Function of honey in disruption of the salmonella typhi biofilm: An in-vitro evaluation

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Abstract

Background: Disruption of biofilm may result due to honey.
Objective: To form and detect the Salmonella enteric serovar Typhi biofilm in microtiter plate and role of honey in prevention and disruption of this biofilm.
Methodology: An experimental research was done at the Department of Microbiology, University of Health Sciences in Lahore, Pakistan in which Multi Drug Resistant (MDR) S. typhi clinical isolate was used, study duration consisted of 6 months, from April to September 2016. ATCC Staphylococcus aureus and ATCC Pseudomonas aeruginosa were used as standard control strains**.** Biofilm formation and detection was done by microtiter plate method. Two different honeys were used in order to see either they inhibits and disrupt the S.Typhi biofilm or not. Data analysis was done by using SPSS 16. Date analysia was done by spss 16.
Results: Manuka and Beri honey inhibited the biofilm formation of S. typhi at 20% (w/v) and both honeys disrupted the established biofilm of S.typhiat concentration greater than 30% (w/v). It wase vident from outcomes that three times to 4 times greater concentration of Beri and of Manuka honey was needed to unsettle established biofilm than that of its inhibition.
Conclusion: S.typhi form moderately adherent biofilm. The conducted research strongly supports that the honey, be it local or outsider, retains noticeable antibiofilm action against the S.typhi.
Key words: Salmonella typhi, Manuka honey, Beri honey, Biofilm, Anti-biofilm activity.

Introduction

The Biofilm is a collection of tiny microorganisms like bacteria, fungus, protozoan, bacteriophage or/and virus having cells attached to one another on either biotic or an abiotic surface.[1] The first biofilm was examined by Casterton in the year 1978.[2] He figured out that the biofilm are strongly held together having the protection of extracellular polymeric substance (EPS) from outside. Some of the vital components of the EPS comprise proteins, peptidoglycans, nucleic acid, lipids or phospholipids and few other constituents of the cell were also existent in the biofilm's matrix.[3]

The construction and development of Biofilm ensues in 2 steps. Firstly, the discrete freely stirring bacteria also known as planktonic attach reversibly to the surface for instance a surface of tooth or perhaps the prosthetic valves of heart. At the primary stage, bacteria are still vulnerable to the antibiotics. Now in the2ndstage they are irreversible adhere with the surface, reproduction, micro-colony establishment and formation of a polymer matrix around the micro-colony of bacteria takes place,[4] later the adult biofilm matures to become thick (approximately 50 μm). A specialized inter-bacterial communication mechanism operates for the controlling of formation that is; Quorum Sensing that is done by *AHL* (N-Acyl homoserine Lactose) particularly in the gram negative strainand the oligopeptides in gram positive strains of bactria.[5] The Biofilms are omnipresent and universal.

The major implants where biofilm are made comprises: the valves of heart, central venous catheters, the ventricular assist device, stent of coronary artery, neurosurgical ventricular shunts, stimulators especially implantable neurological ones, arthro-prostheses, devices used for fracture-fixation, implants of the breast, implants of the cochlea, the intraocular lense/s and also dental implants.[6] It has been studied that biofilm contributed to 80% of all infections in the human body as per the suggestion of Lewis.[7] Tenacious and chronic infections associated with biofilm-populated medical devices or instruments causes uneasiness, irritation and inflammation that warrants either removal or the replacement of the polluted device. The removal of biofilm infected medical instruments not only endangers the patient's health but also

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causes additional cost. Therefore, the bacterial biofilms may weaken cutaneous healing of the wound and reduces topical antibacterial efficacy in the healing process.[8] Such biofilms have shown the capability to carry on in 100 to 1000 times the concentrations of given antibiotics and the biocides which can otherwise impede planktonic cells.[9] Salmonella enteric serovar Typhi is well known for the formation of biofilm in gallbladder and kidney which results in chronic carrier state that has a high risk for dissemination of the infection to the public.[10] The Salmonella carriers having gallstones have proved be non-responsive to the antibiotic treatments.[11]

In order to cure such individuals there is the need for surgery and gallstone removal that is very cost prohibitive. Consequently keeping in view the fiscal circumstances of developing countries like Pakistan, the great cost of antibiotic is not an option for masses and snowballing antimicrobial resistance provoked us to the notion about exploring unconventional modalities for the cure of biofilm related infections with honey.[12,13,14]

In the past honey has been broadly used for curative purposes or a healing agent along with its extensive applications a famous food item.[13] In almost all of the Holy books including Holy Quran, The Holy Bible and The Holy Torah the miraculous and phenomenal healing qualities of honey are mentioned. Furthermore, it is a well-known saying of the Prophet Muhammad (Peace Be Upon Him):

"Honey is a cure for every illness while The Qur'an is a cure for all illness of the mind, consequently I commend to you both remedies, the Qur'an and honey." (Bukhari).[14]

The acidic pH, elevated osmolarity, ability to release hydrogen peroxide (H_2O_2) and plant obtained non-peroxide factors are some of the reasons for the antibacterial abilities of honey.[15] In the recent years it has been identified that some of the antibacterial properties of honey can be because of MGO (methylglyoxal) and the bee's defensing mechanism.[16] A modern study discovered that the quorum sensing can also be inhibited by using honey.[17] Honey acts in effective killing of drug resistant biofilms. Honey is found to have the ability to unsettle the biofilm synthesis by the strains of P. aeruginosa and *S.* aureus.[18] It's unlikely to treat biofilm with conservative antibiotics due to their resistance. Therefore there is a zealous need for exploring such antibacterial agents like honey that can disrupt or unsettle bacterial biofilm.

Methodology

The study type was Experimental in nature; the sudy setting was in the Department of Microbiology in the University of Health Sciences (UHS) Lahore, Pakistan, Form April to September 2016.

The Beri honey and also the Manuka honey UMF25+ (standardized honey / FDA approved) of Pakistan were utilized in this study. Salmonella typhi which is a Multi Drug Resistant strain was used for the study purpose. The clinical isolates obtained had been deposited in micro-bank in the Department of Microbiology, University of Health Sciences (UHS) Lahore, Pakistan. Staphylococcus aureus (ATCC 25923) and Pseudomonas aeruginosa (ATCC 27853) were utilized as the standard control strains. Sterile, Flat-bollonia 96 well polystureneminitive plates (TPP Switzerland) microtiter plates were utilized to cultivate biofilm**.** Salmonella typhi Multidrug-resistant strain was recognized by the standard morphological as well as the cultural featuresplus API-20 E.Salmonella O, Salmonella H and Salmonella Vi antisera (BD Difco™, USA) were used for the serological identification.

The formation of Biofilm and its detection by micro-titer plate technique

To begin with 200 µl of bacterial suspension were filled in 3 wells of each plate. Only nutrient broth was contained in negative control wells. Those plates were later enclosed and incubated aerobically for twenty-four hours at the temperature of 37ºC.250 µl of sterile physiological saline was used to aspirate and wash the contents of each well three times. Vigorous shaking was given to the plates in order to eliminate all non-adherent bacteria. The left over attached bacteria were secured with 200 µl of 99 % methanol per well. After the duration of 15 minutes each plate was emptied and left for drying. Formerly, 0.2 ml of 2% crystal violet was used for Gram staining of each well for 5 min. The plates were then placed under the running tap water in order to be off the excess stain. After which, the plates were left for air drying, resolubilization of the dye bound to adherent cells was done with 160µl of 33 % (v/v) Glacial acetic acid/well. The reason behind resolubilization was that the optical density reader

has the ability to calculate the Optical Density (OD) only in the center of the well. Glacial acetic acid was used to resolubilized the present dye in order to calculate biofilm molded both on the very bottom and the walls of the well.[19] The OD of each of the wells was calculated at 570 nm by the use of an automated *ELISA* reader. Standard deviations (SD) and the mean OD of the negative control were used to define and calculate the cut-off OD (ODc) for the micro-titer plate test. Based upon the ODs of bacterial films the strain was then categorized on the characteristic of adherence capabilities into the following types:

- (0) for non-adherent
- (+) for weakly
- (++) for moderately
- (+++) for strongly adherent,

The explanation of the strains is as follows:

- non-adherent $OD \leq OD_C$
- weakly adherent $OD_C < OD \leq 2 \times OD_C$
- moderately adherent $\times OD_C < OD \leq 4 \times OD_C$
- strongly adherent $4 \times OD_C < OD$

Different concentration of honey that is; 10%, 20%, 30%, 40% and 50% (w/v) were later introduced in subsequent 2 steps;During the first step, where during the method of biofilm synthesis it was observed whether or not honey inhibits the synthesis of the biofilm. Later when biofilm was recognized to observe if it was interrupted/ disrupted or not as demonstrated by the figure II.[20]All of the tests were done thrice and the obtained results were averaged.The estimation of standard deviations (SD) and the mean Optical Density (OD) of the negative control were used for definition of cut-off OD (OD_C) for the micro-titer plate and was calculated as three (SD) beyond the mean OD negative control.

Therefore, ($OD_{C=}$ average OD of negative control + 3 x SD of negative control)

In conclusion Optical Density value of thee stablished strain/s was estimated as:

OD = average OD of a tested strains – OD_C.

The OD_C was estimated for every micro-titer plate individually and ultimate consequences were documented by association with cut-off OD.

T test was used to identify the dissimilarities amongst Manuka and Beri Honey. But results have shown that there was no dissimilarity among the two honey samples. It mean that both the samples of honey have like wise potential to inhibit, impede and disrupt/unsettle the biofilm formed by Salmonellatyphi Data analysis was done by SPSS 16 version. Ethical approval was taken before starting this study.

Results

Biofilms were made by Salmonella. Typhi in 24 hours. The biofilms made by the bacteria were discreetly adherent as it has biofilm absorbance of 1.73 at 570 mm. and "++" biofilm formation. Beri and Manuka honeys prevented the formation of biofilm of the S.typhi strain at around 20% concentration (w/v) and beyond that is, 30%, 40%, and 50 %(w/v) concentrations of honey as illustrated by Table I.

Table I: Effect of Manuka honey and beri honey in preventing biofilm formation .

Concentration of Manuka honey (w/v)%	**S. typhi** **Biofilm absorbance at (570nm)**	
0	1.720	(++)
10	1.659	(++)
20	0.430	(-)
30	0.382	(-)
40	0.366	(-)
50	0.300	(-)
SD	**mean OD**	**OD_C**
0.00432	0.419	0.431
Concentration of Beri honey (w/v)%	***S. typhi*** **Biofilm absorbance at (570nm)**	
0	1.715	(++)
10	1.135	(++)
20	0.428	(-)
30	0.421	(-)
40	0.407	(-)
50	0.384	(-)
SD	mean OD	OD_C
0.00454	0.485	0.498

It is obvious from the given table I that either of Manuka and Beri honey at 10% (w/v) could not

prevent the formation of biofilm. Both types of honey disrupted "established" biofilms of Salmonella .typhi at the concentration beyond 30% (w/v) as given by Table II. The degree of biofilms biomass in every well of micro-titer plate was schemed against diverse concentrations of both types of honey. It is obvious from the figure I and figure II that the declined concentration of honey results in augmented absorbance of light than of the cutoff value.

Table II: Effect of Manuka honey beri and honey on established biofilm

Manuka honey Concentration (w/v)%	***S. typhi* Biofilm absorbance at (570nm)**	
0	1.756	(++)
10	1.748	(++)
20	1.154	(++)
30	0.989	(+)
40	0.441	(-)
50	0.437	(-)
SD	**mean OD**	**OD_C**
0.01893	0.439	0.495
Concentration of Beri honey (w/v)%	***S. typhi* Biofilm absorbance at (570nm)**	
0	1.759	(++)
10	1.739	(++)
20	1.571	(++)
30	1.012	(++)
40	0.450	(-)
50	0.432	(-)
SD	**mean OD**	**OD_C**
0.01516	0.536	0.581

Non adherence: Weakly adherence +; moderately adherence ++; strongly adherence+++

Figure I: Inhibition of *S.* typhi biofilm formation by Manuka and Beri honey

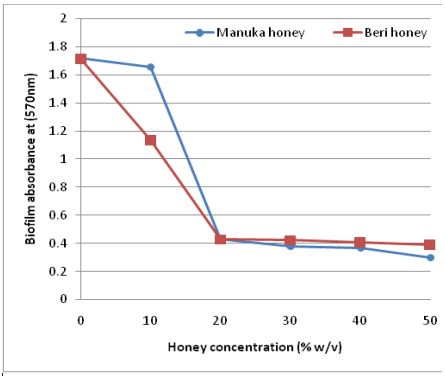
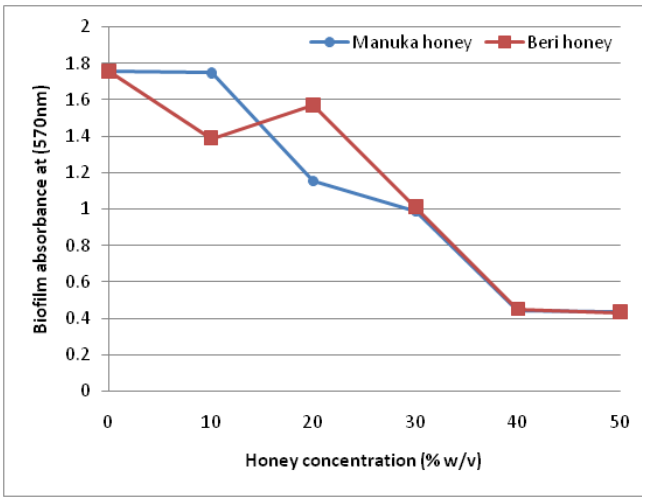


Figure II: Disruption of S. typhi "established" biofilm formation by Manuka and Beri honey



Discussion

Salmonella. typhi, is the chief cause of typhoid or enteric fever and is able to produce biofilms which then subsidizes to its capability to fight off the antibiotic therapy (resistance) and its perseverance in its host. Therefore, this host becomes the carrier; 3% – 5% of such carriers may develop biofilms on gallstones having cholesterol composition and become the permanent source of flaking Salmonella. typhi in the stools specimen obtained from such carriers. These carriers can have on average 8.47 times greater risk of developing gallbladder cancer. The treatment of choice for such individuals having choletithasis along with S.typhi infection is surgical that is cholecystectomy. This situation is further convoluted by the current development of the fluoroquinolone resistance in typhoidal salmonellae.[21] Subsequently, it became extremely tough and costly for health care personnel to cure biofilms, particularly in the developing countries such as Pakistan, where the load of biofilms related ailmentsis very high. It's the supreme need of time to find the modes of disrupting such biofilms via natural products containing antibacterial properties for example honey.

It is noteworthy that up till this date, only 4 such studies have been conducted on this subject, that is, about the effects of honey on the biofilms,[22] but neither of such studies were about typhoidal salmonellae. This research assess the in vitro consequence of honey (both Beri and Manuka) on the disruption of preformed biofilms and on inhibition of its formation. To our belief and best of our understanding perhaps this is the first ever research

Berihoney and Manuka honey associated to Salmonellatyphi. Manuka honey has been used in this study, which is FDA approved and is "active" type of honey having known antibacterial activity viz UMF-25Comvita®, in New Zealand. It is interesting to note that the antibacterial characteristic of Manukahoney corresponds to 21% of that of phenol therefore, it is a registered item/product for treating chronic skin infections, disruptionor inhibition of biofilm and burns. Another type of honey staple to Pakistan is Beri honey which has been assessed as one of the best out of 100 other local types of honey, through a research conducted by the Department of Microbiology. That study demonstrated that that samples of Beri honey obtained from one of Karak district of Khyber Pakhtunkhwa (one of the provinces of Pakistan) has the best antibacterial feature especially against MDR Salmonella, Typhi. It is well known that the dark colored honey have much elevated antioxidant and antibacterial properties, and Beri honey is also dark in color.[23] Moderately adherent biofilmshas been detectedon micro-titerplatein the presence of Salmonella typhi. Previous studies have shown that Salmonella typhihas the ability to make strongly adherent biofilm when in vivo that is, on the cholesterol gallstone in the existence of bile. A study conducted by Abida et al, 2011 established that the presence of Vi capsular antigen has not any relevance to the production of biofilm however pili have a noteworthy part in the formation of biofilm by Salmonella typhi.[24]

The results of this study strongly support those carried out in the past. This study proved that both Manuka and Beri honeys have not only prohibited the "formation" of biofilms but have also repressed or inhibited the already "established" biofilms of all the tested strains.

Relatively higher concentrations of Beri honey was needed to demonstrate inhibition that is it showed inhibition of synthesis/formation of biofilm by Salmonella typhi at 20%(w/v). This is one of the reasons as to why Manuka honey has elevated antibacterial activity in relation with that of Beri honey. This disparity amongst diverse floral Manuka and Beri honey is credited to numerous aspects, for example age, conditions while storing, processing, the procedure, concentration of hydrogen peroxide, plant-derived non-peroxide features etc. The prime objective of this research was the disruption of established biofilm by both types of honey. disruption of already established Salmonella typhi biofilms have been observed at the concentration >30% of both Beriand Manuka types of honey.

In relation to the inhibition of biofilm formation, 3 times to 4 times greater concentration of Beri and Manuka honey was needed in order to disrupt the established biofilms. Rose et al proved in their study that a 500 time increased concentration of antibiotics can be ineffective once the biofilm has been established.[25]

When it comes to the disruption of biofilm honey has proved to be much better than antibiotics. It is vital here to know the mechanism by which honey disrupts the formation of biofilm. In this regard the exact mechanism of inhibition of biofilm by honey (inS.typhi) is unknown and needs further investigations. It is believed that there can be additional factors contributing to anti-bacterial and anti-biofilm activities of honey. It is believed that the overall anti-bacterial feature of honey is a synergistic effect of Methylglyoxal, (H_2O_2), sugar components and phenolic compounds (like flavonoids, acids or minerals). Methylglyoxal and sugar compounds are significant elements for biofilm interference.[22] Additional studies of the effect of honey on the cell cycle, specific sugars in bacterial adhesion, biofilm structure and bacterial communication can be valuable in generating new class of antibacterial.

Conclusion

This study proved that Salmonella typhi is capable of forming moderately adherent biofilm in vitro. The outcomes of present research augment the fact that both the Manuka and the Beri types honey have the potential to inhibit/hinder and disrupt the biofilms formed by Salmonella typhi bacteria.

Thisstudy concludes and intensely supports that honey, be it local or from outside retains excellent anti-biofilm activity.

Authors Contribution: MSH: Conception of work, drafting and final approval. **SS:** Design of work, revising and final approval. **UKC:** Acquisition & analysis of data, revising and final approval**. RA:** Interpretation of data, revising and final approval. **AH:** Analysis of data, revising and final approval. **AR:** Conception of work, revising and final approval.

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