Antibiofilm efficacy of honey and bee-derived defensin-1 on multispecies wound biofilm

Martin Sojka,1,† Ivana Valachova,2,† Marcela Bucekova3,4 and Juraj Majtan1,3,4

Correspondence
Juraj Majtan
juraj.majtan@savba.sk

1Department of Microbiology, Faculty of Medicine, Slovak Medical University, Limbova 13, 833 03 Bratislava, Slovakia
2Faculty of Natural Sciences, Comenius University, Ilkovicova 6, 842 15 Bratislava, Slovakia
3Institute of Zoology, Slovak Academy of Sciences, Dubravska cesta 9, 845 06 Bratislava, Slovakia
4Laboratory of Molecular Apidology and Apitherapy, Institute of Molecular Biology, Slovak Academy of Sciences, Dubravska cesta 21, 845 51 Bratislava, Slovakia

Many clinically relevant biofilms are polymicrobial. Examining the effect of antimicrobials in a multispecies biofilm consortium is of great clinical importance. The goal of this study was to investigate the effect of different honey types against bacterial wound pathogens grown in multispecies biofilm and to test the antibiofilm activity of honey defensin-1 (Def-1) in its recombinant form. A modified Lubbock chronic wound biofilm formed by four bacterial species (Staphylococcus aureus, Streptococcus agalactiae, Pseudomonas aeruginosa and Enterococcus faecalis) was used for evaluation of honey and recombinant bee-derived Def-1 antibiofilm efficacy. Recombinant Def-1 was prepared by heterologous expression in Escherichia coli. We showed that different types of honey (manuka and honeydew) were able to significantly reduce the cell viability of wound pathogens (Staphylococcus aureus, Streptococcus agalactiae and Pseudomonas aeruginosa) in mature polymicrobial biofilm. None of the tested honeys showed the ability to eradicate Enterococcus faecalis in biofilm. In addition, recombinant Def-1 successfully reduced the viability of Staphylococcus aureus and Pseudomonas aeruginosa cells within established polymicrobial biofilm after 24 and 48 h of treatment. Interestingly, recombinant Def-1 did not affect the viability of Streptococcus agalactiae cells within the biofilm, whereas both natural honeys significantly reduced the viable bacteria. Although Enterococcus faecalis was highly resistant to Def-1, Def-1 significantly affected the biofilm formation of Enterococcus faecalis and Streptococcus agalactiae after 24 h of treatment, most likely by inhibiting its extracellular polymeric substances production. In conclusion, our study revealed that honey and Def-1 are effective against established multispecies biofilm; however, Enterococcus faecalis grown in multispecies biofilm was resistant to both antimicrobials.

INTRODUCTION

Chronic wounds are a major worldwide healthcare problem and are associated with decreasing quality of life and significant patient morbidity. The tissue of all chronic wounds is colonized by polymicrobial flora (Smith et al., 2010). Over 90% of chronic wounds contain bacteria and fungi from the skin, oral mucosa, enteric tract or environment. Together, these micro-organisms form a multispecies biofilm construct that protects them from antimicrobial therapy and the patient’s immune system (Price et al., 2009; Attinger & Wolcott, 2012). Biofilm growth and its persistence within wounds have recently been suggested as contributing factors to impaired healing (Rhoads et al., 2007; Bjarnsholt et al., 2008; Davis et al., 2008).

Treatment of bacterial biofilm in the wound is complicated by the mechanisms underlying biofilm growth. Furthermore, mixed-species biofilms have complementary metabolic strategies for obtaining nutrients and degrading host immune molecules (Burmølle et al., 2006). Antibiotics may have only minimal long-term effects on preventing or treating established biofilms, as most antibiotics are designed to target metabolically active planktonic bacterial cells, while bacterial cells embedded in an extracellular polymeric substance matrix are unresponsive

†These authors contributed equally to this work.

Abbreviation: Trx, thioredoxin.
(Costerton & Stewart, 2001). Therefore, there is an urgent need to introduce novel or re-emerging effective approaches to combat bacterial biofilms in chronic wounds.

Honey, a promising biological therapeutic agent, shows antibiofilm activity against biofilms formed by a single species, including *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Streptococcus pyogenes* (Alandejani et al., 2009; Maddocks et al., 2012). However, recent studies have indicated that many clinically relevant biofilms, including wound biofilms, involve multiple species (Elias & Banin, 2012). Polymicrobial biofilm infections display a wound healing impairment compared with wounds infected with single-species bacteria (Dalton et al., 2011). In addition, the bacteria in polymicrobial wound infections display increased antimicrobial resistance and tolerance to different antibiofilm agents in comparison with those in single-species infection. Therefore, examining the effect of honey on bacteria in a multispecies biofilm consortium is of great clinical importance.

A few studies have attempted to identify the active compounds responsible for the antibiofilm activity of honey (Truchado et al., 2009; Lee et al., 2011; Majtan et al., 2014). Most of these compounds take part in the prevention of biofilm formation, but little information is available regarding compounds that are able to disrupt established wound biofilm. One of the potential agents responsible for biofilm destruction is bee (*Apis mellifera*)-derived antibacterial peptide defensin-1 (Def-1), as it has been shown recently that antimicrobial cat-ionic peptides destroy bacterial biofilm (Dosler & Mataraci, 2013; Dosler & Karaaslan, 2014). Def-1 is one of the main regular but quantitatively variable antibacterial components of honey (Kwakman et al., 2011; Majtan et al., 2012).

As several types of honey have been recognized as having therapeutic activity and are routinely used for treatment of chronic wounds, it is important to determine their antibiofilm efficacy against polymicrobial wound biofilms and to elucidate the underlying mechanisms of biofilm eradication. Therefore, the aim of this study was to investigate the effect of different honey types against bacterial wound pathogens grown in multispecies biofilm and to test the antibiofilm activity of honey Def-1 in its recombinant form.

**METHODS**

**Honey samples.** A fir non-sterilized honeydew honey was purchased from Mr Jozef Volansky (Meder apiary, Bardejov, Slovakia). Commercially available non-sterilized manuka honey with unique manuka factor 15 (UMF 15+) imported from New Zealand was purchased from Nature’s Nectar. Artificial honey was prepared by dissolving 39 g d-fructose, 31 g d-glucose, 8 g maltose, 3 g sucrose in 19 ml distilled water and stored in darkness at 2–5 °C as described elsewhere (Majtan & Majtan, 2010).

**Bacterial strains for antibiofilm activity testing.** Bacterial strains of *Staphylococcus aureus*, *Streptococcus agalactiae*, *Enterococcus faecalis*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*, originally isolated from chronic wounds maintained in glycerol cryoprotective medium at −80 °C, were resuscitated on Columbia agar plates supplemented with sheep blood (Oxoid) and incubated at 37 °C for 24 h. Sodium chloride peptone broth (buffered peptone bouillon, BPB; Merck) was used for dilutions and for normalizing the optical density of cultures in all experiments.

**Strains, vectors, enzymes and reagents.** *Escherichia coli* strain JM109 was used as the host for gene cloning and DNA manipulation. *E. coli* Rosetta-gami 2 BL21(DE3) was purchased from Novagen and used as the host for expression of the heterologous protein. Bacteria were grown in Luria–Bertani (LB) medium or LB agar plates with appropriate antibiotics at 37 °C. *E. coli* cells were transformed using a standard heat-shock method. Plasmid pET32a (+) (Novagen) was chosen for the construction and expression of fusion protein. Isis DNA polymerase was purchased from MP Biomedicals. T4 DNA ligase and restriction enzymes were from New England BioLabs and other chemicals were purchased from Sigma-Alrich or Promega.

**Construction of the expression plasmid.** For peptide expression, we used the pET32a (+) plasmid, which provides thioredoxin (Trx) as a fusion partner to increase the solubility of target protein in the *E. coli* cytoplasm. We synthesized the cDNA fragment of mature bee Def-1 with *Kpn*1 and *Xho*1 sites. Primers for the synthesis of the cDNA fragment, which served as the insert for construction of the expression plasmid, were designed based on the cDNA sequence and were used in nested PCRs (Table 1). The synthesized cDNA fragment was purified, digested with *Kpn*1 and *Xho*1 and then ligated into the pET32a (+) vector, which was also digested with these two restriction enzymes, to construct the expression plasmid pET32-maDef. The resulting plasmid was transformed into JM109 *E. coli* and verified by DNA sequencing analysis (GATC Biotech). The correct plasmid encoded a translational fusion peptide containing an N-terminal Trx part, followed by a 6 × His tag, an enterokinase cleavage site and the mature peptide sequence of bee Def-1.

**Expression of recombinant Def-1.** *E. coli* Rosetta-gami host strains can enhance disulfide bond formation. For this reason, the expression vector (pET32-maDef) was transformed into *E. coli* Rosetta-gami 2 BL21(DE3) to generate *E. coli* pET32-maDef. To express recombinant Def-1, an *E. coli* pET32-maDef clone was selected and inoculated into 3 ml LB medium supplemented with appropriate antibiotics and cultured at 37 °C overnight with shaking at 250 r.p.m. The overnight culture was diluted 100-fold into fresh LB medium (the total volume for expression of recombinant Def-1 was 1500 ml) with antibiotics. When the cell culture reached an absorbance at 600 nm (A600) of about 0.5 (mid-exponential phase), the expression of recombinant defensin was induced by the addition of IPTG to a final concentration of 0.8 mM. After an additional 4 h culture in the presence of IPTG at

**Table 1.** Primers used to synthesize the *Apis mellifera* (bee) mature Def-1 insert for construction of expression plasmid

<table>
<thead>
<tr>
<th>PCR</th>
<th>Sense primer (5′→3′)</th>
<th>Antisense primer (5′→3′)</th>
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<tbody>
<tr>
<td>PCR 1</td>
<td>GACGAGCAGCAGCAAGTGAACTTGTGACCTTCTC</td>
<td>CCGTCGAGTTAACCAGAAGTTGTTGCCCA</td>
</tr>
<tr>
<td>PCR 2</td>
<td>CGGGTACCGAGCAGCAGCAAGGGTAACCTTGTGACCTTCT</td>
<td>CCGTCGAGTTAACCAGAAGTTGTTGCCAGAGATCCTTGT</td>
</tr>
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Underlined sequences indicate the restriction enzyme *Kpn*1 recognized site in sense primers and *Xho*1 in antisense primers.
37 °C, the harvested cells were immediately centrifuged at 5000 rpm for 10 min.

Purification of recombinant Def-1. Expressed recombinant Def-1 from the harvested cells was extracted using B-Per Reagent (Thermo Scientific) according to the manufacturer’s instructions. The supernatant (soluble protein fraction) was isolated, and this fraction containing recombinant Def-1 carrying the 6 His tag was then purified using a nickel–nitritotriacetic acid (Ni-NTA) agarose affinity chromatography (Sigma-Aldrich). The recombinant Def-1 bound to the resin was washed with washing buffer [30 mM Tris/HCl (pH 8.0), 0.3 M NaCl, 15 mM imidazole] and then eluted with elution buffer [30 mM Tris/HCl (pH 8.0), 0.3 M NaCl, 250 mM imidazole]. After Ni-NTA chromatography, recombinant defensin was desalinated using a PD-10 desalting column (Sigma-Aldrich), eluted with sterile distilled water, lyophilized and stored at -20 °C. The purity of recombinant Def-1 was analysed by 12% SDS-PAGE. The antibacterial activity of purified recombinant Def-1 was initially determined against laboratory strain Micrococcus luteus ATCC 272.

Antibiofilm activity analysis using polymicrobial biofilm. We used the previously described Lubbock chronic wound biofilm model (Sun et al., 2008), modified according to Kucera et al. (2014). Briefly, 6 ml medium containing Bolton broth base (Sigma), 1% gelatin, 50% porcine plasma and 5% porcine erythrocytes lysed by freeze-thawing was dispensed into sterile 1.6 × 10 cm glass tubes. Cultures of selected bacteria normalized according to optical density were mixed, and a volume of 10 μl mixture containing 10^7 c.f.u. ml^-1 was inoculated into each tube by ejecting the pipette tips along with the bacterial suspension. Inoculated tubes were incubated at 37 °C in an orbital shaker (1500 r.p.m.) for 48 h and the pre-formed biofilms were harvested.

Harvested biofilms were washed with BPB. The biofilms were placed into the artificial wound bed in the nutrient medium and covered with a piece of 100% cotton eight-ply gauze sponge (Batist), soaked with the test substance (100% honey or recombinant Def-1). Biofilms in the artificial wound bed treated with particular substances were incubated at 37 °C for 24 and 48 h, respectively. After treatment, the biofilms were harvested from the artificial wound bed using sterile forceps and a Lang eye spoon, and then homogenized. The number of bacteria per culture was determined using different media as described previously (Kucera et al., 2014) and Brilliance UTI selective medium (Oxoid).

Microtitre plate biofilm formation assay. Ninety microlitres of tryptone soy broth (TSB) medium or TSB medium with recombinant Def-1 was pipetted into the wells of 96-well microtitre plates and inoculated with 10 μl normalized microbial suspension (10^9 c.f.u. ml^-1). The plates were incubated in an orbital shaker (1500 r.p.m.) at 37 °C for 24 and 48 h. After incubation, the biofilm formation was measured using a crystal violet staining method according to our previous study to detect polymeric substances (Majtan et al., 2014). The viability of biofilm-forming cells was measured using resazurin according to van den Driessche et al. (2014).

Statistical analysis. The data are expressed as means ± SEM and were analysed statistically using a two-way ANOVA and multiple comparisons using the Bonferroni method. Data with P < 0.05 were considered statistically significant. All statistical analyses were performed using GraphPad Prism (GraphPad Software Inc.).

RESULTS

Recombinant expression and purification of Def-1

The plasmid pET32-maDef containing the Trx–Def-1 fusion was transformed into E. coli Rosetta-gami 2 BL21(DE3). Upon IPTG induction, Def-1 was expressed predominantly in the soluble cytoplasmic fraction. After SDS-PAGE analysis, there was an obvious recombinant protein band with a molecular mass of about 22.64 kDa, which was consistent with the predicted molecular mass of recombinant Def-1: Trx–6 His–maDef (data not shown). As the recombinant Def-1 contained a 6 × His tag, Ni-NTA agarose resin was used for purification. After purification, desalted recombinant Def-1 was pooled, dried and resuspended in 1 ml sterile distilled water at a concentration of 5 mg ml^-1 and tested for activity. The results of SDS-PAGE showed that the final recombinant Def-1 had a purity of 95% (Fig. 1). The final yield of the recombinant expression was 8.56 mg l^-1. Recombinant Def-1 was active against M. luteus but did not show any antibacterial activity against E. coli.

Effect of different honey types against bacterial wound pathogens grown in multispecies biofilm

In order to assess the antibiofilm activity of different honeys on multispecies biofilm, we used manuka, honeydew and artificial honey at a concentration of 100%. A multispecies biofilm was formed by four bacterial species (Staphylococcus aureus, Streptococcus agalactiae, Pseudomonas aeruginosa and Enterococcus faecalis) and was stable during the experimental time period (up to 48 h). The mature multispecies biofilm was treated with each honey type, and the number of surviving cells was determined

Fig. 1. SDS-PAGE analysis of purified soluble bee recombinant Trx–Def-1 fusion protein. Analysis by 12% SDS-PAGE showed that the final Trx–6 × His–Def-1 was produced to a purity of 95%. Lanes: M, molecular mass marker; 1, soluble cytoplasmic fraction of IPTG-induced E. coli Rosetta-gami 2 BL21(DE3) cells; 2, insoluble cytoplasmic fraction of induced E. coli culture; 3, unbound fraction of soluble protein extract after purification on Ni-NTA column; 4, fraction after wash of Ni-NTA column; 5, Ni-NTA-captured Trx–Def-1 fusion protein; 6, desalted purified Trx–Def-1 fusion protein.
by plate counting on different media (Fig. 2). Both of the natural honeys (manuka and honeydew) showed similar antibiofilm efficacy and were able to significantly reduce cell viability of *Staphylococcus aureus*, *Streptococcus agalactiae* and *Pseudomonas aeruginosa* in the mature biofilm within 48 h. Artificial honey had no significant effect on *Staphylococcus aureus*, *Streptococcus agalactiae* and *Enterococcus faecalis*, whereas *Pseudomonas aeruginosa* showed a significant decrease in viable bacterial counts after 24 and 48 h of treatment. None of the tested honeys could eradicate *Enterococcus faecalis* in multispecies biofilm, and an increased bacterial cell count was observed after 24 h of honey treatment.

**Antibiofilm activity of recombinant Def-1**

Bee Def-1 in a recombinant form at concentrations of 0.1 and 1 mg ml\(^{-1}\) was tested against the same bacterial pathogens grown in multispecies biofilm as described above. Recombinant Def-1 at both concentrations significantly reduced the viability of *Staphylococcus aureus* cells after 24 and 48 h of treatment (Fig. 3). At higher concentrations, it was also able to decrease viable bacterial counts of *Pseudomonas aeruginosa* after 24 and 48 h of treatment. Interestingly, recombinant Def-1 did not affect the viability of *Streptococcus agalactiae* cells within the biofilm, whereas both natural honeys significantly reduced the number of viable bacteria. *Enterococcus faecalis* was highly resistant to honey and Def-1, and its bacterial count did not differ within the biofilm during the treatment.

The most logical approach to prevent bacterial biofilm formation is by inhibiting the initial binding of the bacterium to the tissue or biomaterial. Recombinant Def-1 was investigated for its ability to prevent biofilm formation by *Streptococcus agalactiae* and *Enterococcus faecalis* in a 96-well microtitre plate. Recombinant Def-1 at concentrations of 0.1 and 1 mg ml\(^{-1}\) significantly inhibited the biomass production of both pathogens after 24 h of treatment (Fig. 4). However, recombinant Def-1 did not affect the cell viability of *Enterococcus faecalis*.

**DISCUSSION**

Given the recent confirmation of the presence of multispecies biofilms in wounds and their role in delaying wound healing, we investigated the antibiofilm activity of manuka and honeydew honey against bacterial wound pathogens grown in multispecies biofilm. We found that both honeys exhibited comparable antibiofilm efficacy against *Staphylococcus aureus*, *Streptococcus agalactiae* and *Pseudomonas aeruginosa*. As the artificial honey exhibited no effect on *Staphylococcus aureus* and *Streptococcus agalactiae*, we may speculate that mechanisms other than high

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**Fig. 2.** Antibiofilm effects of different honey types against particular bacterial species after 24 and 48 h of treatment. The data are expressed as means ± SEM and were statistically analysed by ANOVA (*P*<0.001).
osmotic pressure are involved in eradication of these bacteria, unlike the situation for Pseudomonas aeruginosa, which was inhibited even by artificial honey. On the other hand, Enterococcus faecalis grown in multispecies biofilm was resistant to both natural honeys, as well as to artificial honey. Enterococcus faecalis is an important pathogen found in many healthcare-associated infections, including wound and surgical infections (Oliva et al., 2014). The difficulty in Enterococcus faecalis treatment has been attributed to the lack of anti-infective strategies to eradicate its biofilm and to the frequent emergence of multidrug-resistant strains. Several studies have shown that

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**Fig. 3.** Antibiofilm effects of bee recombinant Def-1 at concentrations of 0.1 and 1 mg ml\(^{-1}\) against particular bacterial species after 24 and 48 h of treatment. The data are expressed as means ± SEM and were statistically analysed by ANOVA (*P<0.001, **P<0.05).  

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**Fig. 4.** Effect of bee recombinant Def-1 at concentrations of 0.1 and 1 mg ml\(^{-1}\) on production of extracellular polymeric substances (crystal violet staining, \(A_{570}\)) and on the viability of cells within the formed biofilm (resazurin, \(A_{470}\)). The data are expressed as means ± SEM and were statistically analysed by ANOVA (*P<0.001).
various topical antimicrobial agents including honey and herbal extracts are ineffective for the eradication of Enterococcus faecalis biofilm (Basualdo et al., 2007; Nidadavolu et al., 2012; Hajska et al., 2014). The observations obtained in the present study suggest that, from a clinical point of view, infected wounds associated with Enterococcus faecalis biofilm could be unresponsive to topical honey treatment. In our previous study (Kucera et al., 2014) using the same multispecies bacterial biofilm, we tested two commonly used antimicrobial wound treatments: polyvinylpyrrolidone–iodine complex and cadexomer–iodine complex. Only stabilized iodine at a high concentration (1.8 mg iodine cm$^{-2}$) in the form of cadexomer–iodine significantly reduced the number of biofilm bacteria including Enterococcus faecalis for up to 48 h, whereas cadexomer–iodine complex (0.2 mg iodine cm$^{-2}$) and polyvinylpyrrolidone–iodine (0.2 mg of iodine cm$^{-2}$) appeared ineffective against established polymicrobial biofilms.

Honey has been used successfully in wound care, and its potential antibiofilm properties have recently been indicated, particularly against Staphylococcus aureus and P. aeruginosa (Cooper et al., 2014; Lu et al., 2014). However, little information is available concerning the potential antibiofilm components of honey.

In recent years, antimicrobial peptides have emerged as an attractive target area from which to source new antibiofilm technology solutions. Although no biofilm-active antimicrobial peptides have so far reached the market, substantial developments in their proteolytic stability and design and in minimizing cytotoxicity have been achieved. Some of these antimicrobial peptides are insect derived (Fogaça et al., 2010; Hwang et al., 2013). In this study, we investigated an antibiofilm efficacy of a recombinant honeybee-derived antimicrobial peptide, Def-1, against bacterial wound pathogens grown in multispecies biofilm.

Bee Def-1 (previously referred as a royalisin) is a peptide that belongs to the insect defensin group, and is composed of 51 aa, with a molecular mass of 5.52 kDa. Def-1, together with bumblebee defensin, is the only insect defensin that is amidated at the C terminus. It represents a regular component of royal jelly and honey; however, its concentration varies considerably among the different types of honey (Majtan et al., 2012). The amount of Def-1 in both honey samples used in this study was examined using a newly developed competitive ELISA (unpublished data). Honeydew and manuka honey samples contained 2.0 and 0.7 μg f Def-1 (g honey)$^{-1}$, respectively. Although Def-1 is present in manuka honey, its structure and antibacterial activity are negatively affected by highly reactive methylglyoxal (Majtan et al., 2012).

Def-1 has been shown to be effective against Gram-positive bacteria (Fujiwara et al., 1990; Bachanová et al., 2002; Shen et al., 2012); however, some studies using recombinant Def-1 have also reported its activity against Gram-negative bacteria including Pseudomonas aeruginosa and Salmonella enterica (Tseng et al., 2011; Bilikova et al., 2015). In accordance with these previous studies, Pseudomonas aeruginosa seemed to be resistant to Def-1. In our study, recombinant Def-1 also did not exhibit antibacterial activity against planktonic E. coli. In general, Gram-negative bacteria are suggested to be resistant to insect defensins (Čerovsky & Bém, 2014). Therefore, the antibacterial activity of Def-1 against Gram-negative bacteria remains controversial.

Interestingly, although Def-1 was not able to kill planktonic E. coli cells, it significantly reduced the cell viability of another Gram-negative bacterium, Pseudomonas aeruginosa, in multispecies biofilm after 24 h of treatment. One possible explanation for this observation is based on the ability of Enterococcus faecalis to produce enterocin peptides (e.g. enterocin AS-48), which exhibit strong anti-bacterial activity against Gram-positive bacteria and weak activity against Gram-negative bacteria. Combined treatments of enterocins with heat, chelators or polymyxin B considerably improve the inactivation of Gram-negative bacteria such as E. coli and Salmonella enterica (Grande Burgos et al., 2014). Therefore, combined treatment of Def-1 with enterocin produced by Enterococcus faecalis in multispecies biofilm may have reduced the viability of Pseudomonas aeruginosa in the biofilm.

Due to the high resistance of Enterococcus faecalis and Streptococcus agalactiae in multispecies biofilm to the bacterial activity of Def-1, we investigated the effect of Def-1 on biofilm formation of these two pathogens. Def-1 was shown to significantly inhibit biofilm formation in both Enterococcus faecalis and Streptococcus agalactiae (Fig. 4). However, the viability of biofilm-forming Enterococcus faecalis cells was not affected by Def-1, whereas the viability of Streptococcus agalactiae cells was significantly reduced. Thus, Def-1 could prevent biofilm development either by interfering in bacterial adhesion to a surface or by inhibiting the growth of attached cells in the early biofilm stage, or by alteration of polymeric substances production, as can be seen in Fig. 4.

The recombinant bee Def-1 used in this study is a fusion protein of about 23 kDa using Trx as a fusion partner in order to increase the solubility of recombinant Def-1. Expression of mature Def-1 itself as a soluble peptide in E. coli was unsuccessful (Klaudiny et al., 2012). According to the studies of Shen et al. (2010, 2012), mature Def-1 from Apis cerana fused with glutathione S-transferase was active against Staphylococcus aureus and Bacillus subtilis, suggesting that the fusion partner is important for the solubility of the recombinant peptide and prevents peptide aggregation.

In conclusion, our study revealed that both honeydew and manuka honey are effective against established multispecies biofilm; however, Enterococcus faecalis grown in poly-microbial biofilm was resistant to both natural honeys. This needs to be taken into consideration in clinical practice. In addition, we found that bee Def-1 may take part in the antibiofilm activity of natural honey against wound pathogens, particularly Staphylococcus aureus, and is also active in its recombinant form.
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