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Preliminary Analysis of the Anti-biofilm Efficacy of Manuka Honey on Extended Spectrum B-lactamase Producing *Escherichia Coli* Tem-3 and *Klebsiella Pneumoniae* Shv18, Associated with Urinary Tract Infections.

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Abstract

Urinary Tract Infections (UTIs) are one of the most common infections in the UK and many other parts of the world. The prevalence of the Extended Spectrum β-Lactamases (ESBLs) producing UTIs, combined with their ability to form a bio film, has significantly risen and is limiting therapeutic options. This study investigated the anti-bio film activity of Manuka honey on two ESBL producing pathogens, Escherichia coli TEM-3 and Klebsiella pneumonia SHV18, commonly found in UTIs. The ESBL production was confirmed by the double disk synergy method used to confirm the ESBL production. The antibacterial activity of Manuka honey was determined using the agar well diffusion method. The Minimum Inhibitory Concentration (MIC) was established using serially diluted honey ranging from 50% to 1.56%. The effect of Manuka honey on the pathogen bio films was analysed using the Tissue Culture Plate method, with an established MIC and under 24h incubation with the honey. The results indicated that K. pneumonia SHV18 is a stronger bio film producer than E. coli TEM 3. 50% (w/v) MIC Manuka honey appears to fully prevent the plank tonic growth of both strains. A significant reduction of 81% of the E. coli TEM3 ($p < 0.001$) and 52% of the K. pneumonia SHV18 ($p = 0.001$) bio film biomass was observed. The E. coli bio films were found to be more sensitive to the 50% (w/v) honey dilution than those produced by K. pneumonia. The study indicated the anti-bio film potency of Manuka honey and its potential to become an alternative treatment for the ESBL producing pathogens associated with UTIs.

Keywords: Bio films, Manuka honey, ESBLs, biomass, Urinary Tract Infections

Introduction

Prevalence of Urinary Tract Infections

Urinary Tract Infections (UTIs) are among the most common bacterial infections in humans and are rapidly increasing in different parts of the world, adding high medical costs to the healthcare community (Pitout, 2012 and Patwardhan et al., 2017). UTIs affect approximately 150 million

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people annually worldwide, and thus have become one of the most common human infections (Dash et al., 2018). UTIs are a substantial cause of morbidity, especially in females, elderly men and infants (Foxman, 2003). The complications of UTIs can be serious, involving recurrent infections, pyelonephritis with sepsis, pre-term birth and renal damage in children. (Reid, 2018). The most common bacterial species isolated from UTIs are Escherichia coli, Klebsiella pneumonia, Pseudomonas aeruginosa and Proteus mirabilis. More than 80% of UTIs are caused by uropathogenic E. coli strains (Trautner et al., 2003). UTIs caused by K. pneumonia were considered to be hospital-acquired, due to its ability to attach to plastic medical instruments such as urethral catheters (Anes et al., 2017), but they was later found in outpatients spread around the community (Mazzariol, 2017).

Antibiotic resistance and biofilm formation ability of UTI pathogens

In the early 1960s, two of the clinically important plasmid mediated Beta Lactamase enzymes, encoded by the TEM and SHV genes, were identified within E. coli and K. pneumonia, respectively (Shaikh et al., 2015). Those strains quickly became resistant to 3rd generation antibiotics and became widely known as Extended Spectrum Beta Lactamases (ESBL) (Mazzariol, 2017). Clinically, the most important class of ESBLs include the TEM, SHV and CTX-M (ur Rahman et al., 2018 and Reid, 2018). A cross-sectional hospital based study on the prevelence of resistance among UTIs, carried out in Leicestershire in the UK by the author's research team found that 76% of CTX-M, 3.8% of TEM and 3.8% of SHV account for the UTI isolates tested. (Reid et al., 2018). ESBLs produce betalactamase and carbapenemase enzymes, which easily hydrolyse the Beta-Lactam ring commonly shared by many antibiotics, such as penicillin, carbapenems, cephalosporin's and aztreonams, used in UTI treatments (Baho et al., 2018). In recent years many have reported that the rapid dissemination of these ESBL producers among UTIs is due to the pathogens' increased ability to form bio film structures (Patwardhan et al., 2017). Hence it is vital to understand the ability of bio film formation of ESBL producing E. coli and K. pneumonia to develop an effective treatment for UTIs caused by these pathogens. Bacterial bio films are organized bacterial communities embedded in an exopolysaccharide matrix on biotic and abiotic surfaces, and are a major problem as they interfere in bacterial eradication with antibiotics (Dufour et al., 2010). Bio film formation is a five-step process, as illustrated in Figure 1. Biofilm-associated cells are physiologically and phenotypic ally different from free living cells in terms of the cell densities, nutrients, oxygen supply, waste product concentration, and gene expression. The increased resistance to antimicrobials is an important characteristic of these bio film associated cells, which can reach up to 1000-fold more free living cells. This resistance can be implemented through several mechanisms; resistance gene exchange within the community, efflux pump expression, modifying pH values and metal ion concentration that leads to inactivation of the antibiotic, restriction of the antibiotic diffusion through the matrix, and furthermore, the presence of metabolically inactive persister cells that have a high tolerance to antibiotics (Prigent-Combaret et al., 1999, Parsek and Greenberg, 2005, Xu et al., 1998, Mikkelsen et al., 2007, Stewart and Costerton, 2001, Lewis et al., 2001). Hence collectively, bio film formation represents a significant threat to infection treatment and for many antimicrobial treatments fail, as nearly 65-80% of all infections are presumably related to the bio

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film formation ability of pathogens associated with UTIs (Hall-Stoodley et al., 2004, Costerton et al., 1999, Parsek and Singh, 2003).

Demand for alternative methods for UTI treatment.

As indicated above, the development of bio films in ESBL producing pathogens among UTIs is an issue that needs attention and alternative methods for treatment need to be sought urgently. In the last few years, studies have shown the potential of plant based anti-microbials on Gram-negative pathogens, and the potential to lower the risk of resistance development, as many of these plant based products do not affect the growth and fitness of the pathogens, and hence do not exert an associated selection pressure for the tested pathogens (Ravichandiran et al., 2013 and Tay & Yew, 2013). Of the plant-based products analysed, Manuka honey has demonstrated antimicrobial activity against both Gram-positive and Gram-negative bacteria (Bouacha et al., 2013). Specifically for Gram- negative bacteria, Manuka honey has demonstrated its antimicrobial activity in targeting the bacterial outer membrane proteins, leading to instability and disruption of the membrane, followed by the cell lysis (Camlin and Maddocks, 2014). The antimicrobial activity of Manuka honey is attributed to several factors, including its high concentration of sugar and acidity, and the presence of non-peroxide components, such as methylglyoxal (MGO), which have been widely explored in many studies (Carter et al., 2016). The high level of MGO in Manuka honey is due to the conversion of the dihydroxyacetone (DHA) present in the nectar of Leptospermum flowers and is considered the main reason for its non-peroxide activity (Kwakman and Zaat, 2012). Manuka honey has played a significant role in wound treatment and healing for many years, and, very recently, it was officially approved as an alternative antimicrobial for clinical use (Morroni et al., 2018).

Although studies have showed the antimicrobial activity on many Gram-negative pathogens, its anti-bio film efficacy is not well documented, specifically for Gram-negatives such as ESBL producing pathogens. Therefore, the objective of the present study was to analyse the anti-bio film efficacy of Manuka Honey on E. coli (TEM-3) and K. Pneumonia (SHV-18), which are associated with UTIs. Firstly, the study evaluated the direct antimicrobial activity of Manuka honey on the selected strains. Secondly, it evaluated the bio film forming ability of the two strains and thirdly, it analysed the effect of Manuka honey on bio film reduction in E. coli (TEM-3) and K. Pneumonia (SHV-18).

Materials and Methods

Bacterial Strains and genotypic detection.

All of the test and control strains were obtained from the Public Health England, UK, National Collection of Type Cultures: NCTC 13351(E. coli TEM-3), NCTC 13368 (K. Pneumonia SHV-18) and NCTC 12241 (E. coli) and NCTC 9633 (K. pneumonia) were used as controls two antibiotics resistant ESBL bacteria strains. The genotypic identification of the ESBL producing genes TEM-3 and SHV-18 was confirmed by the multiplex PCR method, as outlined in our previous work (Reid et al., 2018).

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Phenotypic detection of ESBL

A Double disk synergy test was performed to confirm the ESBL production of the test strains according to the MAST ESBL ID double disk synergy method, specified in the manufacturer's instructions (MAST, 2018). Three sets D62C, D52C and D64A were used to identify the ESBL strains. Semi- confluent growth cultures were prepared from an overnight culture according to the British Society of Antimicrobial Chemotherapy (BSAC) standards (BASC, 2013). The discs contained Cefotaxime, Ceftazidime and Cefpodoxime with an ESBL inhibitor Clavulanic acid. The plates were incubated at 37°C for 18 hours. A zone of inhibition difference of ≥ 5mm between the ESBL producer and the inhibitor indicated a positive result in terms of ESBL production.

Manuka Honey sample

Unpasteurized and cold press Steen's Manuka Honey UMF 24+ produced in New Zealand (Steenshoney.com, 2019) was chosen as a sample. UMF24+ is regarded as having a minimum of 1122 mg/kg of Methylglyoxal, which is the key antimicrobial component of Manuka honey tested.

Antimicrobial activity of Manuka honey by Agar Well diffusion

An Agar Well diffusion assay was performed to determine the antimicrobial effect of the honey, based on the protocol described by Boorn et al. (2010), with modifications: Three 8mm wells were cut into each agar plate using a sterile cork borer. 150-200µl of 50% (w/v) Manuka honey was added to fill each well. The honey dilution was prepared prior to the experiment by mixing 2g of Manuka honey with 2ml of sterile water. Dilution of the neat honey was needed as the honey was considered dense and adjusted according to the test. Bacterial strains grown on nutrient broth were streaked onto Mueller-Hinton Agar (MHA) and incubated with Manuka honey at 37°C for 18 hours. After incubation the diameters of the inhibition zones were measured.

Minimum Inhibitory Concentration (MIC)

In order to determine the concentration of Manuka honey to use in the Bio film assays, the MIC for both test strains (TEM3 and SHV18) was established, as described previously (Sherlock et al., 2010) using sterile 96-well microtiter plates. A starting concentration of 50% (w/v) Manuka honey was prepared. Serial dilutions in duplicate of the following concentrations, 25%, 12.50%, 6.25%, 3.13%, 1.56%. were created in an LB medium. An overnight cultures of test strains were diluted 0.5 McFarland standard in an LB medium and 200µl of this diluted culture was added to each concentration. The plates were incubated at 37°C for 18 hours and the shaking incubator at 1000rpm. A positive control, consisting of an LB medium and Test strain, and a negative control, consisting of an LB medium only, were included in each assay. The MIC was determined as the lowest concentration with no visible growth using a spectroscopic assay, as described previously (Patton et al., 2006). The optical density (OD_{600}) of the wells was measured using the plate reader SPECTRA max Plus at

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600nm, prior to incubation (T0) and after incubation (T24). The recorded measurements were subtracted (T24- T0) for each of the replicates to see the bacterial growth. To calculate the percentage of inhibition in each well, the equation % of inhibition = 1 - (OD test well/OD of corresponding control) x 100 was used. Maximal Bacterial Growth (MBG) was performed to confirm the MIC of the honey, by spreading 20µl of the suspension from the wells with no turbidity present. The MIC was reported as lower honey concentration resulted in 100% of inhibition and no MBG.

Detection of bio film formation

The bio film formed by each test strain was assessed by a Tissue Culture Plate (TCP) assay, as described previously (Baho et al., 2018), with modifications: the overnight cultures of the test strains were diluted in LB to the ratio of 1:100 to achieve the confluence of 105 cells. 3ml of each test strain and the LB medium only, were cultured in triplicate in two separate plates. The plates were then incubated at 37°C in a static incubator for 24h. After incubation the cultures were discarded carefully and the wells were washed with saline water to remove unattached cells to eliminate the background staining. Each well was fixed with 4ml of 99% methanol for 15min; then the plates were emptied and left to dry at room temperature for 30 min in an inverted position. After that each was further stained with 4ml of 2% crystal violet for 10min. The stain was rinsed with sterile water until clean and the dye was re-solubilised with 4ml of 33% glacial acetic acid. After 30min of incubation at room temperature, the optical density of each well was measured using spectrophotometer (HλLIOS) at 570nm to evaluate the amount of bio film formation.

Effect of Manuka honey on formed bio films

To determine the effect of Manuka honey on established bio films (growth under 24 hours in a total volume of 2ml in TCP plates) they were treated with MIC of Manuka honey previously established for planktonic bacteria. Diluted (1:100) bacterial cultures in a volume of 2ml were inoculated in sixapulate together with and LB medium only as a negative control in triplicate in a volume of 2ml. After incubation the bacterial cells were discarded and the wells were washed with saline water as described previously (Cooper et al, 2011). After that, three wells containing the bacterial culture were filled with 2ml of 50% Manuka honey and the next three with 2ml LB medium only. The negative control wells were re-filled with 2 ml of LB medium only. The plates were then incubated for a further 24 hours. The bio film formation was determined as described previously. The average optical density values of the bio films treated with Manuka honey were compared with the untreated ones to determine the reduction in bio film biomass after 24 hours of Manuka honey treatment.

Statistical analysis

Each experiment was performed twice for each test organism and the representative values were the average of the triplicates. The data collected were approximately normally distributed. An

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Independent T test was run to analyse the statistical difference between the control and treated groups in SPSS. A P-value <0.05 indicated statistical significance in the reduction of the bio film biomass.

.**Results**

ESBL detection and Agar well diffusion.

The presence of ESBL activity was confirmed by the Double Disk Synergy method for the test strains, E. coli TEM-3 and K. pneumonia SHV-18. Both strains showed sensitivity to Cefotaxime and Ceftazidime combined with Clavulanic acid (Figure 2). The size of the zone of inhibition was measured in millimetre units, recorded on a table and interpreted using MAST standard instructions, as shown in Table 1. The agar well diffusion assay demonstrated antimicrobial activity of 50% (w/v) Manuka honey for both the test and control strains. However, the inhibition of the growth was significantly lower for the ESBL producing strains compared to the controls. Furthermore, we observed that K. pneumonia SHV-18 is more sensitive to honey compared to E. coli TEM-3. The inhibition of the growth by Manuka honey for all of the strains was represented as an average zone of inhibition, as shown in Table 2.

MIC of Manuka honey for E. coli TEM-3 and K. pneumonia SHV-18.

The MIC of Manuka honey for both test strains, E. coli TEM-3 and K. pneumonia SHV-18 was observed as the 50% (w/v) honey dilution based on the calculation established by Patton et al. (2006), and the results are indicated in Table 3. A massive drop in the inhibition ability of Manuka honey was observed for K. pneumonia SHV-18 - below 25% (w/v), whereas for E. coli TEM-3, a gradual decrease in inhibition was observed in lower honey concentrations. The negative values of inhibition indicated the growth of bacteria. Hence in low Manuka concentrations there was increased growth of K. pneumonia SHV-18 growth compared to E. coli TEM-3. These results were further confirmed by the Maximal Bacterial Growth (MBG) assay for the bacterial cultures treated with 50% (w/v) and 25% (w/v) Manuka honey. MBG has shown that the 50% (w/v) honey as the MIC for both ESBL producing strains, as there was no bacterial growth on the plates observed. The bacterial cultures consisting of a 25% dilution of honey exhibited small growth for E. coli TEM-3, and for K. pneumonia SHV-18 normal growth was observed.

Bio film formation of the E. coli TEM-3 and K. pneumonia SHV-18.

The ESBL strains were assessed for their bio film-forming ability prior to the Manuka honey treatment. The tested strains were classified according to their OD570 values regarding their adherence capacity, as mentioned in Baho et al. (2018). An OD570 of less than 0.2 was considered as weakly adherent, 0.2-0.4 was considered as moderately adherent, while greater than 0.4 was considered as strongly adherent (Table 4). Both strains were identified as having the ability to attach and form bio films on a plastic surface in-vitro. The mean amount of bio film formed in static conditions by E. coli TEM 3, expressed by the OD570 reading was 0.430,

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whereas by K. pneumonia SHV18 the OD570 reading was 0.548, which indicates strong adherence ability. K. pneumonia produced almost 20% more biomass than E. coli, indicating its stronger bio film formation ability.

The Effect of Manuka honey on E. coli TEM-3 and K. pneumonia SHV-18 bio films.

To analyse the efficacy of Manuka honey on E. coli TEM-3 and K. pneumonia SHV-18 bio films (grown under 24 h condition), strains were treated with 50% (w/v) Manuka honey and incubated for further 24 h period. For the E. coli TEM-3 samples, which were not treated with Manuka honey, an OD570 of 0.453 was observed, and for the Manuka honey treated samples, an OD570 of 0.084 was observed, indicating an 81% reduction in OD570 in the presence of Manuka honey (Figure 3). The mean OD570 for K. pneumonia SHV18 was 0.544, whereas for the treated groups it was 0.26, indicating a 52% reduction in OD570 when treated with Manuka honey (Figure 4). The results show that both strains are sensitive to Manuka honey treatment after 24 hours' incubation; however E. coli TEM-3 displayed a very sensitive profile, as only 19% of the bio film biomass remained after treatment. The significant reduction in the bio film was confirmed by an Independent T test, as the p value of TEM-3 was reported to be lower than 0.001 (t value = 119.529; df = 2.048) and the P value of SHV18 was found to be equal to 0.001 (t value = 8.261; df = 4).

Discussion

Bio film development is a field of intense research for many pathogenic microorganisms, associated with bio film infections. However, very few studies have investigated the inhibitors of bio film associated ESBL producing UTI pathogens. Therefore, the present study evaluated the bio film inhibition activity of Manuka honey on the ESBL producing pathogens, E. coli TEM-3 and K. pneumonia SHV18, which are commonly associated with UTIs. Our study found that Manuka honey exhibited substantial activity in terms of eradicating the biomass of the bio films formed by both of the ESBL strains tested. This behaviour may be accounted for by the inhibition and annihilation ability of Manuka honey against the bio film-formation process, as previously reported in regard to medically important antibiotic resistant Gram-negative strains (Emineke et al., 2017 and Okhiria et al., 2009). A greater reduction in the biomass of E. coli TEM-3 was observed compared to K. pneumonia SHV-18. Our observation of the ability of low inhibition of bio films by Manuka honey on K. pneumonia SHV-18, could be attributed to its higher bio film formation ability. This characteristic of K. pneumonia could account for its faster kinetics in the bio film formation process; thus it has recently been reported as a strong bio film forming pathogen (Surgers et al., 2019). The observed behaviour on greater inhibition by Manuka honey on E. coli TEM-3, similarly agreed with other ESBL producing E. coli pathogens, as reported previously (Idris and Afegbua, 2017).

The study undertaken on E. coli by Neupane et al. (2016) highlighted that ESBL producing strains have a greater capacity to form bio films, which was further confirmed in our previous studies, in relation to the bio film formation ability of non ESBL and ESBL producing pathogens (Baho et al., 2018). Similarly, in this study, we have shown that ESBL producing E. coli TEM-3

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and K. pneumonia SHV-18 also have a strong adherence capacity to form bio films. Hence the increased ability of these cells to produce bio films may correlate with the increased resistance to many antimicrobials and prevalence in many bio film associated infections, including the prevalence of E. coli TEM-3 and K. pneumonia SHV-18 in UTIs. As the above findings and our present study, emphasize the correlation between higher bio film production and increased antibiotic resistance, it is necessary to find alternative methods or treatments to disturb the bio films produced by ESBL producing pathogens.

The agar well assay was performed in this study to determine the bactericidal activity of Manuka honey indicated that the honey has a lower capacity of inhibition against ESBL producing, drug resistant strains compared to non – ESBL antibiotic susceptible controls (Table 2). This reduced effect of the honey was similarly observed in a previous study on multi-drug resistant, bio film associated pathogens (Kwakman and Zaat, 2012).The spectroscopic analysis to determine the efficacy of Manuka honey, on tested ESBL strains, indicated that K. pneumonia SHV18 is more sensitive to Manuka honey than E. coli TEM-3, as lower concentrations of honey was able to inhibit any visual growth of the K. pneumonia SHV18 strain (Table 3). The TCP method was chosen for this study as this method has been used as a standard to detect the efficacy of Manuka honey, and the method is also the most appropriate in estimating bacterial growth in lower concentrations, as reported previously. For example, the highest bacteriogenic activity on ESBL-producing K. pneumonia was observed at a lower concentration of 30-40% (w/v) in a study by Shah Pratibha J, Williamson TM (2015), and similarly a lower concentration of 30% (w/v) was noted in a study on metallo-β- lactamases (NDM-1), ESBL (Qamar et al., 2018). However, instead of using serial dilutions, a series of dilutions would allow more precise values in a wider range of concentrations, which would improve our current bio film efficacy analysis further.

In the present study, we observed the biomass reduction of the bio films for both E. coli TEM-3 and K. pneumonia SHV18, thereby showing the potential anti-bio film efficacy of Manuka honey on ESBL producing UTI pathogens. Understanding the mechanism of the honey in bio films infections is important, as there is little research available on mechanism of action of Manuka honey on E. coli and K. pneumonia bio film associated UTIs. It was reported that Gram- negative being targeted by honey in two different anti-bio film mechanism patterns (Camplin and Maddocks, 2014). The first mechanism was attributed to honey's direct biocidal activity, and the second one was attributed to honey's anti-virulent activity (Camplin and Maddocks, 2014). A previous study on the effect of Manuka honey on the Gram-negatives, E. coli, and P. merabilis, reported a 15% to 70% biomass reduction due to impaired adhesion within these cells (Emineke et al. 2017). Similarly, the biocidal activity of Manuka honey (a reduction of bio film biomass up to 85%) due to impaired adhesion was also observed in P. aeruginosa bio films when exposed to a 40 % (w/v) Manuka honey concentration (Okhiria et al., 2009).

Understanding the potency of Manuka honey is also important in these types of bio film analysis, as the potency of honey can vary as much as 100-fold and many studies have reported that honey concentrations by (w/v) or (v/v) do not represent the true density of the honey considered (Halstead et al., 2016). The degree of dilution is crucial for retaining the

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antimicrobial activity and this varies between honeys with peroxide and non-peroxide properties (Olaitan et al., 2007). Literature argued that which component of Manuka honey is a major antagonist of developing bio films as it is necessary to determine how this component in Manuka honey interferes with bio film integrity. One possible suggestion regardless of the fact that the non-peroxide concentration may vary between Manuka honey brands and further dilution can affect its activity, is the ability of Manuka honey to create a high osmotic pressure, which could lead to destroying the extracellular matrix of bio films, allowing the active components of the honey to penetrate and interact with bacteria on the molecular level.

Conclusion

The main results of the present work demonstrate the anti- bio film efficacy of Manuka honey on the ESBL producing strains E. coli TEM-3 and K. pneumonia SHV18 and that Manuka honeys has the potential to be an effective bio film inhibitor for both strains. The ability to develop bio films was confirmed in both E. coli TEM-3 and K. pneumonia SHV18. Furthermore, the bio films established by E. coli TEM-3 were found to be more sensitive to Manuka honey activity compared to those established by K. pneumonia SHV18, as a greater biomass reduction was observed for the E. coli TEM-3. Collectively the results obtained indicate that Manuka honey may be a promising antibacterial for the treatment and modulation caused by ESBL pathogens, specifically the ESBL producers associated with UTIs. Unlike conventional antibiotics, Manuka honey has an extra advantage, as it appears to act on multiple bacterial targets, and hence it has the lowest propensity for inducing further resistance among ESBL associated UTI pathogens. However, the time and dose dependent manner of biomass reduction need to be further tested, specifically the response of the biofilms to different concentrations of Manuka honey over time; both shorter and prolonged incubation with Manuka honey need be further analysed. Furthermore, the inhibitory effect and influence on bacterial adhesion need to be addressed by adding a honey dilution together with bacterial cultures prior to the biofilm development process, as shown by a previous study (Cooper et al., 2011). Moreover, the molecular mechanism through which Manuka honey mediates anti-biofilm activity requires further investigation. It is not yet well known what the impact of Manuka honey is on the expression of virulence-related genes, with an emphasis on the genes involved in biofilm formation, quorum sensing (cell dependent bacterial communication) and stress survival. Hence it is necessary to further explore the impact of Manuka honey on the expression of virulence-related genes, in these ESBL producing UTI pathogens.

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Author Contribution

SS has conceived and designed and wrote the manuscript. PC and SB preformed experiments and helped manuscript preparation.

References

Anes, J., Hurley, D., Martins, M. & Fanning, S. (2017) Exploring the genome and phenotype of multi-drug resistant Klebsiella pneumonia of clinical origin. Frontiers in microbiology, 8, 1913.

Baho S, Reid R and Samarasinghe S. (2018) Adaptability to Various Growth Conditions of Bio film Associated Extended-Spectrum-Beta-Lactamases Producing Bacteria. Journal of Infectious Diseases and Diagnosis, 3(1), 1-9.

BASC (2013) Methods for Antimicrobial Susceptibility Testing. BASC British Society for Antimicrobial Chemotherapy. Available from: http://www.bsac.org.uk.

Boorn, K. L., Khor, Y. Y., Sweet man, E., Tan, F., Heard, T. A., & Hammer, K. A. (2010) Antimicrobial activity of honey from the stingless bee Trigona carbon aria determined by agar diffusion, agar dilution, broth micro dilution and time-kill methodology. Journal of applied microbiology, 108(5), 1534-1543.

Bouacha, M., Ayed, H. and Grara, N. (2013) Honey Bee as Alternative Medicine to Treat Eleven Multidrug-Resistant Bacteria Causing Urinary Tract Infection during Pregnancy. Scientia Pharmaceutical, 86 (2), 14.

Camplin, A.L. and Maddocks, S.E. (2014) Manuka honey treatment of bio films of Pseudomonas aeruginosa results in the emergence of isolates with increased honey resistance. Annals of Clinical Microbiology and Antimicrobials, 13(1), 19.

Carter, D. A., Blair, S. E., Cokcetin, N. N., Bouzo, D., Brooks, P., Schothauer, R., & Harry, E. J. (2016) Therapeutic manuka honey: no longer so alternative. Frontiers in microbiology, 7, 569.

Cooper, R., Jenkins, L. and Rowlands, R. (2011) Inhibition of bio films through the use of manuka honey. Wounds UK, 7 (1), 24-32.

Costerton, J. W., Stewart, P. S. & Greenberg, E. P. (1999) Bacterial bio films: a common cause of persistent infections. Science, 284, 1318-22.

Dash, N. R., Albataineh, M. T., Alhourani, N., Khoudeir, A. M., Ghanim, M., Wasim, M., & Mahmoud, I. (2018) Community-acquired urinary tract infections due to extended-spectrum β-lactamase-producing organisms in United Arab Emirates. Travel Medicine and Infectious Disease, 22, 46-50.

Dufour, D., Leung, V. & Lévesque, C. M. (2010) Bacterial bio film: structure, function, and antimicrobial resistance. Endodontic Topics, 22, 2-16.

Emineke, S., Cooper, A. J., Fouch, S., Birch, B. R., & Lwaleed, B. A. (2017) Diluted honey inhibits bio film formation: potential application in urinary catheter management? Journal of Clinical Pathology, 70(2), 140-144.

Foxman, B. (2003) Epidemiology of urinary tract infections: incidence, morbidity, and economic costs. Disease-a-month, 49(2), 53-70.

Hall-Stoodley, L., Costerton, J. W. & Stoodley, P. (2004) Bacterial bio films: from the natural environment to infectious diseases. Nature reviews microbiology, 2, 95-108.

Halstead, F. D., Webber, M. A., Rauf, M., Burt, R., Dryden, M., & Oppenheim, B. A. (2016) In vitro activity of an engineered honey, medical-grade honeys, and antimicrobial wound dressings against bio film-producing clinical bacterial isolates. Journal of wound care, 25(2), 93-102.

Idris A. R. and Afegbua A.S. (2017) Single and joint antibacterial activity of aqueous garlic extract and Manuka honey on extended-spectrum beta-lactamase-producing Escherichia coli. Transactions of the Royal Society of Tropical Medicine and Hygiene, 111 (10), 472-478.

Kwakman, P.H.S. And Zaat, S.A.J. (2012) Antibacterial components of honey. IUBMB Life, 64 (1), 48-55.

Lewis, H. A., Furlong, E. B., Laubert, B., Eroshkina, G. A., Batiyenko, Y., Adams, J. M., Bergseid, M. G., Marsh, C. D., Peat, T. S. & Sanderson, W. E. (2001) A structural genomics approach to the study of quorum sensing: crystal structures of three LuxS orthologs. Structure, 9, 527-537.

MAST, G. (2015) ESBL and AmpC Detection Disc Sets. In: LTD., M. G. (ed.). Bootle: Mast House.

Mazzariol, A., Bazaj, A., & Cornaglia, G. (2017) Multi-drug-resistant Gram-negative bacteria causing urinary tract infections: a review. Journal of Chemotherapy, 29(sup1), 2-9.

Mikkelsen, H., Duck, Z., Lilley, K. S. & Welch, M. (2007) Interrelationships between colonies, biofilms, and planktonic cells of Pseudomonas aeruginosa. Journal of bacteriology, 189, 2411-6.

Morroni, G., Alvarez-Suarez, J. M., Brenciani, A., Simoni, S., Fioriti, S., Pugnaloni, A., Giampieri, F., Mazzoni, L., Gasparrini, M., Marini, E & Mingoia, M. (2018) Comparison of the antimicrobial activities of four honeys from three countries (New Zeeland, Cuba, and Kenya). Frontiers in microbiology, 9, 1378.

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ISSN: 2581-3366

Neupane, S., Pant, N. D., Khatiwada, S., Chaudhary, R., & Banjara, M. R. (2016) Correlation between biofilm formation and resistance toward different commonly used antibiotics along with extended spectrum beta lactamase production in uropathogenic Escherichia coli isolated from the patients suspected of urinary tract infections visiting Shree Birendra Hospital, Chhauni, Kathmandu, Nepal. Antimicrobial resistance and infection control, 5(1), 5.

Okhiria, O. A., Henriques, A. F. M., Burton, N. F., Peters, A., & Cooper, R. A. (2009) Honey modulates biofilms of Pseudomonas aeruginosa in a time and dose dependent manner. Journal of Api Product and Api Medical Science, 1(2), 6-10.

Olaitan, P.B., Adeleke, O.E. and Iyabo, O.O. (2007) Honey: a Reservoir for Microorganisms and an Inhibitory Agent for Microbes. African Health Sciences, 7 (3), 159-165.

Parsek, M. R. & Greenberg, E. P. (2005) Sociomicrobiology: the connections between quorum sensing and bio films. Trends in microbiology, 13, 27-33.

Parsek, M. R. & Singh, P. K. (2003) Bacterial bio films: an emerging link to disease pathogenesis. Annual Reviews in Microbiology, 57, 677-701.

Patton, T., Barrett, J., Brennan, J., & Moran, N. (2006) Use of a spectrophotometric bioassay for determination of microbial sensitivity to manuka honey. Journal of Microbiological Methods, 64(1), 84-95.

Patwardhan, V., Kumar, D., Goel, V., & Singh, S. (2017) Changing prevalence and antibiotic drug resistance pattern of pathogens seen in community-acquired pediatric urinary tract infections at a tertiary care hospital of North India. Journal of laboratory physicians, 9(4), 264.

Pitout, J.D. (2012) Extra intestinal Pathogenic Escherichia coli: A Combination of Virulence with Antibiotic Resistance. Frontiers in microbiology, 3, 9.

Prigent-Combaret, C., Vidal, O., Dorel, C. & Lejeune, P. (1999) Abiotic surface sensing and biofilm-dependent regulation of gene expression in Escherichia coli. Journal of bacteriology, 181, 5993-6002.

Ravichandiran, V., Shanmugam, K., & Princy Solomon, A. (2013) Screening of Sdi A inhibitors from Melia dubia seeds extracts towards the hold back of uropathogenic E. coli quorum sensing-regulated factors. Medicinal Chemistry, 9(6), 819-827.

Reid, R. (2018) Beta-lactam resistant urinary tract infections: prevalence, the development of rapid diagnostics and novel treatments. Unpublished thesis (PhD.), De Montfort University.

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ISSN: 2581-3366

Reid, R., AL-Bayati, M. & Samarasinghe, S. (2018) Genotypic Identification of Extended-Spectrum β-Lactamase (ESBL) Producing Enterobacteriaceae from Urinary Tract Infections in the Leicestershire Area, United Kingdom: A One Health Prospective. Journal of Infectious Diseases and Diagnosis, 3(2), 1-8

Shaikh, S., Fatima, J., Shakil, S., Rizvi, S. M. D., & Kamal, M. A. (2015) Antibiotic resistance and extended spectrum beta-lactamases: Types, epidemiology and treatment. Saudi journal of biological sciences, 22(1), 90-101.

Sherlock, O., Dolan, A., Athman, R., Power, A., Gethin, G., Cowman, S., & Humphreys, H. (2010) Comparison of the antimicrobial activity of Ulmo honey from Chile and Manuka honey against methicillin-resistant Staphylococcus aureus, Escherichia coli and Pseudomonas aeruginosa. BMC complementary and alternative medicine, 10(1), 47.

Steenshoney.com (2019) UMF 24+ Raw Manuka Honey 250G [Online] steenshoney.com. available from https://steenshoney.com/products/steens-raw-manuka-honey-umf-24-cold-pressed [accessed 02/02/2018].

Stewart, P. S. & Costerton, J. W. (2001) Antibiotic resistance of bacteria in bio films. The lancet, 358, 135-8.

Surgers, L., Boyd, A., Girard, P. M., Arlet, G., & Decré, D. (2019) Bio film formation by ESBL-producing strains of Escherichia coli and Klebsiella pneumonia. International Journal of Medical Microbiology, 309(1), 13-18.

Tay, S., & Yew, W. (2013) Development of quorum-based anti-virulence therapeutics targeting Gram-negative bacterial pathogens. International journal of molecular sciences, 14(8), 16570-16599.

Trautner, B. W., Hull, R. A. & Darouiche, R. O. (2003) Escherichia coli 83972 inhibits catheter adherence by a broad spectrum of uropathogens. Urology, 61, 1059-1062.

Ur Rahman, S., Ali, T., Muhammad, N., Umer, T., Ahmad, S., Ayaz, S. & Han, B. (2018) Characterization and mechanism of dissemination of extended spectrum beta lactamase producers Escherichia Coli in food producing animals in Pakistan and China. 2018 15th International Bhurban Conference on Applied Sciences and Technology (IBCAST), IEEE, 203-214. Abstract retrieved from https://ieeexplore.ieee.org/abstract/document/8312224

Xu, K. D., Stewart, P. S., Xia, F., Huang, C. T. & Mcfeters, G. A. (1998) Spatial physiological heterogeneity in Pseudomonas aeruginosa bio film is determined by oxygen availability. Applied and Environmental Microbiology, 64, 4035-9.

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Figures and Tables

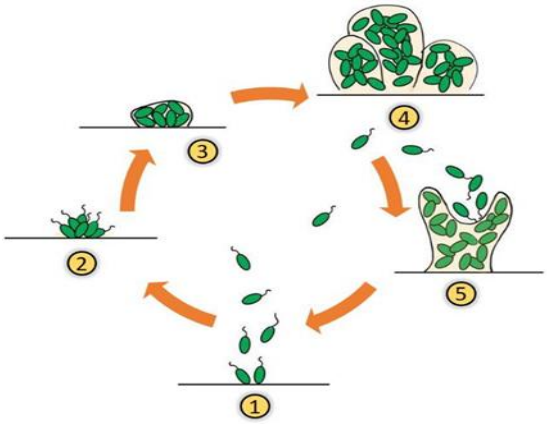

Figure 1. Bio film formation process. 1) Reversible attachment; 2) Irreversible attachment and formation of exopolysaccharide matrix; 3) Maturation under developed matrix; 4) 3D structure formation attained with micro channel; 5) Detachment of plank tonic bacteria.

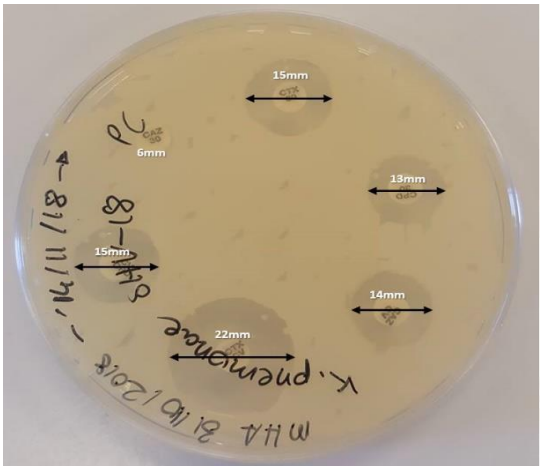

Figure 2. Identification of the ESBL via Double disk synergy. Illustration of the method used - K. pneumonia SHV18 as example.

Table 1. Interpretation of the results from MAST disc Synergy. Strain is regarded as Extended Spectrum β Lactamase (ESBL) if zone of inhibition between combination disk with clavulanicacid and corresponding antibiotic differs ≥ 5mm, in one or more pairs. Only E. coli TEM-3 and K. pneumonia SHV18 were positively recognised as ESBL, showing a difference in inhibition zones between combination disk and corresponding antibiotic. CPD – Cefpodoxime; CTX – Cefotaxime; CAZ – Ceftazidime; CV - Clavulanic Acid.

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Strain	D52C		D62C		D64A		ESBL
	CPD30	CPD CV	CTX30	CTX CV	CAZ30	CAZ CV	
E. coli TEM3	6	9	9	18	6	15	yes
E. coli NCTC 12241	22	21	26	26	19	18	no
K. pneumonia SHV18	13	15	15	22	6	14	yes
K. pneumonia NCTC 9633	26	24	26	26	20	21	no

Table 2. Well diffusion assay performed on Muller Hilton agar plated to check activity of 50% (w/v) of Manuka honey on semi-confluent cultures of control and pathogenic strains. The table represent zones of inhibition diameters (mm).

Strain	Well 1	Well 2	Well 3	Average
E. coli TEM-3	15	14	13	14.0
E. coli NCTC 12241 (control)	26	26	27	26.3
K. pneumonia SHV18	11	12	12	11.7
K. pneumonia NCTC 9633 (control)	26	28	27	27.0

*200µm of honey solution was used per well (8mm diameter).

Table 3. Minimum Inhibitory Concentration (MIC) established for ESBL strains. Values were obtained via Spectroscopic assay for MIC of honey according to (Patton et al., 2006). The optical density (OD) of wells prior incubation were subtracted from optical density after incubation. Percent of inhibition is equal 1 - (OD test well/OD of corresponding control) x 100. Positive values indicate inhibition of plank tonic bacteria, whereas negative values represent growth of bacteria in the culture. SD was calculated to evaluate distribution, from the mean values obtained.

Concentration of Manuka honey (%)	*E. coli* TEM3		*K. pneumoniae* SHV18	
	Average % of inhibition	SD	Average % of inhibition	SD
50%	109%	0.06	107%	0.02
25%	103%	0.17	12%	0.02
12.5 %	37%	0.21	-14%	0.02
6.25%	36%	0.15	-27%	0.02
3.13%	25%	0.12	-28%	0.06
1.56%	26%	0.11	-29%	0.07

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Table 4 Adherence ability of tested strains before and after treatment with Manuka honey. B (Before treatment), A (After treatment).

OD_{570} value	TEM-3		SHV-18	
	B	A	B	A
<0.2	-	√	-	
0.2-0.4	-	-	-	√
>0.4	√	-	√	-

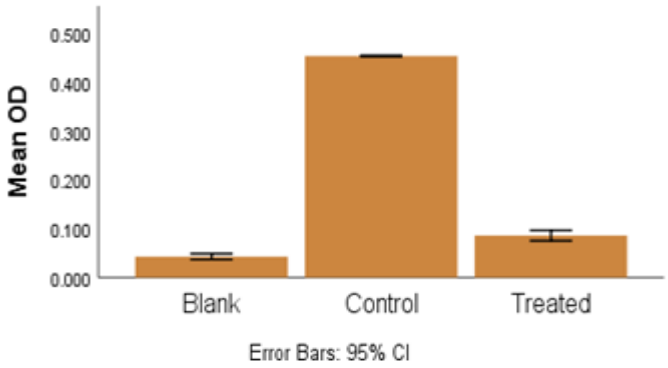

Figure 3. 24h bio film formation and reduction in bio film biomass of E. coli TEM-3 after treatment with 50% (w/v) of Manuka honey. Optical density of re-solubilised dye remaining on bio film was measured at 570nm. Bar chart shows 81% of bio film reduction. An Independent T-test were performed for control and treated bio films showing significant difference in biomass reduction (p value <0.001). Error bars show deviation form mean values.

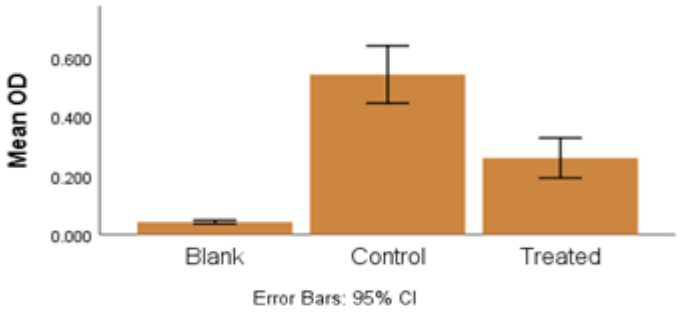

Figure 4. 24h bio film formation and reduction in bio film biomass of K. pneumonia SHV18 after treatment with 50% (w/v) of Manuka honey. Optical density of re-solubilised dye remaining on bio film was measured at 570nm. Bar chart shows 52% of bio film reduction. An Independent T-test were performed for control and treated bio films showing significant difference in biomass reduction (p value = 0.001). Error bars show deviation form mean values.