and Rose A. Cooper

Cardiff School of Health Sciences, Cardiff Metropolitan University, Western Avenue, Llandaff, Cardiff, CF5 2YB

Running header: *P. aeruginosa* flagella and honey

Keywords: motility, virulence, wound infection

*Correspondence: Sarah Maddocks, Cardiff School of Health Sciences, Cardiff Metropolitan University, Western Avenue, Llandaff, Cardiff CF5 2YB, United Kingdom. Tel: 44-2920-20-5607. Email: smaddocks@cardiffmet.ac.uk

Synopsis

Manuka honey is bactericidal against *Pseudomonas aeruginosa* by destabilizing the cell wall. In this study additional inhibitory effects were investigated using a combination of 2D electrophoresis, quantitative RT-PCR, transmission electron microscopy and motility assays. Exposure of *P. aeruginosa* to manuka honey reduced both swarming and swimming motility. Moreover this was as a consequence of de-flagellation of the bacterial cell, which was correlated with decreased expression of the major structural flagellin protein, FliC and concurrent suppression of flagellin associated genes, including *fliA, fliC, flhF, fleN, fleQ* and *fleR.* The differential expression of the flagellar regulon in the presence of manuka honey was mapped schematically. Flagella are integral to bacterial adhesion, the initiation of infection and biofilm formation, and swarming has been associated with increased virulence. By limiting motility *in vitro*, we infer that manuka honey impacts on the virulence of *P. aeruginosa.* This deduction must now be tested *in vivo*.

Introduction

Pseudomonas aeruginosa is a Gram negative opportunistic pathogen that demonstrates exceptional environmental versatility and extensive antimicrobial resistance. More recently, it has gained notoriety in nosocomial settings due to the emergence and persistence of multidrug resistant (MDR) strains. Between 1993 and 2002, for example, the number of multi-drug-resistant (MDR) strains isolated from intensive care units (ICUs) with resistance to >3 antimicrobials quadrupled (n = 13,999) and this trend has continued with time. *P. aeruginosa* has been implicated in both acute and chronic infections. In wounds, *P. aeruginosa* is problematic in burns and neutropenic patients and approximately 12% of chronic wounds are colonised.[1,2,3] Venous leg ulcers are particularly susceptible to *P. aeruginosa* infection resulting in larger wound sites with delayed healing.[4] Furthermore, chronic venous leg ulcers that are colonised with *P. aeruginosa* exhibit a marked decrease in the success rate of skin grafts.[5]

Initiation of infection depends on bacterial adhesion within the wound environment which is facilitated by altered motility and cell surface interactions. Many virulence determinants have been associated with *P.* aeruginosa; in acute infections the bacterium is usually invasive and relies on the secretion of an array of toxins and proteases with the involvement of flagella, type II and III secretion systems and type IV pili. Conversely chronic infections tend to be non-invasive involving the formation of biofilms.[7] During the initial stages of cutaneous wound infection, bacterial cells interact with sub-cutaneous surfaces, resulting in one of two distinct surface-associated behaviours: translocation or attachment.[8] Surface translocation usually precedes attachment; it facilitates the spread of bacterial cells within the wound bed prior to their adherence and growth into a multicellular community. Various motility appendages and a sophisticated chemotaxis system aligns cells along concentration gradients, enabling *P. aeruginosa* to traverse various types of surface by swarming, swimming or twitching, depending on the solidity of their environment.[9,10,11]

Chronically infected wounds can be difficult to resolve and multidrug resistance means that many currently available antimicrobial treatments are not effective. Innovative antimicrobial interventions for wound infections that have been introduced into conventional medicine during the past decade have included medical grade honey; one example that is often used in the UK and North America is manuka honey. Bactericidal activity of manuka honey against planktonic cultures of *P. aeruginosa* isolated from wounds has been reported.[12] Using electron microscopy this honey was shown to cause structural changes in the cell surface which led to lysis.[13] These effects were confirmed by confocal microscopy and atomic force microscopy and decreased expression of OprF (an outer membrane protein which is involved in cell wall stability, diffusion and virulence) was implicated.[13] Additionally, manuka honey inhibits siderophore production and adhesion to human keratinocytes.[14,15] This study aimed to investigate the effects of manuka honey on the motility of *P. aeruginosa* with a view to better understanding its potential to impact on virulence.

Materials and Methods

Bacterial strain and culture conditions

A reference strain of *P. aeruginosa* ATCC 9027 (also known as NCIMB 8626) was used throughout this study maintained on nutrient agar (NA; Oxoid, Cambridge, UK) and incubated aerobically at 37^{o}C unless otherwise stated. The Minimum Inhibitory Concentration (MIC) of this organism to manuka honey is 12% (w/v).[13]

Manuka honey

Gamma irradiated medical grade manuka honey (Advancis Medical, Nottingham, UK) was used throughout this study. The MIC of *P. aeruginosa* against this honey has been shown to be 12% (w/v).[13]

Hydrophobicity assays

Cell surface hydrophobicity of test organism was determined using a modified MATH test.[16] Essentially mid-exponential phase cells were diluted 1:1 with nutrient broth (NB; Oxoid, Cambridge, UK) and NB containing manuka honey (6%, 12%, and 24% final concentrations respectively), and incubated with shaking (100 rpm) at 37°C for 5 hours. At 30 minute intervals, 4 ml samples were collected and centrifuged at 4,700 rpm for 5 minutes (Heraeus Multifuge X3 Benchtop Centrifuge; Thermo Scientific, Waltham, MA, USA). The supernatant was discarded and the pellet washed twice in sterile PBS. After two washes the pellet was resuspended in 3 ml PBS and the absorbance at λ540 nm was measured (pre-hexadecane OD). To this washed cell suspension, 0.8 ml of n-hexadecane was added and vortexed for 30 seconds. The mixture was allowed to separate into an aqueous and n-hexadecane phase over 20 minutes and the absorbance of the aqueous phase was measured at λ540 nm (post-hexadecane OD). The percentage of adherence to hydrocarbons was determined a using the following formula:

$$\% \text{ of adherence} = \frac{100 \times (OD_{540}\text{pre-hexadecane} - OD_{540}\text{post-hexadecane})}{OD_{540}\text{pre-hexadecane}}$$

Hydrophobicity was evaluated using the percentage cell adherence cut off values previously described.[16] Hence <25%, 25-75%, and >75% were interpreted as negative, intermediate, and strong-positive hydrophobicity, respectively.

Preparation of cell free extracts

Exponential cultures of *P. aeruginosa* were obtained by diluting an overnight culture 1 in 50 with NB and incubating at 37°C for 5 hours. To cultures either 50 ml of NB or 50 ml NB containing 24% w/v manuka honey was added and incubated for a further 3 hours. Cells were harvested by centrifugation at 10,000 g (Sorvall RC5B, DuPont Instruments, Cincinnati, USA) for 5 minutes and resuspended in 3 ml NB. Cell samples were sonicated (VCX 500, Sonics, Newtown, USA) at 40% amplitude for 2.5 mins and the cellular debris harvested by centrifugation at 13,000 g (MSE, London,

UK) for 4 minutes was discarded. The supernatant containing cellular proteins was and stored at -20ºC for no more than 14 days and the protein concentrations were determined using the BioRad Bradford kit (BioRad, Hertfordshire, UK) according to the manufacturers' instructions.

Two dimensional electrophoresis

Methods were derived from Bernhardt et al (1999).[17] Briefly 190 µg protein samples were diluted to 200 µl with re-hydration buffer and absorbed into a 11cm pH 3-10 ReadyStrip™ immobilised pH gradient (IPG) strip (BioRad) during 60 min at room temperature as per the manufactures' instructions. The strip was then covered with a thin layer of paraffin oil (Fisher Scientific, Loughborough, UK) to prevent evaporation and the IPG strips were incubated at room temperature overnight. IPG strips were blotted to remove excess paraffin oil and loaded into a PROTEAN® Iso-Electric focusing (IEF) tray (BioRad) as per the manufacturers' instructions. Paper wicks soaked with 10 µl of deionised (18 MΩ-cm) water were used to connect the IPG strips with the electrode wire. Paraffin oil (2 ml) was overlaid onto the IPG strip and the PROTEAN ® IEF tray was loaded into a PROTEAN® IEF cell (BioRad). First dimension separation of proteins was achieved at 50 µA per strip for 35,00 volt hours at 20ºC, holding at 500 v upon completion. Second dimension gel electrophoresis was achieved by removing IPG strips from the PROTEAN ® IEF tray and blotting with wet blotting paper to remove excess paraffin oil. The IPG strip was placed in a 11 cm rehydration tray, overlaid with 3 ml equilibration buffer 1 and incubated on an orbital shaker (10 rpm) at room temperature for 30 min. This process was repeated with equilibration buffer 2. IPG strips were dipped in MOPS running buffer to remove excess equilibration buffer and loaded into Precast 4-12% Criterion™ XT Bis-Tris polyacrylamide gel cassettes (BioRad) using the manufacturers'instructions. IPG strips were sealed in place with bromophenol blue agarose and the polyacrylamide gels were loaded into a 2DGE tank (BioRad). The tank was filled with MOPS running buffer and second dimension separation of proteins was achieved at 200 v for 45 min.

Identification of differentially expressed proteins

IPG strips were discarded and polyacrylamide gels removed from their plastic casing. Polyacrylamide gels were washed three times by adding 100 ml 18 MΩ-cm water, microwaving for 1 min at 950 W, and discarding the water. Polyacrylamide gels were then stained with 25 ml SimplyBlue™ SafeStain (Life Technologies, Paisley, UK), microwaved for 1 min at 950 W, and incubated on an orbital shaker (10 rpm) at room temperature for 10 min. SimplyBlue™ SafeStain was discarded and the gels were washed with 100 ml 18 MΩ-cm water and incubated for a further 10 mins. After incubation, 20 ml 20% w/v sodium chloride (Fisher Scientific) was added to each gel and incubated for a further 10 min. The sodium chloride was discarded and the gels were washed with 100 ml 18 MΩ-cm water and incubated for a further 60 min.

Stained protein spots were photographed using a BioSpectrum® Advanced Imaging System (UVP, Upland, USA) and the images were analysed using PDQuest™ Basic 2-D gel analysis software (BioRad). Gels containing protein samples from untreated and manuka honey cells were compared using an untreated control. Gels were aligned to the control gel and the number of protein spots up/down regulated calculated. Only protein spots that showed >2 fold expression differences were counted during analysis. Select protein spots that demonstrated significant differences between the two gels were analysed by MALTI-ToF MS.

Mass spectrometry

MALDI-ToF MS was completed using a MALDI ToF/ToF mass spectrometer (4800 MALDI ToF/ToF Analyzer, Applied Biosystems, Foster City, USA) and a solid state laser (200 Hz) operating at 355 nm.[18,19,20,21] Peptide mass fingerprinting was conducted using Global Proteome Server Explorer software (v3.6) in conjunction with the MASCOT database search engine (v2.1) on the Swiss-Prot. database (downloaded 10/12/2010).[22] Searches were restricted to the *Pseudomonas* genus and proteins identified were based on high quality tandem MS data for ≥2 peptides (E value $p < 0.05$ for each peptide, overall $p < 0.0025$).

Quantitative PCR

To investigate the expression of flagella associated genes (*fliA, fliC, flhF, fleN, fleQ* and *fleR*) primers were designed (Table 1) and *rpoD* was used as a housekeeping gene. *P. aeruginosa* ATCC 9027 cells treated with and without 12% and 24% (w/v) manuka honey for 24 h underwent RNA extraction, cDNA conversion, and qPCR expression analysis as described previously.[13]

Swim and swarm plate assays

Fastidious anaerobe broth (FAB, Sigma Aldrich, Dorset, UK) supplemented with either 0.3% or 0.5% (w/v) noble agar was used to detect swimming or swarming motility, respectively.[10] Mid-exponential phase *P. aeruginosa* culture was diluted 1:1 with either nutrient broth (NB; Oxoid Cambridge, UK) or NB containing manuka honey (3, 6 and 12% w/v), and incubated aerobically at 37^{o}C for 3 hours. After 3 hours incubation, 1.5 µl of each sample was injected at a ~45^{o} angle, under the surface of each of swim and swarm plates and incubated aerobically at 37^{o}C for 24 hours to allow the migration of bacteria away from the point of inoculation. Then the diameter (mm) of each zone of bacterial migration was measured using digital callipers (Mitutoyo) and mean diameters were calculated. Triple replicates were performed on nine separate occasions.

Motility agar assay

Motility agar (Mast Laboratories Ltd, UK) was prepared containing manuka honey at final concentrations of 0, 1.5, 3, 6 and 12% (w/v). Colourless tetrazolium salts in the agar are taken up by viable bacteria and reduced to produce formazan which is a red insoluble compound. Bacterial growth is indicated by a deep pink colour. Overnight cultures of *P. aeruginosa* were inoculated into the agar using the stab method. Plates were incubated at 37^{o}C for 24 h and the pink zones observed.

Transmission electron microscopy

Mid-exponential phase *P. aeruginosa* cells were diluted 1:1 with either NB or NB containing manuka honey (12% w/v final concentration), and incubated aerobically at 37ºC for 3 hours. Samples were transferred to 400-mesh carbon-coated nickel grids fixed in 2.5% (w/v) glutaraldehyde (Fisher Scientific, Loughborough, UK) for 10 mins, washed six times for 1 min in sterile filtered deionised water, and stained with 4% (w/v) aqueous uranyl acetate for 10 mins. Cells were visualised using transmission electron microscopy (TEM; FEICM12, Hillsboro, USA) operating at 80 kV and images were collected using a digital camera (SIS MegaView III, Olympus, Münster, Germany). The percentage of flagellated cells in each sample was then calculated.

Statistical analysis

The statistical change of hydrophobicity using the MATH assay was determined using the One-way ANOVA test. The statistical changes in qPCR products were determined using the Student's T-Test (Two tailed, two sample - equal). Finally, significant changes in swim/swarm colony diameters were determined using ANOVA. The statistical changes in the percentage of flagellated and non-flagellated cells in untreated and manuka honey treated samples were determined using the Student's T-Test (Two tailed, two sample - unequal).

Results

Manuka honey results in consistently negative hydrophobicity

In the modified MATH assay the adherence of untreated *P. aeruginosa* cells to n-hexadecane gradually increased over five hours (Fig. 1), indicating that hydrophobicity changed from negative to intermediate. In comparison, adherence to n-hexadecane of all manuka honey treated samples (6%, 12%, and 24% w/v) was significantly reduced over time ($p<0.05$), consistently demonstrating negative hydrophobicity. Differences between hydrocarbon adherence in sub-inhibitory and

inhibitory concentrations were observed, and determined to be significantly different ($p<0.05$). A dose response in hydrophobicity values between the two inhibitory manuka honey concentrations (12% and 24% w/v) was not observed during the 5 hour treatment period, but the lower values of treated cells compared to untreated cells suggests that exposure to manuka honey disrupts non-specific hydrophobic interactions and reduces adherence efficiency.

Manuka honey treated P. aeruginosa *exhibits differential protein expression*

Following treatment with manuka honey, the expression of 142 proteins was significantly ($p<0.05$) altered (100 down regulated and 42 up regulated). Of those, only 111 proteins (86 down regulated and 25 up regulated) were differentially regulated by a factor of 2-fold or more. Two representative images, one from untreated and one from honey-treated cells, were overlaid to find proteins with differential regulation and ten spots (Fig. 2) were selected for identification by mass spectrometry. Five proteins were identified (Table 2); four were at reduced levels, of which three were identified as FliC. Dihydrolipoamide dehydrogenase (DldH2) was also down regulated in manuka honey treated samples and a heat shock protein (DnaK) was up-regulated in response to manuka honey.

Expression of flagella-associated genes is suppressed following manuka honey treatment

Four (*fliA, fliC, fleN* and *fleR*) of the six flagella-associated genes investigated showed a statistically significant reduction in gene expression following treatment with 12% (w/v) manuka honey (Table 3) and all (*fliA, fliC, flhF, fleN, fleQ* and *fleR*) were significantly reduced, compared to the housekeeping gene, following treatment with 2xMIC (24% w/v) (Table 3). This suggests that manuka honey inhibits flagella gene expression impacting both on regulatory (*fliA, fleN fleQ* and *fleR)* and structural genes (*fliC, flhF*), and supports the protein expression data.

Manuka honey results in reduced swimming and swarming capacity

Kearns (2010) described colonies on swim and swarm plates that typify swimming and swarming motility as colonies with a bullseye-like appearance surrounded by three or with two concentric rings, respectively. Such colonies were seen with untreated *P. aeruginosa* samples on swim (Fig. 3a) and swarm (Fig. 4a) plates. Compared to untreated samples, mean colony diameters of samples pre-treated for 3 h with 3%, 6%, and 12% (w/v) manuka honey (Fig.3b, c and d, respectively) on swim plates were significantly ($p<0.05$) smaller in size by between 15 and 20%. Similarly swarming was reduced by manuka honey and mean swarm colony diameters were also significantly smaller (17 and 20%) for samples pre-treated with 3%, 6%, and 12% (w/v) manuka honey (Fig. 4b, c and d, respectively) ($p<0.05$) Loss of motility function did not appear to be dependent on manuka honey concentration at or below the MIC (12% w/v).

Manuka honey treatment results in reduced motility

Tetrazolium motility agar impregnated with concentration of manuka honey of 0%, 1.5%, 3%, 6% and 12 % (w/v) showed clearly a dose dependent reduction in motility in response to manuka honey treatment (Figure 5). Movement through the agar was impeded as concentration of honey increased, and at 12% (w/v) manuka honey there was no motility observed at all. Between 1.5-6 % (w/v) manuka honeys, movement through the agar was retarded but not completely abrogated.

Manuka honey treatment promotes de-flagellation

Using transmission electron microscopy (TEM) flagellated cells of *P. aeruginosa* were readily detected and counted (Fig. 6a). Whilst the number of flagellated cells in untreated and honey treated samples remained similar, double the numbers of non-flagellated cells were observed in manuka honey treated sample (Fig. 6b). The difference in cellular flagellation was statistically significant ($P<0.05$) as 79% of cells in untreated samples were flagellated, decreasing to 62% in manuka honey treated samples (Fig 6 c).

Discussion

Detailed information on the ways in which bacteria are affected by manuka honey has been obtained by proteomic and genomic investigations.[23,24,25,26] These approaches were therefore applied to *P. aeruginosa* treated with and without manuka honey to discover further inhibitory effects. Following treatment with manuka honey swarming, swimming and general motility responses by *P. aeruginosa* were impeded. Protein expression analysis identified differential expression of FliC in the presence of manuka honey. Swarming and swimming are both flagellum-dependent; FliC is the key structural component of the flagella filament therefore reduced expression following manuka honey treatment could explain the observed reduction in motility.[27]

Moreover gene expression analysis identified dose dependent suppression of multiple key regulatory genes within the flagella regulon following exposure to manuka honey, including *fliC.* Transcription of *fliC* is governed by intracellular concentrations of sigma factor (FliA), and the anti-activator of the sigma factor (FlgM).[28] It is likely that the suppression of *fliC* in response to manuka honey (12% w/v) resulted from *fliA* suppression, however, doubling the concentration of manuka honey suppressed *fliC* further whilst having no further effect on *fliA*. This suggests that manuka honey affects various aspects of the flagellar regulon, resulting in the differential suppression of *fliC* in both a FliA-dependent and independent manner. The basal body of flagella is formed from Class III genes of the flagellar regulon which reside under the transcriptional control of FleR and FleS. Manuka honey activity was observed to significantly suppress the expression of *fleR* and whilst *fleS* expression was not examined, the contiguous arrangement of *fleR* and *fleS* on a single operon suggests parallel suppression could also occur.[29]

The ability of *P. aeruginosa* cells to produce a single flagellum located at the polar cap is governed by the Class II genes: *flhF* and *fleN*.[30] Suppression of both genes was observed to occur in

response to manuka honey activity (12% and 24% w/v); however, *flhF* suppression was not significant at lower manuka honey concentrations (12% w/v). Insignificant changes in the expression of *fliF* correlates with the observation of flagellated cells containing a polar flagellum when viewed using TEM. MATH data also supported a loss of flagella as *P. aeruginosa* treated with manuka honey were seen to have reduced adhesive properties. The suppression of *flhF* with higher concentrations of manuka honey (24% w/v) suggests that should flagellated cells be observed, the flagellum may be misaligned, resulting in poor motility. However, it is most likely that cells unable to produce flagella will also have reduced expression of *flhF* and thus flagellated cells will have a fully functioning regulon.

FleN functions to negatively regulate FleQ, providing a critical feedback loop, ensuring *P. aeruginosa* only produces a single flagellum. Suppression of *fleN* following exposure to manuka honey (12% and 24% w/v) should result in the parallel suppression FleN, increasing the relative activity of FleQ. This suggests a conflict whereby reduced *fleN* expression should concomitantly result in its own increased expression. However, manuka honey activity suppresses the expression of *fleQ*. This would cause suppression of *fleN* expression without the contradictory effect observed here, and we suggest that this occurs in manuka honey treated samples.

The bacterial flagellum has been shown to be important in adhesion and virulence and conditions that favour swarming in *P. aeruginosa* have been linked to the up-regulation of many virulence genes.[31,32] Therefore we propose that via the repression of flagella associated genes, manuka honey mediates de-flagellation of *P. aeruginosa* in a dose dependent manner, resulting in reduced motility, adherence and virulence (Figure 7). In addition to the bactericidal effect of manuka honey against this organism, findings here reiterate the antipathogenic and antiadhesive properties of manuka honey that have been demonstrated in MRSA (Jenkins et al 2013) and *Streptococcus pyogenes,* respectively.[13,27,33] Antimicrobial agents exhibiting antipathogenic properties are becoming an attractive alternative to antibiotic-mediated inhibition because attenuation of virulence will result in failure of that organism to establish successful infection before it is

successfully cleared by the host. This hypothesis must now be tested in animal models and ultimately *in vivo,* as it was outside the scope of this study.

Acknowledgments

Electron microscopy was performed with the help of Dr Jan Hobot at the Electron Microscopy Unit in Cardiff University and mass spectrometry was done with the aid of Dr Ian Brevis at the Central Biotechnology services at Cardiff University.

Funding

This work was supported by a Vice Chancellor's Doctoral Award at Cardiff Metropolitan University (formerly University of Wales Institute Cardiff)

Transparency declaration

The authors declare no conflict of interest

References

1. Obritsch MD, Fish DN, MacLaren R *et al*. National surveillance of antimicrobial resistance in Pseudomonas aeruginosa isolates obtained from intensive care unit patients from 1993 to 2002. *Antimicrobial Agents and Chemotherapy* 2004; **48** 4606–4610.
2. Kerr KG, Snelling AM. Pseudomonas: a formidable and ever-present adversary. *J Hosp Infect* 2009; **74** 338-344
3. Frank DN, Wysocki A, Specht-Glic *et al.* Microbial diversity in chronic open wounds. *Wound Repair Regen* 2009; **17** 163–172.
4. Madsen SM, Westh H, Danielsen L *et al*. Bacterial colonization and healing of venous leg ulcers. *APMIS* 1996; **104** 895–899.
5. Høgsberg T, Bjarnsholt T, Thomsen JS *et al.* Success rate of split-thickness skin grafting of chronic venous leg ulcers depends on the presence of *Pseudomonas aeruginosa*: a retrospective study. *PLoS One.* 2011; **6** e20492
6. Coggan KA, Wolfgang MC. Global regulatory pathways and cross-talk control *Pseudomonas aeruginosa* environmental lifestyle and virulence phenotype. *Curr Issues Mol Biol* 2012; **14** 47-70
7. Cooper RA, Halas E, Molan PC. The efficacy of honey in inhibiting strains of *Pseudomonas aeruginosa* from infected patients. *J Burns Care Rehabil* 2002; 23 366-370
8. Caiazza NC, Merritt JH, Brothers KM *et al*. Inverse regulation of biofilm formation and swarming motility by *Pseudomonas aeruginosa* PA14. *J Bact* 2007; **189** 3603–3612.
9. Burrows, L. L. *Pseudomonas aeruginosa* twitching motility: Type IV Pili in Action. *Microbiology* 2012; **66** 493–520.

10. Kearns DB. A field guide to bacterial swarming motility. *Nature Revi Microbiol* 2010; **8** 634–644.

11. Taguchi K, Fukutomi H, Kuroda A, *et al*. Genetic identification of chemotactic transducers for amino acids in *Pseudomonas aeruginosa*. *Microbiology* 1997; **143** 3223–3229.

12. Henriques AF, Jenkins RE, Burton NF *et al.*) The effect of manuka honey on the structure of Pseudomonas aeruginosa *Eur J Clin Microbiol Infect Dis* 2011; **30** 167-71.

13. Roberts AEL, Maddocks SE, Cooper RA. Manuka honey is bactericidal against *Pseudomonas aeruginosa* and results in differential expression of *oprF* and *algD*. *Microbiology* 2012; **158** 3005–3013.

14. Kronda JM, Cooper RA, Maddocks SE. Manuka honey inhibits siderophore production in pseudomonas aeruginosa. *J Appl Microbiol* 2013; **115** 86-90

15. Maddocks SE, Jenkins RE, Rowlands RS , Purdy KJ, Cooper RA Manuka honey inhibits adhesion and invasion in medically important wound bacteria in vitro. *Future Microbiol* 2013; **8** 1523-36.

16. Das SC, Kapoor K N. Effect of growth medium on hydrophobicity of *Staphylococcus epidermidis*. *Indian Journal of Medical Research* 2004; **119** 107–109.

17. Bernhardt J, Büttner K, Scharf C *et al.* Dual channel imaging of two-dimensional electropherograms in *Bacillus subtilis. Electrophoresis.* 1999 **20** 2225-40

18. Bienvenut WV, Déon C, Pasquarello C, *et al*. Matrix-assisted laser desorption/ionization-tandem mass spectrometry with high resolution and sensitivity for identification and characterization of proteins. *Proteomics* 2002; **2** 868–876.

19. Brennan P, Shore A, Clemen M, *et al*. Quantitative nuclearproteomics reveals new phenotypes altered in lymphoblastoid cells. *Proteomics Clinical Applications* 2009; **3** 359–369

20. Glückmann M, Fella K, Waidelich D, *et al*. Pre-validation of potential protein biomarkers in toxicology using iTRAQ reagent technology. *Proteomics* 2007; **7** 1564–1574.

21. Medzihradszky KF, Campbell JM, Baldwin MA, *et al*. The characteristics of peptide collision-induced dissociation using a high-performance MALDI-TOF/TOF tandem mass spectrometer. *Anal Chem* 2000 **72** 552–558.

22. Perkins DN, Pappin DJ, Creasy DM *et al*. Probabilitybased protein identification by searching sequence databases using mass spectrometry data. *Electrophoresis* 1999; **20** 3551–3567.

23. Jenkins RE, Burton NF, Cooper RA. The effect of manuka honey on the expression of universal stress protein A in meticillin resistant Staphylococcus aureus. *Int J Antimicrob Agents* 2011; **37** 373 - 376.

24. Packer JM, Irish JL, Herbert BR, *et al.* Specific non-peroxide antibacterial effect of manuka honey on the *Staphylococcus aureus* proteome. *Int J Antimicrob Agents* 2012; **40** 43-50.

25. Blair S, Cokcetin N, Harry E, Carter D. The unusual antibacterial activity of medical-grade *Leptospermum* honey: antibacterial spectrum, resistance and transcriptome analysis. *Eur J Clin Microbiol Infect Dis* 2009; **28** 1199–1208.

26. Jenkins R, Burton N, Cooper R. Proteomic and genomic analysis of methicillin-resistant *Staphylococcus aureus* (MRSA) exposed to manuka honey in vitro demonstrated down-regulation of virulence markers. *J Antimicrob Chemother.* 2014; 69 603-15

27. Winstanley C, Coulson MA, Wepner B, *et al*. Flagellin gene and protein variation amongst clinical isolates of *Pseudomonas aeruginosa. Microbiology* 1996; **142** 2145–2151.

28. Frisk A, Jyot J, Arora SK *et al*. Identification and functional characterization of *flgM*, a gene encoding the anti-sigma 28 factor in *Pseudomonas aeruginosa*. *J Bact* 2002; **184** 1514–1521.

29. Winsor GL, Lam DKW, Fleming L, *et al. Pseudomonas* Genome Database: improved comparative analysis and population genomics capability for Pseudomonas genomes. *Nucleic Acids Research* 2011; **39** D596–D600.

30. Murray T S, Kazmierczak BI. FlhF Is Required for Swimming and Swarming in *Pseudomonas aeruginosa*. *J Bact* 2006; **188** 6995–7004.

31. Haiko J, Westerlund-Wikström B. The role of bacterial flagellum in adhesion and virulence *Biology* 2013; **2** 1242-1267

32. Overhage J, Bains M, Brazas MD. *et al*. Swarming of *Pseudomonas aeruginosa* is a complex adaptation leading to increased production of virulence factors and antibiotic resistance. *J Bact* 2008; **190**, 2671–2679.

33. Maddocks SE, Salinas Lopez M, Rowlands, RS, Cooper RA. Manuka honey inhibits the development of *Streptococcus pyogenes* biofilms and causes reduced expression of two fibronectin binding proteins. *Microbiology* 2012; **158** 781–790.

Table 1. Primers used for Q-PCR of flagellar associated genes.

Gene name	Gene locus tag	Forward primer	Reverse primer	Amplicon (bp)
fliA	PA1455	CTCCAATTGAGCCTCGAAGA	TTCGTTGTGACTGAGGCTGG	192
fliC	PA1092	GCTTCGACAACACCATCAAC	AGCACCTGGTTCTTGGTCAG	121
flhF	PA1453	CGAGCCTGAACGTGAAGAAT	GCCTCGTCCAGCTTAGTCA	127
fleN	PA1454	GAGCCGTATACGAGGCATTC	GTGTTGGACCAGTCGTTCG	137
fleQ	PA1097	AAGGACTACCTGGCCAACCT	CCGTACTTGCGCATCTTCTC	134
fleR	PA1099	ACAGCCGCAAGATGAACCT	TGGATGGCGTTGTCGAGTT	109

Table 2. Differentially regulated proteins in response to manuka honey treatment; identified using Mass Spectrometry in combination with the UniProt database

SSP ID	Protein ID	Accession No.	Product	Total No. of Peptides	Top Peptide E-Values		Mascot Score
					No. 1	No. 2	
402	DldH2	DLDH2_PSEAE	Dihydro-lipoamide dehydrogenase	4	2.9×10^{-15}	3.2×10^{-10}	473
7603	FliC	FLICA_PSEAE	Flagellin	2	1.3×10^{-14}	4.9×10^{-8}	435
7604	FliC	FLICA_PSEAE	Flagellin	3	2.9×10^{-8}	7.0×10^{-5}	396
7605	FliC	FLICA_PSEAE	Flagellin	2	1.6×10^{-8}	1.0×10^{-3}	250
7901	DnaK	DNAK_PSEAE	Heat shock protein	2	3.8×10^{-9}	1.5×10^{-8}	519

Table 3: The effect of manuka honey treatment on the expression of flagellar associated genes in planktonic organisms.

Planktonic: fold changes in flagellar associated genes following manuka honey treatment		
Gene	**12% (w/v) honey**	**24% (w/v) honey**
fliA	-2.0*	-2.0*
fliC	-2.5*	-8.3*
flhF	-1.2	-2.0*
fleN	-1.6*	-3.8*
fleQ	-0.25	-2.2*
fleR	-1.8*	-2.7*

* denotes a statistically significant change in the level of expression as compared to the untreated ($p<0.05$)

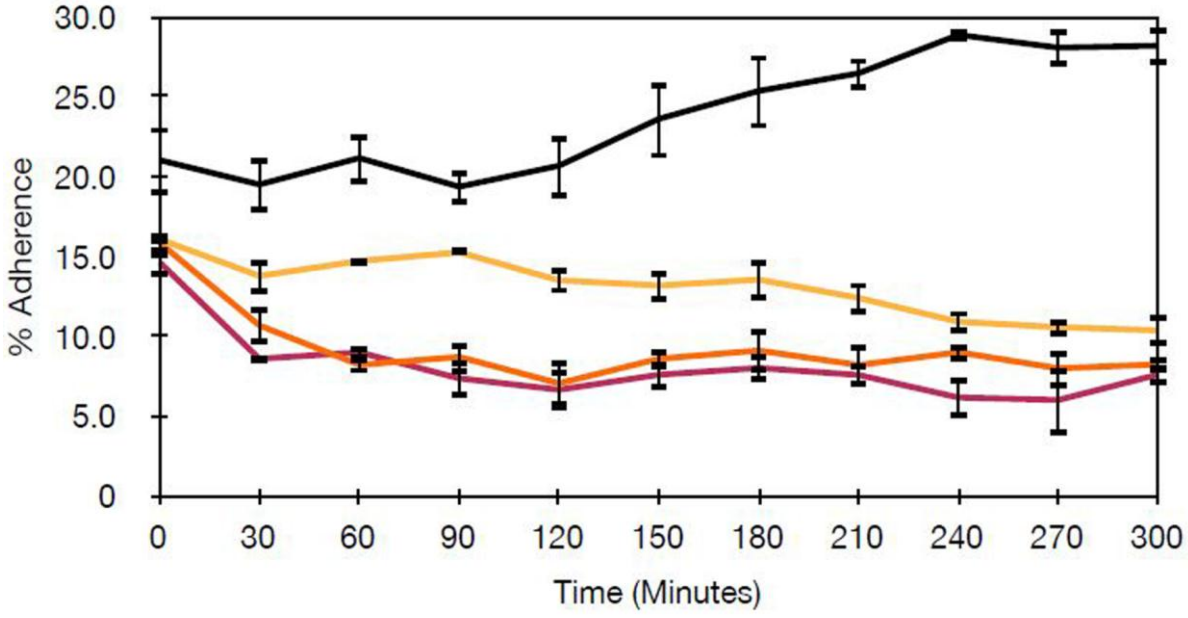
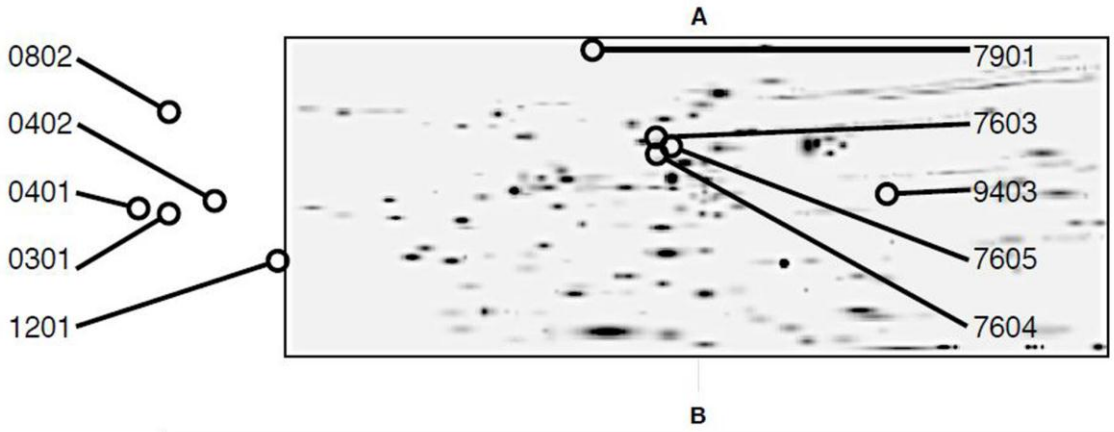


Figure 1. Adherence of *P. aeruginosa* cells to n-Hexadecane when grown with and without manuka honey. Percentage of *P. aeruginosa* cells adhered to n-Hexadecane in absence (black) and presence of 6% (yellow), 12% (orange), and 24% (red) (w/v) manuka honey for 5 hours.



B

SSP ID	Relative Quantity in Untreated Gel Images	Ratio	Relative Quantity in Treated Gel Images	Ratio
0301	266*	1	1987	7.45
0401	273*	1	3903	14.27
0402	462	1	4140	8.95
0802	266*	1	4580	17.18
1201	942	1	8056	8.55
7603	2413	1	145*	0.06
7604	2341	1	142*	0.06
7605	2008	1	145*	0.07
7901	1157	1	11461	9.90
9403	10512	1	145*	0.01

Figure 2. Differential regulation of proteins from *P. aeruginosa* cells grown with and without manuka honey. Ten proteins on the master gel (A) were differentially regulated (B) in samples treated with 12% (w/v) manuka honey. (*relative quantity as determined by PDQuest 8.0 due to the absence of a corresponding protein spot).

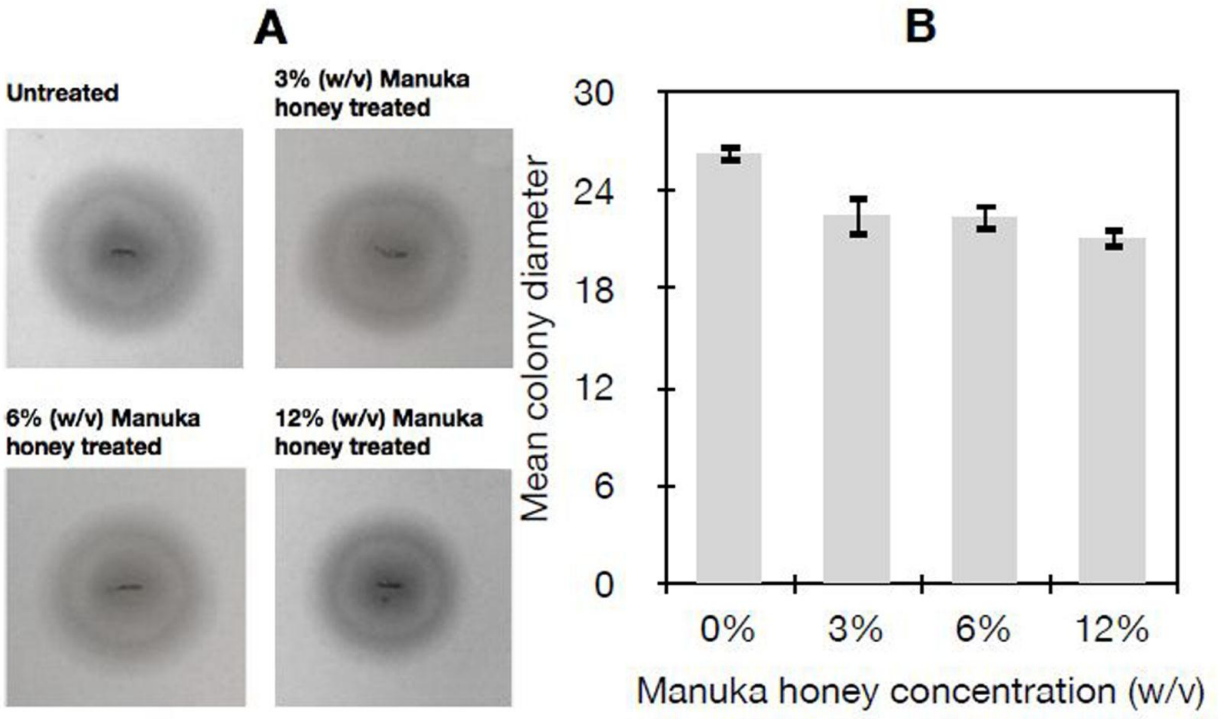


Figure 3. Swimming motility of *P. aeruginosa* cells treated with and without manuka honey. Swim colonies of *P. aeruginosa* cells inoculated onto FAA containing 0.3% noble agar (A), following pre-treatment with or without 3%, 6%, or 12% (w/v) manuka honey for 3 hours, were measured, and significant ($P<0.05$) reductions in mean colony diameters observed (B).

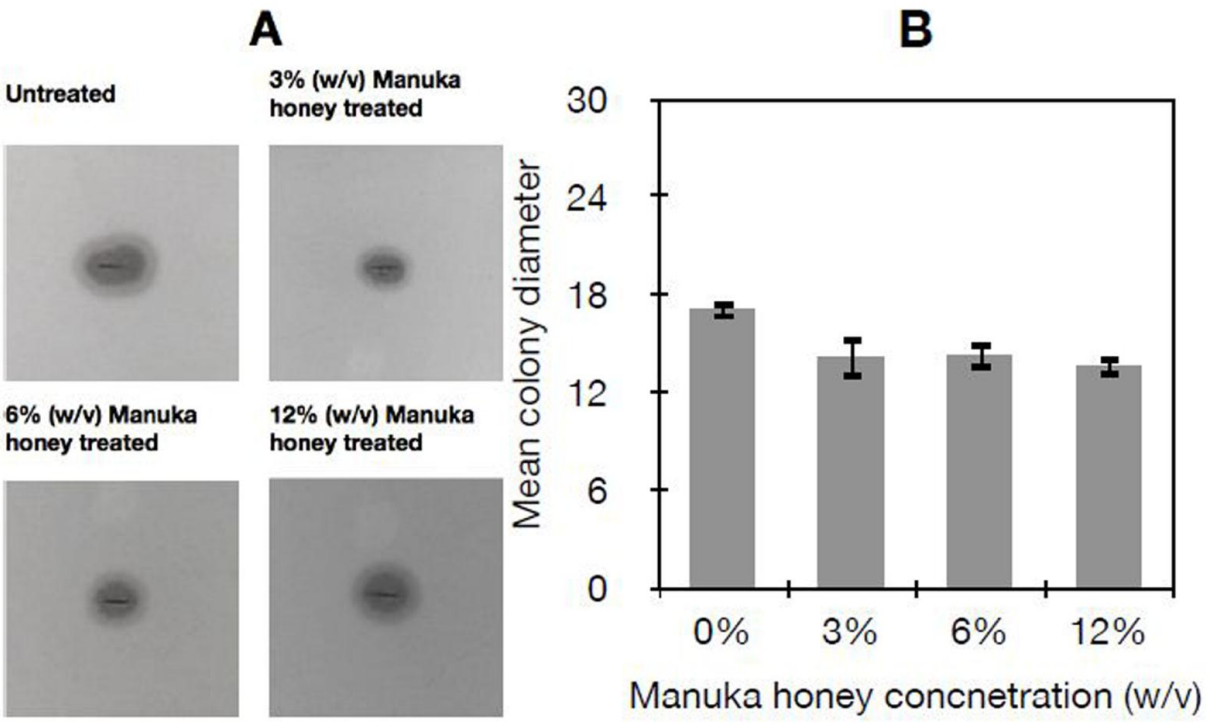


Figure 4. Swarming motility of *P. aeruginosa* cells treated with and without manuka honey. Swarm colonies of *P. aeruginosa* cells inoculated onto FAA containing 0.5% noble agar (A), following pre-treatment with or without 3%, 6%, or 12% (w/v) manuka honey for 3 hours, were measured, and significant ($P<0.05$) reductions in mean colony diameters observed (B).

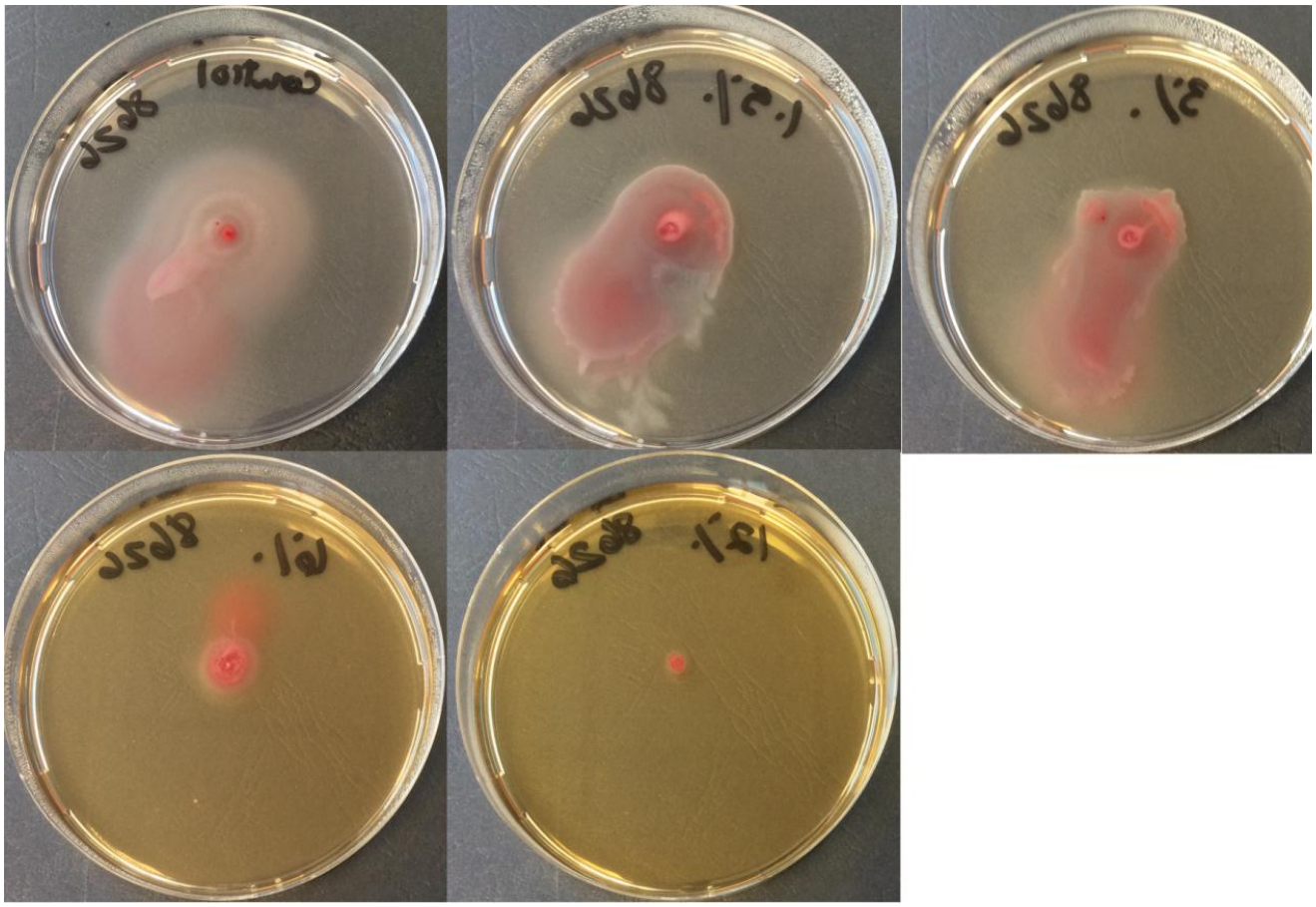


Figure 5. Dose dependent reduction in motility of *P. aeruginosa* in response to manuka honey treatment. Top (Left to right: Control (0% w/v manuka honey); 1.5% (w/v) manuk ahoney; 3% (w/v) manuk ahoney; 6% (w/v) manuka honey and 12% (w/v) manuka honey.

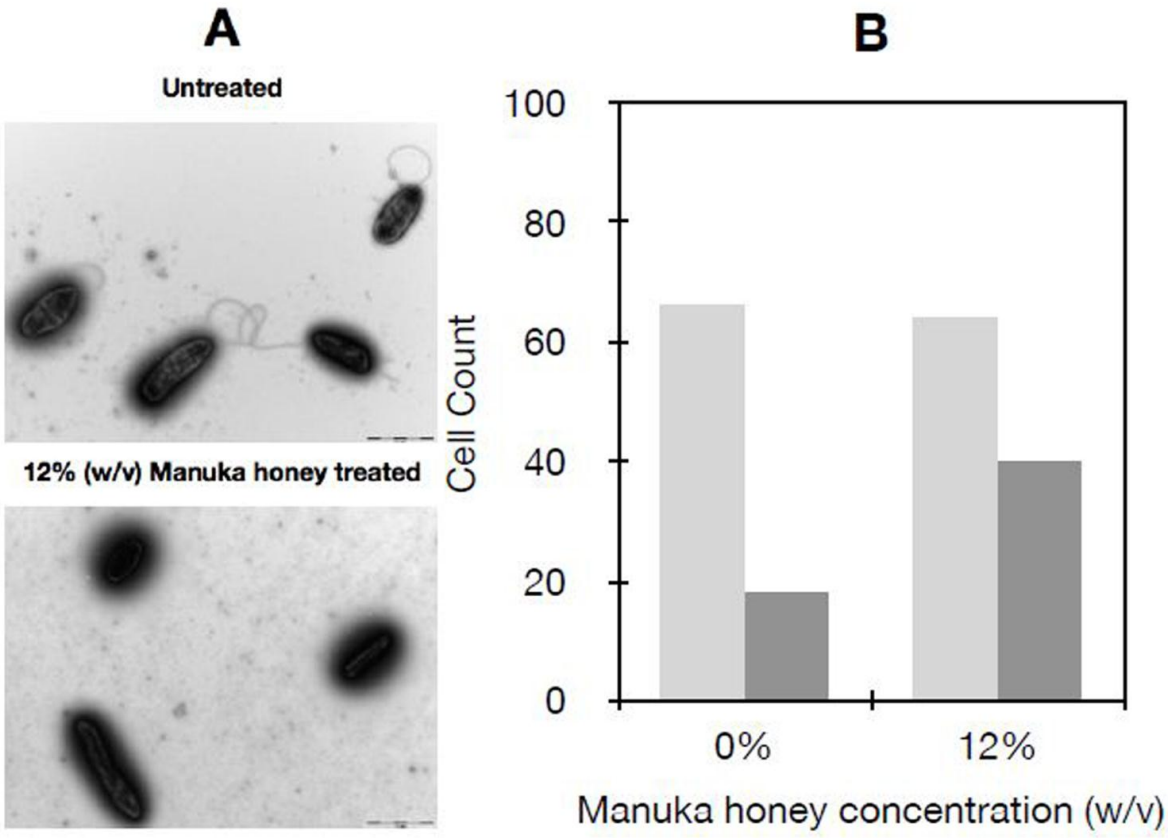


Figure 6. Flagellated state of *P. aeruginosa* cells grown with and without manuka honey. TEM images (n = 102) of *P. aeruginosa* grown with and without 12% (w/v) manuka honey for 3 hours (A), were used to calculate the number of flagellated (light grey) and non-flagellated (dark grey) cells (B). (*P<0.05).

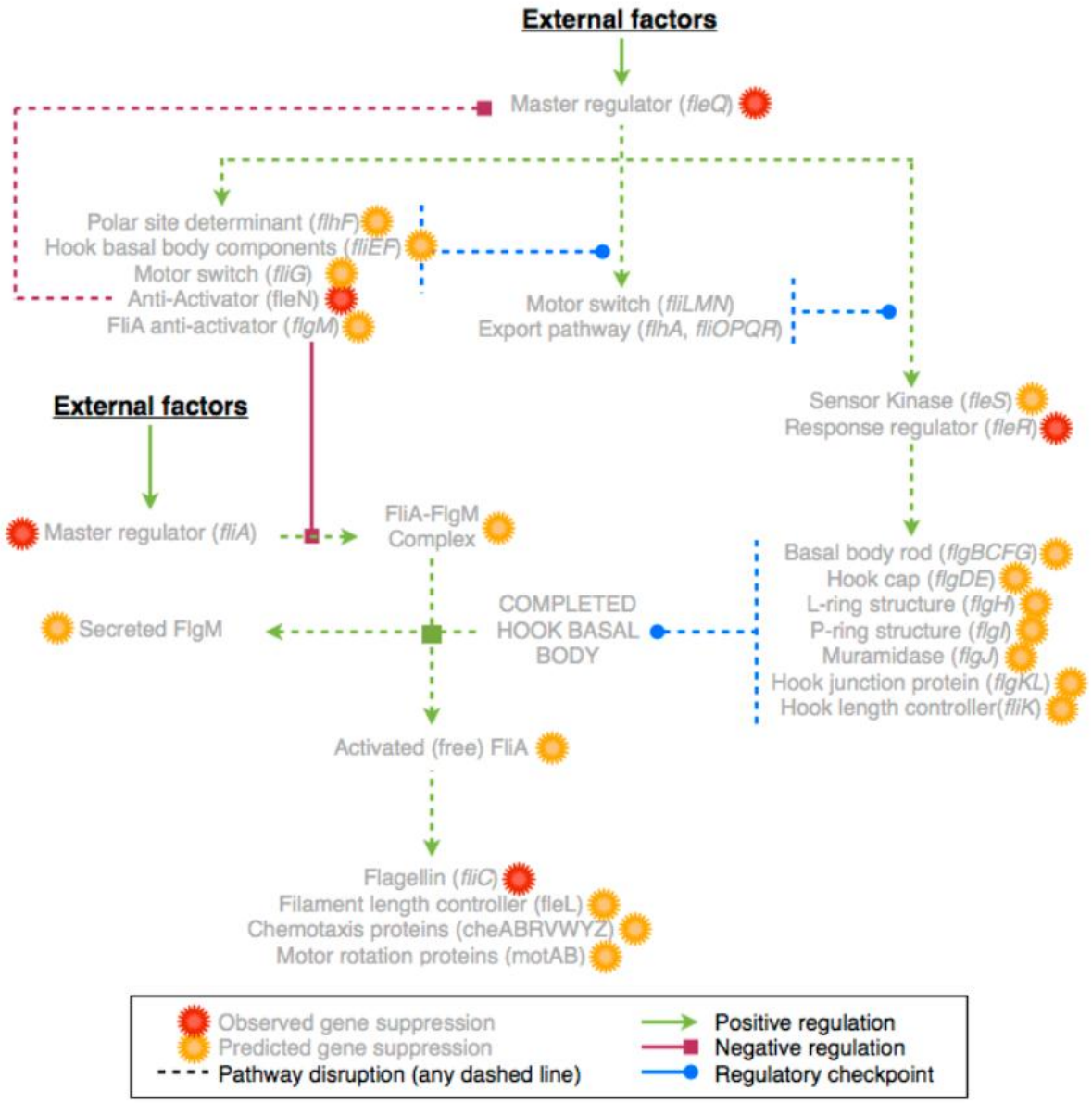


Figure 7. Differential expression of the flagellar regulon in *P. aeruginosa* cells grown with inhibitory concentrations of manuka honey. Observed (red star) and suspected (yellow star) suppression of flagella gene expression in *P. aeruginosa* cells treated with manuka honey for 3

hours. Suspected gene expression alterations were based on regulatory check points, regulator suppression and operon structure.