Anti-Biofilm Properties Exhibited by Different Types of Monofloral Honey †

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† Presented at the 1st International Electronic Conference on Microbiology, 2–30 November 2020; Available online: https://ecm2020.sciforum.net./

Abstract: Our aim was to evaluate the susceptibility of bacterial biofilm formation and the metabolic changes occurring in the bacterial cells by the action of ivy, strawberry tree, lavender, sulla and tree of heaven monofloral honeys. Listeria monocytogenes was the most sensitive bacteria with percentages of biofilm inhibition up to 72.20%. Pseudomonas aeruginosa was less sensitive, but tree of heaven and sulla honey caused an inhibition of biofilm up to 40.41% and 35.85%, respectively. The tree of heaven honey acted on the P. aeruginosa metabolism (75.24%). Staphylococcus aureus, majorly resistant to the biofilm-inhibitory action of the honey, was more sensitive at the metabolic level (61.63% inhibition in the presence of the tree of heaven honey).

Keywords: honey; biofilm; Pseudomonas aeruginosa; Listeria monocytogenes; Staphylococcus aureus

1. Introduction

Biofilm formation represents a self-protective mechanism of bacteria where bacteria aggregate to create a complex structure to resist the harsh conditions. This gives rise to an increase in their surface attachment ability, and a higher population density, with the production of extracellular polymeric substances (EPS) and with a subsequent range of physical, metabolic, and chemical processes, which take place also due to an increase in pathogenicity. [1]. The formation of biofilm determines higher tolerance to the conventional antimicrobial agents and resistance to phagocytosis so that they become more difficult to eradicate from living hosts [2]. The problem has provoked intensive efforts from scientists to develop better strategies to prevent, inhibit and demolish biofilm formation. Since the prehistoric age, honey has been used in curing ailments, in preventing the onset of ailments [3] and in folk medicine it is used to treat some types of infections. In the last few decades, the modern clinical practices, cost, and difficulty of chronic wound care have led to the desire for better and cost-effective remedies [4]. Honey has demonstrated that it is effective in inhibiting the formation of biofilm of Klebsiella pneumoniae and Pseudomonas aeruginosa [5], oral streptococci [4,6], Proteus mirabilis and Enterobacter cloacae [7]. Among monofloral types, manuka honey is one of the most studied, demonstrating its capacity to inhibit the biofilm formation of Clostridium difficile [8], Staphylococcus aureus [9] and Candida albicans [10] among all. The aim of our work was to evaluate the susceptibility of bacterial biofilm formation and the metabolic changes occurring in the bacterial cells of the ivy, strawberry tree, lavender, sulla and tree of heaven Italian monofloral honeys. We
considered three bacteria, *Listeria monocytogenes*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*, as tester strains, as infections are becoming more difficult to treat as further evolution of drug resistance occurs within them.

2. Material and Methods

Different types of Italian organic monofloral honey were purchased from an Italian company (Thun, Trento, Italy). The Luria Bertani culture medium, Phosphate-buffered saline (PBS), dimethyl sulfoxide (DMSO) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide or MTT were provided by Sigma (Milano, Italy).

2.1. Microorganisms and Culture Conditions

*Listeria monocytogenes* ATCC 7644, *Pseudomonas aeruginosa* DSM 50071 and *Staphylococcus aureus subsp. aureus* ATCC 25923 were used as test bacterial strains. Bacteria were cultured in LB broth for 18 h at 37 °C and 80 rpm (Corning LSE, Pisa, Italy).

2.2. Minimal Inhibitory Concentration (MIC)

The MIC values were calculated using the resazurin microtiter-plate assay [11]. Multiwell plates were prepared in triplicate and incubated at 37 °C for 24 h. The lowest concentration at which a color change occurred (from dark purple to colorless) revealed the MIC value.

2.3. Biofilm Inhibitory Activity

The effect of the honeys on bacterial ability to form biofilm was assessed according to the method of O‘Toole and Kolter [12] in flat-bottomed 96-well microtiter plates, using two sub-inhibitory volumes of honey (previously dissolved in sterile PBS), precisely 5.71 μL/mL and 11.42 μL/mL. The overnight bacterial cultures were adjusted to 0.5 McFarland with fresh culture broth. Then, 10 μL of the diluted cultures was distributed in each well, and the samples of the honeys and sterile Luria Bertani broth were added, to reach a final volume of 250 μL/well. To avoid the evaporation of the samples, microplates were then completely covered with parafilm tape, and incubated for 48 h at 37 °C. Planktonic cells were removed and the attached cells were gently washed twice with sterile physiological saline. Then, 200 μL of methanol was added to each well, and left for 15 min to fix the sessile cells. After discharge of methanol, each plate was placed under laminar flow cap until complete dryness of samples. The staining of the adhered cells was obtained through the use of 200 μL of 2% *w/v* crystal violet solution to each well, which were then left for 20 min. Wells were gently washed with sterile PBS and left to dry. The release of the bound dye was obtained through the addition of two hundred microliters of glacial acetic acid 20% *w/v*. The absorbance was measured at λ = 540 nm (Varian Cary spectrophotometer model 50 MPR, Cernusco sul Naviglio, Italy). The percent value of biofilm inhibition was calculated with respect to control (cells grown without the presence of samples assuming % = 0). The average results from triplicate tests were taken for reproducibility.

2.4. Metabolic Activity of Biofilm Cells

The effect of two volumes, 5.71 μL/mL and 11.42 μL/mL, of the honeys (prepared as described above) on the metabolic activity of biofilm cells was evaluated through the MTT colorimetric method [13,14] using 96-well microtiter plates. The overnight bacterial cultures were adjusted to 0.5 McFarland and treated as previously described. Bacterial suspension, representing the planktonic cells, was removed after 48 h incubation. Then, 150 μL of sterile PBS and 30 μL of 0.3% MTT (Sigma, Milan, Italy) were added in each well, keeping the microplates at 37 °C. After 2 h, the MTT solution was removed, two washing steps were performed gently with 200 μL of sterile physiological solution, then 200 μL of DMSO was added to obtain the dissolution of the formazan crystals, which were measured at λ = 570 nm (Varian). Triplicate tests were carried out and the average results were taken for reproducibility.
3. Results and Discussion

The potential effect that the monofloral honeys had on the formation of biofilm of some pathogenic bacteria and on the metabolism of the bacterial cells included in the biofilm was assessed using sublethal amounts of the samples. Results are shown in Tables 1 and 2, respectively.

The results show that the honeys exhibited a remarkable capacity to inhibit the formation of the bacterial biofilm.

**Table 1.** Inhibitory action of the different types of monofloral honey on the formation of biofilm. Results are reported as percent of inhibition with respect to the control (which % was assumed = 0). They are the mean (±SD) of three experiments. TH: tree of heaven honey; I: ivy honey; L: lavender honey; S: sulla honey; ST: strawberry tree honey. LM: Listeria monocytogenes; PA: Pseudomonas aeruginosa; SA: Staphylococcus aureus.

| CV Test % of Biofilm Inhibition | TH 5.71 μL/mL | TH 11.42 μL/mL | I 5.71 μL/mL | I 11.42 μL/mL | L 5.71 μL/mL | L 11.42 μL/mL | S 5.71 μL/mL | S 11.42 μL/mL | ST 5.71 μL/mL | ST 11.42 μL/mL |
|---------------------------------|---------------|----------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|
| LM                              | 65.82 (2.81)  | 66.07 (1.57)  | 53.65 (1.35)  | 63.37 (1.57)  | 24.17 (0.57)  | 51.40 (1.25)  | 56.78 (1.12)  | 72.20 (2.44)  | 57.68 (1.12)  | 60.99 (1.22)  |
| PA                              | 30.12 (0.57)  | 40.41 (1.54)  | 33.39 (0.57)  | 35.32 (1.12)  | 0 (0)         | 6.15 (0.57)   | 0 (0)         | 35.85 (1.57)  | 8.96 (0.57)   | 10.28 (1.67)  |
| SA                              | 24.05 (2.02)  | 26.13 (0.57)  | 20.20 (0.57)  | 23.93 (1.12)  | 0 (0)         | 20.09 (1.57)  | 0 (0)         | 17.53 (0.57)  | 20.79 (1.67)  |

**Table 2.** Metabolic activity exhibited by the cells present within the bacterial biofilms in the presence of different volumes of the monofloral honeys. Results are reported as percentage of inhibition with respect to the control (which % of inhibition was assumed = 0). They are the mean (±SD) of three experiments. TH: tree of heaven honey; I: ivy honey; L: lavender honey; S: sulla honey; ST: strawberry tree honey. LM: Listeria monocytogenes; PA: Pseudomonas aeruginosa; SA: Staphylococcus aureus.

| MTT Test % of Inhibition | TH 5.71 μL/mL | TH 11.42 μL/mL | I 5.71 μL/mL | I 11.42 μL/mL | L 5.71 μL/mL | L 11.42 μL/mL | S 5.71 μL/mL | S 11.42 μL/mL | ST 5.71 μL/mL | ST 11.42 μL/mL |
|--------------------------|---------------|----------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|
| LM                       | 8.81 (0.57)   | 16.71 (1.13)   | 10.02 (0.57)  | 15.67 (0.57)  | 12.04 (1.67)  | 13.46 (0.57)  | 14.44 (1.12)  | 16.76 (1.12)  | 19.98 (2.02)  | 18.01 (0.57)  |
| PA                       | 61.72 (1.57)  | 75.22 (1.12)   | 46.04 (1.44)  | 57.98 (2.01)  | 60.71 (1.12)  | 61.89 (1.12)  | 57.58 (1.40)  | 62.60 (1.12)  | 63.12 (1.44)  | 64.32 (1.67)  |
| SA                       | 27.11 (1.57)  | 61.63 (1.67)   | 36.15 (2.05)  | 1.91 (0.30)   | 36.73 (1.57)  | 47.64 (2.02)  | 38.12 (1.44)  | 39.03 (1.47)  | 22.97 (1.57)  | 24.89 (1.57)  |

Many studies have ascertained that honey has antibacterial effects, which is due to its high values of osmolarity, as well as its low pH, hydrogen peroxide content, and content of other compounds that are still uncharacterized [15,16]. In some cases, the action of honey in inhibiting the growth of pathogenic microorganisms is caused by a low water activity of honey, but this is not the only explanation for its antimicrobial activity; in fact, by studying the effect of sugar syrups with the same water activity, it was found that they exhibit less strength as antimicrobial agents [17]. We studied different types of monofloral honey, some of them, such as the honey of tree of heaven, have never been studied before under this viewpoint.

All types of honey affected the formation of biofilm of Listeria monocytogenes, with the percentage of inhibition up to 72.28%, determined by the presence of 11.42 μL/mL of sulla honey, and never more inferior than 24.17% (determined by the lavender honey). On the whole, the most resistant bacteria to the action of honey seemed S. aureus. This bacterium was resistant to the biofilm-inhibitory action of the honeys, with percentages not exceeding 26.13% (in the presence of the tree of heaven honey). P. aeruginosa exhibited an intermediate behavior, resulting in being sensitive to almost all types of honey, with a capacity to form biofilm decreasing at 40.41%, 35.85% and 35.32% in the presence of tree of heaven, sulla and ivy honey, respectively. The honey of strawberry tree showed its antibiofilm properties against Pseudomonas aeruginosa and St. aureus, and mainly against St. aureus, similarly to
the results obtained by da Silva et al. [18]. To our knowledge, this was the first time wherein the activity of some types of honey, such as that of sulla and that of tree of heaven, was observed.

The behavior exhibited by the honeys to affect the metabolism of the cells present within the biofilm differed in respect to the inhibitory effect on the biofilm formation. Thus, *L. monocytogenes*, which resulted in being the most sensitive to the action of all honeys, was conversely the most resistant to the action of the honeys affecting its metabolism when the biofilm was formed, with percentages of inhibition not superior to 18.01%. On the contrary, all honeys, although demonstrating less or no strength in inhibiting the formation of biofilm of *P. aeruginosa* and *S. aureus*, were more effective in inhibiting their metabolism once the biofilm was formed. This could let us hypothesize that in the case of *L. monocytogenes*, the action of the honeys did not particularly interest the metabolism of the cells, as they were only partially affected by the presence of the honey. On the contrary, the honeys, although less effective in inhibiting the formation of biofilms of *P. aeruginosa* and *S. aureus*, were more effective in inhibiting the metabolism of their cells within the biofilm. This demonstrated once again the wide range of action of natural substances in fighting the pathogenicity of bacteria [1].

4. Conclusions

Clinical studies have ascertained the use of honey for several infected cutaneous wounds, where it quickly clears the infection from the wound, improving tissue healing too. Several in vitro studies have confirmed the wide-spectrum antimicrobial and antiviral properties of honey, due to several mechanisms [19]. The antimicrobial efficacy of honey is very dependent on the type of flower, region, and season. We confirmed that not all honeys have the same antibacterial potency [20,21], and we are trying to biochemically characterize the honeys herein evaluated for their antibacterial activities. In each case, unlike most conventional local drugs, honey does not lead to the development of antibiotic-resistant bacteria, and it may be used continuously.

**Author Contributions:** F.N., V.D.F., R.C.: conceptualization. F.N., A.d., F.F., M.N.O., and L.C.: investigations. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research received no external funding.

**Conflicts of Interest:** The authors declare no conflict of interest.

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