Antimicrobial properties and therapeutic benefits of honey in the quest for more efficient antimicrobial agents

C. Faustino and **L. Pinheiro**

[1] iMED.ULisboa Research Institute for Medicines, Faculty of Pharmacy, Universidade de Lisboa, Avda. Professor Gama Pinto, 1649-003 Lisbon, Portugal

The currently global and urgent need for alternative antimicrobial strategies to fight the continuous rise of microbial resistance led to a re-evaluation of the therapeutic use of ancient medicines such as honey. The antimicrobial (antibacterial, antifungal, and antiviral) properties of honey are dependent on physicochemical parameters, floral and entomological origin, harvesting process and storage conditions. Large clinical evidence concerning the use of honey in wound care, where the rationale of its therapeutic use takes account of the debridement, anti-inflammatory action, antioxidant activity and immunomodulatory role, is based on several studies demonstrating the broad antibacterial activity. Honeys selected for use in medical devices are chosen on the basis of their antibacterial effectiveness, the most well-known being manuka honey. This mini-review covers the antimicrobial properties of honey produced by *Apis mellifera* (honeybee) from floral sources (unifloral or multifloral) and discusses some of the proposed mechanisms underlying its antimicrobial action.

Keywords: honey; antimicrobial properties; wound dressing

1. Introduction

Honey, a natural product formed from the nectar of flowers by honeybees, has been used as a medicine throughout the ages and has recently been rediscovered mainly due to the development of bacterial resistance to conventional modern therapeutic agents [1-4]. Laboratory studies and clinical trials have shown that honey is an effective broad-spectrum antibacterial agent, and much of the research to date has addressed the antibacterial properties of honey and its effects on wound healing. When ingested, honey also shows antibacterial activity and promotes healing by decreasing prostaglandin levels, elevating nitric oxide levels, and exerting prebiotic effects [5]. These factors play a major role in controlling inflammation and promoting microbial control and healing processes. Several studies support the use of honey-based products in wound care, endorsing their efficacy, cost-effectiveness and safety. Thus, there has been a sizeable renaissance in the use of honey as a topical treatment for a wide range of wounds, together with those that do not respond to the conventional therapy, i.e., antibiotics and antiseptics. This resurgence has brought an array of new honey-based wound products into the market place. From these, the best known is manuka (*Leptospermum scoparium*) honey from New Zealand which has an inhibitory effect over more than 60 different species of bacteria, including methacilin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococcus* (VRE) and multi-drug resistant (MDR) *Pseudomonas aeruginosa* and *Acinetobacter baumanni* [4]. More recently, investigations on the nutritional and antiseptic properties of Scottish heather honey, which presents the same consistency and rheological behaviour of the manuka honey, are being carried out after its antimicrobial activity for horse wound treatment was disclosed [6].

2. Honey composition

2.1 Chemical composition

Honey's ingenious elaboration starts with the nectar collected from plants, which honeybees (*Apis mellifera*) transform and mix with their own specific substances, store and leave to mature in honeycombs. This natural product is a supersaturated solution of sugars (predominantly fructose, glucose and sucrose) with low water content and low concentrations of other bioactive compounds, including phenolic compounds (phenolic acids, flavonoids and polyphenols), amino acids (mainly proline), peptides, proteins, enzymes, vitamins (especially B complex and vitamin C), minerals (Ca, Cu, Fe, K, Na, Mg, Zn, Mn, P, S), organic acids (predominantly gluconic acid from enzymatic oxidation of glucose), lipids (including waxes), aroma compounds, carotenoid-like substances, and pollen grains [5].

The three main honey enzymes are diastase (amylase), decomposing starch or glycogen into smaller sugar units, invertase (sucrase, α-glucosidase), decomposing sucrose into fructose and glucose, and glucose oxidase, producing hydrogen peroxide and gluconic acid from glucose [3]. Other additional and relevant antimicrobial components present in some honeys are methylglyoxal, the glycoside leptosin, and peptide bee defensin-1.

Factors influencing the antimicrobial effectiveness of honey include the high osmolarity, low water activity (below 0.60), the acidic pH (usually between 3.2 and 4.5), the high viscosity, the hydrogen peroxide content and the presence of phytochemical elements [7,8]. Osmotic effects of sugars seem to account for non-specific activity of some honeys, while in other honeys undefined phytochemical components are the key of a more specific growth inhibitory effect on

bacterial strains. The huge variation in the phytochemical content, according to floral sources and climate conditions, contributes to the diversity of colors, flavors, aromas, and bioactivities found in honey.

The specific chemical composition and physical properties of honey depend on many factors, such as the nectar composition of the source plant, bees' species, the climate, environmental and seasonal conditions, agricultural practices and treatment of honey during extraction and storage [9,10]. Thus, the bioactivity of honey varies and depends on the floral and entomological origin, harvesting process, subsequent storage conditions, and time impact on physicochemical characteristics.

2.2 Antimicrobial components

The high sugar content and physicochemical properties of honey (e.g. high osmolarity, low water activity, acidity) that hinder microbial growth can only partially explain the antimicrobial properties since artificial honeys, with the same sugar composition of natural honeys, show lower antimicrobial activity. Honeys are known to contain several minor components in variable amounts, depending on the bee, the floral source and geographical location, some of which contribute to the antimicrobial activity of honey, like lysozyme, glucose oxidase, methylglyoxal, bee defensin-1 and phytochemical components [11,12].

The bee enzyme glucose oxidase (GOX) that catalyzes the conversion of glucose to gluconic acid during the ripening process is of considerable interest since hydrogen peroxide (H_2O_2) is produced in the reaction, which not only stabilizes the ripening of nectar against spoilage but also has a microbicidal action.

The discovery of hydrogen peroxide as an intrinsic compound of honey brought an assumption that H_2O_2 is a main factor underlying the broad antimicrobial activity of this natural product [11-13]. The levels of H_2O_2 in honey depend on the relative rates of its production by GOX and its hydrolysis in the presence of catalase of pollen origin [13]. The efficiency of H_2O_2 production by GOX increases with honey dilution, which facilitates access of the enzyme to its glucose substrate and prevents milieu acidification responsible for GOX inhibition. The constant production of H_2O_2 in the diluted honey produces an enduring antiseptic effect that is most required in wound infections [13]. The enzyme becomes inactivated upon exposure to excessive heat or light, thus careful packaging and storage of honey are required to maintain its bioactivity.

In most honeys, a substantial correlation has been found between the antimicrobial activity and H_2O_2 content, however the values found in honey, typically between 0.4 and 2.6 mmol/L, are much below the biocidal levels (which are approximately 800 mmol/L in a 3% H_2O_2 solution commonly used as an antiseptic agent). Therefore, it has been suggested that hydroxyl radicals, generated from H_2O_2 by the metal-catalyzed Fenton-type reaction, are the main cytotoxic species underlying honey antibacterial activity [14-16].

Some honeys, like manuka honey, are known to retain their growth inhibitory action even after removal of H_2O_2 following catalase addition [7,13]. Methylglyoxal (MGO) has been extensively found in manuka honey, and its levels (which can reach concentrations of 828 mg/kg [17] compared with approx. 24 mg/kg in other honeys) directly correlate with the non-peroxide antibacterial activity of this honey [18]. MGO is a ketoaldehyde formed by a non-enzymatically conversion of dihydroxyacetone present at exceptionally high concentrations in the nectar of *L. scoparium* flowers [19] that is able to activate macrophages through superoxide and nitrite production in the course of the mitogen-activated protein kinase (MAPK)/nuclear factor (NF)-κB signaling pathway [1].

Therefore, honeys can be classified in two main groups regarding the main component involved in their antibacterial activity: peroxide honeys, which include European and American honeys whose activity is catalase-sensitive and shows substantial correlation with H_2O_2 content; and non-peroxide honeys, such as those from *Leptospermum* spp., whose activity is H_2O_2-independent but instead correlates with MGO levels. More importantly, neither type of activity is influenced by sterilizing procedures using γ-irradiation [11,12].

Although MGO has been considered the major factor underlying the non-peroxide antimicrobial activity of manuka honey [20], other antimicrobial compounds have recently been identified, such as leptosin, a glycoside derivative of methyl syringate and a chemical marker that contributes to the overall antimicrobial properties of this unique honey [8,21].

Compounds influencing the non-peroxide antimicrobial activity have also been found in honeys other than manuka, including kanuka, heather, lavender and kamahi honeys [13]. The cationic antimicrobial peptide bee defensin-1 (also known as royalisin) previously identified in bee hemolymph, head, and thoracic glands, enters honey via bee saliva during the regurgitation process of honey making [22]. Bee defensin-1 was discovered in Revamil® (Bfactory Health Products, Rhenen, the Netherlands) source (RS) after the enzymatic neutralization of the inhibitory effects of both H_2O_2 and methylglyoxal [8]. The peptide exhibits a potent action against Gram-positive bacteria that is part of bee's immune response, including *Bacillus subtilis*, *S. aureus* and *Paenibacillus larvae* [1], by a mechanism not yet described with clarity. Interestingly, bee defensin-1, which is a regular but quantitatively variable component in honeys of different botanical origins, has not been identified in manuka honey [1]. A recent study by Majtan *et al*. [23] revealed that treatment of a solution of defensin-1 with high amounts of MGO caused a time-dependent loss of its antibacterial activity. The same study suggested that MGO could also have negative effects on the structure and function of other peptides or proteins in manuka honey, such as glucose oxidase, which could explain the low H_2O_2 content of this honey [23]. Hence, H_2O_2, MGO and bee defensin-1 are distinct mechanisms involved in the bactericidal activity of honey.

Phenolic compounds with antioxidant properties originating from plant nectar, namely flavonoids (quercetin, luteolin, pinocembrin, chrysin, galangin, apigenin, pinobenksin, kaempferol) and phenolic acids (syringic, ferulic, cinnamic, benzoic, caffeic acids), also contribute to the non-peroxide antimicrobial activity of honeys [1,5]. Indeed, a correlation has been found between antioxidant and antimicrobial activities, and the concentration of phenolic compounds in honey, which also correlates to the colour of the honey; usually, the darker the honey (e.g. buckwheat, sidr, clover, manuka, heather and borage), the higher the concentration of polyphenols and the higher the antioxidant and antimicrobial activities [24].

Oxidation of polyphenols by atmospheric oxygen, which can occur during honey harvesting from the comb and during storage, leads to reactive intermediate species that target bacterial macromolecules, including cell membrane proteins and lipids, enzymes and nucleic acids, leading to membrane injury and DNA damage. On the other hand, complexation of oxidized polyphenols with bioactive molecules present in honey can lead to a decrease in its antioxidant and antimicrobial properties, which depend on the balance between antioxidant and pro-oxidant activities of polyphenols [15].

Protection against oxidation processes in a given organism, through the presence of free radicals and reactive oxygen species (ROS), is a key-factor in the prevention of the pathogenesis of chronic inflammatory disorders. Sugars and proteins may interact through Maillard reaction, causing non-enzymatic browning in honeys, and leading to the formation of several complex compounds with antioxidant and antibacterial properties [8]. This is the origin of melanoidins found in a series of Canadian honeys, as a consequence of heating or aging processes [25]. The radical scavenging property of melanoidins enables them to sequester Maillard reaction-like products formed in honey, resulting in a gain-and-loss of antioxidant and antimicrobial properties of honey [25]. The antioxidant activity of melanoidins is related to the polyphenolic content, with unheated dark honey containing a significantly higher amount of melanoidins than other honeys. Depending on the melanoidin content in unheated honeys, heat-treatment can either accelerate formation of melanoidins with an increase in the radical scavenging activity (light and medium honeys) or accelerate degradation of melanoidins with reduction of antioxidant activity (dark honeys) [25].

3. Antimicrobial activity

3.1 Antibacterial

Honey has a broad-spectrum of antimicrobial activity, being effective against more than 70 strains of bacteria, including Gram-positive and Gram-negative microorganisms, some fungi and viruses. However, the antimicrobial potency depends on the type of honey, floral source and geographical location [11,12].

Allen *et al*. [26] studied the variation in antibacterial activity of honey against *S. aureus* using 345 samples of unpasteurized honey from New Zealand, most of them unifloral from 26 different floral sources, and found that the difference between floral sources highly influenced antibacterial potency. Kanuka (*Kunzea ericoides*), manuka (*Leptospermum scoparium*), ling heather (*Calluna vulgaris*) and kamahi (*Weinmannia racemosa*) were shown to be sources likely to give honey with high antibacterial activity.

Willix *et al*. [27] compared the non-peroxide activity of manuka honey with that of a peroxide-producing honey against several pathogenic wound bacteria. The response of the bacterial species showed no significant difference between the two types of activity overall, but marked differences existed in the ranking order of sensitivity. Although both types of honey completely inhibited the growth of all tested bacteria at concentrations below 11% (v/v), a concentration of only 1.8% (v/v) of manuka honey was enough to completely inhibit the growth of *S. aureus* after 8 h of incubation [27].

The antibacterial activity of several honey types, among them manuka honey, was assessed against 10 equine pathogens recovered from the wounds of horses, including MRSA, methicillin-resistant *Staphylococcus epidermis* (MRSE), *Streptococus equi*, *Acinetobacter baumannii* and *P. aeruginosa* [6]. From the 11 samples tested, 8 were effective against all bacterial isolates at concentrations between 2% and 16% (v/v). The Scottish heather honey from *Calluna vulgaris* (family Ericaceae), which has the same consistency and thixotropic properties of manuka honey, performed best among the tested honey samples, inhibiting bacterial growth at concentrations ranging from below 2% to 6% (v/v) [6].

Recently, the antibacterial activity of a Chilean honey originating from the ulmo tree (*Eucryphia cordifolia*) was tested against selected strains of bacteria and compared with manuka honey [28]. Although the ulmo honey had higher antibacterial activity against the five MRSA isolates tested, similar activities were observed against *E. coli* and *P. aeruginosa*, with equivalent MICs of 12.5% (v/v) being obtained for both honeys. Unlike manuka, the antibacterial activity of ulmo honey is mainly due to H_2O_2 production.

3.2 Antifungal

Besides microorganisms, honey inhibits also the growth of fungi like *Candida albicans*. The mechanism by which honey exerts its antifungal activity is not entirely understood. However, this bioactivity has been generally attributed to hydrogen peroxide, flavonoids, methylglyoxal and the high sugar content [2,29].

The efficacy of various honeys (Medihoney® Antibacterial Honey Barrier, Comvita® Wound Care 18+, an unprocessed jarrah honey with hydrogen peroxide activity, and an artificial honey) was determined against clinical isolates of *C. albicans*, *C. glabrata* and *C. dubliniensis* [30]. The hydrogen peroxide-type honey was found to have a higher antifungal effect, inhibiting the growth of the tested yeasts between 15 and 30% (w/v).

The first study concerning the antifungal effect of unifloral lavender honey against *C. albicans*, *C. krusei*, and *Cryptococus neoformans* showed that the growth of all yeasts were reduced in the presence of this honey [31]. The honey concentration inhibiting 10% of yeast growth was determined as 31.0%, 16.8% and 23.0% (w/v), respectively.

Recent studies involving the antifungal potential found in Portuguese unifloral *Erica* sp. heather honeys, against *C. famata*, *C. albicans*, *C. krusei* and *C. neoformans* (in the absence or presence of catalase), seems to be leading to promising yet limited results [32]. MIC values ranged approximately from 13 to 20% (w/v), and from 15 to 26 (w/v), for honey without and honey with catalase, respectively. The most important inhibiting factor seems to be H_2O_2.

Antifungal properties of honey and starch were combined in the treatment of superficial mycoses (*C. albicans*). Starch, which is the substrate of the diastase and also a microbial nutrient, was found to have a synergistic action on the antifungal activity of honey [33]. MIC ranged between 40-45% (v/v) and 7-25% (v/v), without and with starch, respectively. A negative correlation has been established between the MIC drop and the diastase number (DN).

Among fungi, *C. albicans* is the most common pathogen associated with fungal biofilm infections. Studies involving the *in vitro* effects of natural jujube honey on planktonic states of *C. albicans* and detachment of biofilm-embedded states, proved the growth inhibition and biofilm inhibition, for concentrations higher than 10% w/v [29]. The results also indicated that honey also caused changes to the cell wall and exopolysaccharides, pointing out the higher fungistatic, fungicidal and antibiofilm potential when compared with the most commonly used antifungal agents.

3.3 Antiviral

Following the actual requirements for the urgent development of novel anti-influenza virus drugs, several studies have been performed aiming the evaluation of anti-influenza activity of honey from different sources [34]. Manuka honey, amid other honeys, showed a remarkable action inhibiting the virus replication, which could be very promising for a future medicinal application. For the evaluation of the virucidal effects, plaque inhibition experiments were carried out and Madine-Darby canine kidney (MDCK) cells were used. Results also showed that the antiviral action of manuka honey had a synergistic effect with that of neuraminidase inhibitors such as zanamivir and oseltamivir [34]. Even though the influenza-inhibitory constituents of honey remain unknown, a number of antibacterial constituents may contribute to this antiviral activity, as hydrogen peroxide, flavonoids rutin and chrysin (reported in buckwheat and acacia honeys) and methylglyoxal [2,34].

Other reports in the literature refer to the *in vitro* anti-viral effect of manuka and clover honeys on a clinical isolate of varicella zoster virus [35], with both types showing significant antiviral properties. For the screening of the antiviral actions, plaque inhibition experiments were accomplished and a human malignant melanoma (MeWo) cell line was used.

Some different types of manuka honey have also been reported to reveal antiviral activities against herpes simplex and respiratory syncytial viruses, with results suggesting some correlation between a honey's antibacterial activity and its antiviral activity. The antiviral activity could be linked with the amount of methylglyoxal present in honeys as to the high levels of phenolic compounds. The evaluation was performed using susceptible A549 cell line and viral isolates of Adenovirus serotypes, and Herpes simplex virus serotypes [36]. Additional investigations would be necessary to identify the active antiviral components in honeys and to determine eventual synergistic effects with known antiviral drugs.

Antiviral effects of honey, royal jelly and acyclovir on Herpes Simplex Virus-1 (HSV-1) were compared in an extra-somatic environment [37]. Using the plaque assay technique and Vero cells, results were obtained showing that honey, royal jelly and acyclovir presented the highest inhibiting effects on HSV-1 at concentrations of 500, 250 and 100 mg/mL, respectively. These are encouraging reports suggesting the use of honey and royal jelly as alternatives to acyclovir drug in the treatment of herpetic lesions provided that efficacy may be substantiate.

3.3 Antibiofilm

Medical device-related nosocomial infections associated with microbial biofilm formation are becoming a public health concern with the rapid emergence of multi-drug resistant strains. Microbial biofilms are composed mainly of surface adherent microbial consortia enclosed in a self-produced extracellular polysaccharide (EPS) matrix, which provides protection against the host immune response(s) and also against antibiotic therapy. The decrease in antibiotic susceptibility of microbial biofilms mainly results from the failure of conventional antimicrobial agents to achieve biofilm penetration at appropriate therapeutic levels.

The effects of sidr and manuka honeys against methicillin-susceptible *S. aureus* (MSSA), MRSA and *P. aeruginosa* isolates, involving both planktonic and biofilm-grown bacteria, were studied *in vitro* [38]. Both honeys were effective in killing 100% of the isolates in the planktonic form while the bactericidal rates against MSSA, MRSA and *P. aeruginosa*

biofilms were 63, 73 and 91% for sidr honey and 82, 63 and 91 for manuka honey, respectively, being higher than those observed with single antibiotics commonly used against *S. aureus*.

The activity of manuka honey, in combination with antibiotics, was also assessed *in vitro* against *S. aureus* and *P. aeruginosa* biofilms. Manuka honey has a synergistic interaction with vancomycin against *S. aureus* (IDRL-4284) biofilms and an additive interaction with gentamicin against *P. aeruginosa* (PAO1) biofilms. Both biofilms, when exposed to manuka honey, exhibited distorted cell morphologies and a decrease in the extracellular matrix for *P. aeruginosa* was evidenced by scanning electron microscopy (SEM). The synergistic action between manuka honey and oxacillin was also able to re-sensitize MRSA to oxacillin treatment *in vitro* [39].

Biofilm formation by enterohemorrhagic *E. coli* O157:H7 was reduced by a low honey concentration of 0.5% (v/v) while the growth of planktonic cells and commensal *E. coli* K-12 biofilm formation were not inhibited [40]. Transcriptome analysis showed that honey repressed curli genes (*cgsBAC*), quorum sensing genes and virulence genes, with glucose and fructose in the honeys being key components in the process. Honey was also able to decrease the colonization of *E. coli* O157:H7 on human HT-29 epithelial cells, suggesting that sublethal concentrations of honey can be used for reducing the colonization and virulence of pathogenic bacteria.

Chestnut honey was found to inhibit the production *N*-acyl-L-homoserine lactones (AHLs), which have been identified as main signaling molecules in Gram-negative bacteria, resulting in impaired biofilm formation in *Erwinia carotovora*, *Yersinia enterocolitica* and *Aeromonas hydrophila* [41]. The carbohydrates in honey were also associated with the process. The sugar components in honey, including glucose and fructose, can provide a mechanical barrier that prevents surface adhesion of bacteria.

Moreover, the high fructose content and the presence of mannosylated glycoproteins in honey were found to act as competitive blockers of the fucose-binding lectin of *P. aeruginosa*, which also binds fructose and mannose [Lerrer2007]. This adhesive protein mediates cell-to-cell interactions essential for biofilm formation and adhesion of *P.aeruginosa* to animal cells, highlighting the potential of honey in antibacterial adhesion therapeutic strategies aimed at abrogating microbial adhesion to host cells, preceding infection establishment [42].

The antiadhesive properties of honey also play an important role in the prevention of biofilms of the oral cavity that comprise the dental plaque. Besides being bactericidal for *Streptococcus mutans*, the main causative organism of dental caries that is known to adhere to the salivary pellicle on the tooth surface via specific surface adhesins, manuka honey is able to prevent the growth of *S. mutans* on glass surfaces and saliva-coated hydroxyapatite discs [43].

Manuka honey also has bactericidal effects in both planktonic cultures and biofilms of *Streptococcus pyogenes* (M28), a wound-associated pathogen, although higher concentrations were needed to inhibit biofilms [44]. Abrogation of adherence and intercellular aggregation was observed, with manuka honey permeating established biofilms of *S. pyogenes*, resulting in significant cell death and disruption of the biofilm. Sublethal concentrations of manuka honey were associated with a reduced expression of the genes encoding two major fibronectin-binding surface adhesins, Sof and Sfbl, thus inhibiting bacterial binding to the host protein fibronectin and preventing initial colonization [44].

Biofilm growth and its persistence within wounds are major factors contributing to impaired healing. Several honeys of different botanical origin were found to substantially reduce biofilm development of *Proteus mirabilis* and *Enterobacter cloacae* wound isolates, at a sub-inhibitory concentration of 10% (w/v) [45]. Among the tested honeys, manuka honey showed the most potent antibiofilm properties. Furthermore, MGO, the main antimicrobial component of manuka honey, was observed to penetrate and disrupt the biofilms, being able to kill the biofilm-embedded wound bacteria [45].

4. Mechanism(s) of action

The precise mechanisms of action of honey(s) against microbial pathogens are still not completely understood, however recent advances in proteomics and genomics techniques allowed the identification of some cellular targets for honey.

The antimicrobial activity of Canadian buckwheat (*Fagopyrum esculentum*) honeys, which are known to contain high amounts of H_2O_2 and antioxidant polyphenols, has been assayed against *E. coli*, *B. subtilis* and antibiotic-resistant clinical isolates of MRSA and VRE [14,16]. The antimicrobial activity was found to arise from increased oxidative stress correlated with the formation and accumulation of hydroxyl radicals that inhibit bacterial growth in a concentration-dependent manner. Hydroxyl radicals are short-life powerful oxidants known to cause oxidative injury to bacterial membrane lipids, proteins and nucleic acids, leading to impaired permeability of cell membranes and cell proliferation. Hydroxyl radicals can be formed from H_2O_2 via the metal-catalyzed Fenton reaction. Honey is known to contain traces of metals (e.g. Cu, Fe), and addition of Cu^{2+} to honey enhanced bacteriostatic action with a drop in the MIC values against both antibiotic-sensitive and -resistant clinical isolates. Addition of catalase prior to supplementation with Cu^{2+} restored bacterial growth [14]. A concentration-dependent bactericidal effect associated with extensive degradation of bacterial DNA was also observed. This effect depended on the ratio of honey concentration to bacterial load indicating that honey dose was critical for the bactericidal effect [16]. Moreover, polyphenols can contribute to the process since in the presence of transition metal ions they can behave as powerful pro-oxidants driving the generation of hydroxyl radicals from H_2O_2 via the Fenton reaction [15].

The effect of kanuka, manuka and clover honeys on bacterial growth dynamics and cellular morphology of *B. subtilis*, *E. coli*, *S. aureus* and *P. aeruginosa* has been studied, and the effectiveness of growth inhibition followed the general trend manuka > kanuka > clover [46]. Moreover, each species had a unique response profile to the tested honeys and cell morphology analysis showed a varied set of responses to honeys that involved cell length changes, cell lysis and alterations in DNA conformation. These responses are likely to reflect the different regulatory circuits of the organisms that are activated by the stress of honey treatment, suggesting that some cellular targets might be broadly specific for Gram-positive or Gram-negative microorganisms [46].

Manuka and pasture honeys have been found to inhibit the bacterial growth of several MRSA and vancomycin-sensitive enterococci, isolated from infected wounds, and also of vancomycin-resistant enterococci strains isolated from hospital environmental surfaces, with MIC values below 10% (v/v) [47,48]. Moreover, comparison of the MIC values of antibiotic-sensitive strains with their corresponding -resistant strains showed similar susceptibility to honey [48].

A bactericidal mode of inhibition for manuka honey on *S. aureus* was established and transmission electron microscopy (TEM) showed marked structural changes in MRSA cells after honey treatment, with a significant increase in the number of whole cells with completed septa, suggesting that honey interferes with the cell cycle of MSRA [49,50]. Sugars were not implicated in the process. Moreover, downregulation of universal stress protein A (UspA), which is involved in the stress stamina response, was shown to occur in honey-treated cells, helping to explain the inhibition of MSRA by manuka honey [51].

Manuka honey is also bactericidal against *P. aeruginosa*, with MIC and minimal bactericidal concentration (MBC) values of 12% (w/v) and 16% (w/v), respectively [52]. Atomic force microscopy (AFM) and confocal microscopy showed extensive cell lysis after only 1 h exposure to inhibitory concentrations of manuka honey, which was attributed to damages to the cell envelope. Genomic analysis revealed differential expression of *oprF* and *algD*, two key microcolony-forming genes, after honey treatment, with a decrease in the expression of *oprF* encoding an integral membrane protein involved in the structural stabilization of the cell envelope in Gram-negative bacteria [52].

Transcriptome analysis was used to obtain the pattern of gene expression in *E. coli* upon exposure to a sublethal dose of a medical-grade *Leptospermum* honey [53]. It was found that 2% of the genes were upregulated and 1% were downregulated, with many of the upregulated genes being involved in stress responses while most of the downregulated genes were associated to protein synthesis. Furthermore, resistance to honey could not be induced under conditions that rapidly lead to antibiotic resistance [53,54]. This lack of resistance has been attributed to the multifactorial action of honey, which has more than one target site thus reducing the likelihood of microbial acquired resistance [53,54].

In addition to inducing changes in microbial cellular structure and metabolism, honey can also affect the virulence of bacterial pathogens. The antibacterial properties of honey have been shown to result not only from the presence of bactericidal components but also of quorum sensing inhibition, which weakens bacterial communication, coordination and virulence [55]. Low concentrations of honey have been shown to inhibit the expression of the transcriptional regulator MvfR and the *las* and *rhl* regulons in *P. aeruginosa*, which resulted in reduced expression of the associated virulence factors. The sugar content of honey played an important role in the quorum sensing inhibition process [55].

Another factor contributing to the virulence of *P. aeruginosa* is its ability to sequester iron from the host, during infection, by the synthesis and secretion of siderophores, which are iron-chelating molecules regulated by quorum sensing that play a central role in bacterial proliferation. Exposure of *P. aeruginosa* to manuka honey at sub-MIC concentrations inhibited siderophore production, suggesting that manuka honey may impact on bacterial iron homeostasis and providing an additional target for manuka honey in *P. aeruginosa* [56].

The interference with bacterial quorum sensing is thus an attractive therapeutic strategy for the treatment of microbial infections since it impacts on the virulence of pathogenic bacteria and also on biofilm development. Moreover, the antivirulent effect has been observed at honey concentrations far below the MIC and does not pose the same selective evolutionary survival pressure that promotes the emergence of resistance.

5. Therapeutic uses of honey

5.1 Honey in wound healing

Honey has been used as a topical treatment in wound care since ancient times due to its antimicrobial, antioxidant and anti-inflammatory properties. Natural honey stimulates tissue regeneration and angiogenesis, debrides necrotic tissues, removes malodour, reduces scarring, edema and inflammation and promotes rapid wound healing. Honey usage has declined after the development of antibiotics in the last century, however the continuous emergence of antibiotic resistance led to a renewed interest in the medicinal properties of honey.

The wound moist environment, although essential for the healing process, is an ideal growth medium for bacteria. Infection has been identified as a major factor in impaired healing, with the rising in microbial resistance to currently available antibiotics posing further concerns. Burns and chronic wounds are particularly prone to infection, which is often polymicrobial and involves mainly Gram-positive cocci (Streptococci and Staphylococci) but also Enterococci, Enterobacteriaceae anaerobes (*Escherichia*, *Klebsiella*, *Enterobacter*) and Pseudomonadaceae family of bacteria [57,58].

Wound infections are largely treated with antibiotics in conventional medicine and although antibiotic therapy may be required for systemic or progressive infections, it is often inappropriate in cases such as burns and chronic wounds, where reduction in blood flow prevents access of the antibiotic to the target area and may even encourage colonization by resistant microorganisms. Advances in dressing technology have provided alternative options that include alginate, foam, hydrogel and hydrocolloid dressings incorporating topical antimicrobial agents such as honey, silver and iodine. These bioactive dressings control the level of wound hydration while providing not only a physical barrier against infection but also antimicrobial properties that promote the healing process. Honey-based treatments are preferable to silver and iodine dressings due to its comparative lack of cytotoxicity [58].

The high osmolarity and low pH of honey inhibits the growth of most pathogenic bacteria within wounds. Natural honeys are also bactericidal or bacteriostatic against a wide variety of pathogens, including most wound pathogens, with MICs in the range 10%–20% (v/v), making them effective in preventing and controlling infection even after dilution by wound exudates [57,58]. The high sugar content of honey also helps deodorize infected wounds since bacterial degradation of amino acids from dead cells and serum that cause malodour, such as sulphur compounds, ammonia, and amines, are replaced by lactic acids resulting from preferential degradation of glucose and fructose.

Additionally, honey maintains a moist wound environment, thereby aiding fibroblast migration and epithelialisation, reducing scarring and total wound healing time, while its high viscosity provides a physical barrier to external pathogens [59]. Honeys with a high wax content form a semisolid medium offering a better wound contact that helps prevent biofilm development. Natural honey has a low adherence to the wound bed upon removal therefore minimizing pain and preserving the newly forming tissues [58,59].

The beginning of the healing process is patented by the body's inflammatory response. However, a long-lasting inflammatory response, often associated with high levels of exudate, can inhibit healing. Honey's skilfulness as wound dressing contributes to its anti-inflammatory action [60]. In clinical terms, there have been a huge amount of observations reporting the reduction of oedema, exudate and scarring, and a soothing effect on inflamed wounds and burns.

Honey has also been shown to be able to reduce the activities of inflammatory mediators cyclooxygenase (COX)-1 and COX-2 [4]. Furthermore, the immunomodulatory properties of honey, which are capable of inducing the release of pro-inflammatory cytokines from the monocytes, are relevant for wound healing. Honeys such as manuka, jelly bush and pasture honey, have been found to stimulate the monocytes to secrete tumor necrosis factor (TNF)-α, interleukin (IL)-1β and IL-6 [61]. Additionally, glycosylated proteins in honey can also induce TNF-α secretion by macrophages. Recently, activation of human keratinocytes incubated with honey was also observed leading to increased levels of mRNA for TNF-α, IL-1β, transforming growth factor β (TGFβ) and matrix metalloproteinase-9 (MMP-9) that may contribute to wound healing [62].

Anti-inflammatory effects of raw honey in humans were studied after ingestion of 70 g on a daily basis [5]. Inflammation indicators such as the plasma concentrations of prostaglandin (PG) E_2, $PGF_{2\alpha}$ and thromboxane B_2 were decreased by 63%, 50% and 48%, respectively, after 15 days. Other studies allude to a reduced inflammation process in an experimental model of inflammatory bowel disease in rats [5], plus being as effective as prednisolone treatment in an inflammatory model of colitis [4]. Prevention of free radicals released from the inflamed tissues seems to be the mechanism of action, and the reduction of inflammation may possibly be due to the antibacterial effect or to a direct anti-inflammatory effect [5,60]. The latter hypothesis was supported in animal studies, where anti-inflammatory effects of honey were observed in wounds with no bacterial infection [5,57].

Honey has been referred in traditional folklore as a remedy for diarrhea, gastritis, peptic ulcers and other gastrointestinal complaints, suggesting that honey may promote the repair of damaged intestinal mucosa, stimulate the growth of new tissues and act as a local anti-inflammatory agent. Moreover, manuka honey has been shown to inhibit the growth of *Helicobacter pylori* with a MIC of 5% (v/v) being established *in vitro*, but insufficient time of contact with the microorganisms in the stomach and dilution to ineffective concentrations by bodily fluids can hamper its biological effects [63]. Although manuka honey has been regarded as a promising functional food for the treatment of stomach ulcers, no improvements were seen in a small number of patients with *H. pylori* gastric ulcers which were given a tablespoon of manuka honey 4-times a day for 6 weeks relatively with the members of the control group given a conventional treatment [64].

Moreover, the unusually high levels of MGO in manuka honey, which is a highly reactive precursor in the formation of advanced glycation endproducts (AGEs) associated with aging and some age-related chronic diseases, have recently raised concerns regarding the potential toxicity of dietary MGO in honey [65], although manuka honey has a history of safe use [58].

5.2 Medical-grade honey

A wide range of honey-based products have recently been developed as topical medical preparations, including gels, ointments and honey-impregnated dressings, mainly made of manuka, buckwheat, chestnut or clover honeys. Large discrepancies have been reported by hospitals using honey, most likely due to the variability in potency of the different

honeys used. The variation in physicochemical properties and antimicrobial efficacy of honey and the incomplete knowledge of the mechanisms underlying its antimicrobial activity hamper medicinal applicability.

Moreover, concerns about topical usage of honey in wound healing regarding the potential risk of infection due to honey microbial contamination, namely by *Clostridium botulinium*, have arise. Carnwath *et al*. [6], when assessing the antimicrobial activity of 29 honey products against equine wound bacterial pathogens, including γ-irradiated and non-irradiated commercial medical-grade honeys, supermarket honeys, and honeys from local beekeepers in the UK, found evidence for contamination with aerobic bacteria or fungi among 18 samples. Therefore, non-sterile honeys must be avoided in wound treatment due to the potential risk of wound contamination with pathogenic microorganisms eventually present in non-sterilised honey samples.

The use of honey as an antimicrobial agent requires safe preparations and standardized honeys with defined antibacterial activity. The two major medical-grade honeys commercially available are manuka honey from New Zealand (Medihoney®, Comvita®) and Revamil® honey form the Netherlands, their formulations sterilized by γ-irradiation being licensed as medical devices and approved for the management of wounds and burns. The source for Revamil®, RS honey, is produced in greenhouses under standardized conditions but further details about the origin of the honey have not been disclosed yet. Being produced under controlled conditions, Revamil® has standardized antibacterial activity while manuka honey shows large batch-to-batch variation.

The properties of honey underlying its bioactivity and contributing to its therapeutic applications are summarized in Fig. 1.

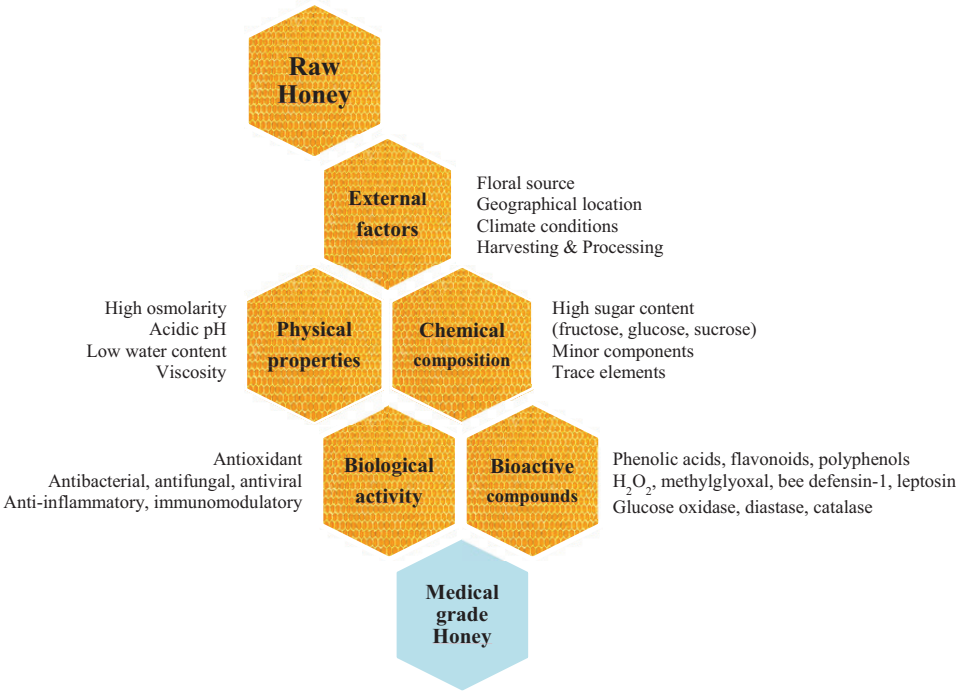

Fig. 1 Honey properties contributing to its bioactivity and therapeutic use.

Medical-grade manuka honey carries a Unique Manuka Factor (UMF) value based on its activity against *S. aureus* in an agar well diffusion assay in reference to phenol as a standard [26]. Therefore, a UMF value of 10, for example, corresponds to the same antibacterial activity of a 10% solution of phenol. Generally, only manuka honeys with a UMF of 10 or higher are used in medical preparations.

The bactericidal activity and modes of action of medical-grade manuka honey and RS honey against *B. subtilis*, *E. coli*, *P. aeruginosa* and MRSA strains have been studied and compared [66]. Both honeys killed all tested bacteria, including MRSA, after 24 h of incubation, with manuka honey retaining activity up to higher dilutions than Revamil®. Manuka honey killed *B. subtilis* within 2 hours, while in the same period Revamil® also killed *E. coli* and *P. aeruginosa*.

RS honey has been reported to accumulate up to 5.62 mmol/L H_2O_2 and to contain 0.25 mmol/L of MGO [67]. The presence of bee defensin-1 was also detected in RS honey, but not on manuka honey. The different antimicrobial compounds in RS honey were sequentially neutralized in order to determine their individual effects on the different strains of bacteria [67]. Catalase was used to inhibit production of hydrogen peroxide, glyoxyalase 1 to inhibit MGO, and sodium polyanethol sulfonate to neutralize bee defensin-1. After enzymatic neutralization of H_2O_2 and MGO, RS

honey still retained substantial activity due to the presence of bee defensin-1. Following the neutralization of the antimicrobial peptide, RS honey had only minimal activity left, and subsequent adjustment of the honey pH from 3.3 to 7.0 reduced the activity to that of sugar alone.

Moreover, H_2O_2, MGO, and bee defensin-1 differed in their activity against specific bacterial strains suggesting that there are synergistic and redundant components in honey that depend on the type of microorganism [67]. The rapid bactericidal activity of Revamil® was attributed mainly to bee defensin-1 and H_2O_2, which were not detected in manuka honey. On the other hand, manuka honey contained much higher levels of MGO, which was considered the non-peroxide factor responsible for the antimicrobial activity of the honey. However, neutralisation of MGO in manuka honey abolished antimicrobial activity against *S. aureus* but not against *E. coli* and *P. aeruginosa* [66]. Therefore, MGO cannot be fully responsible for the non-peroxide antimicrobial activity of manuka honey, and other antimicrobial components, namely antibacterial phenolic compounds, such as methyl syringate, were suggested as possible contributors to the overall antibacterial activity [66]. Later on, Kato *et al*. [21], using chromatographic techniques, identified a novel glycoside derivative of methyl syringate, called leptosin, which positively correlated with the bactericidal activity of manuka honey.

In summary, the differences in bactericidal activity of medical-grade manuka honey and RS honey mainly reside on their different chemical composition and on the presence of different antimicrobial components in both honeys, viz. H_2O_2, MGO and bee defensin-1 in RS honey, and higher levels of MGO in manuka honey and eventually other bioactive compounds, such as leptosin.

6. Conclusion and future perspectives

Honey can provide an alternative and effective antimicrobial strategy against the continuous emergence of antibiotic-resistant microorganisms. Honey has a broad-spectrum activity against pathogenic and food-spoiling bacteria, including Gram-positive and Gram-negative microorganisms, and also against fungi, viruses and biofilms. Antibiotic-sensitive and -resistant strains, including MRSA, MRSE and VRE, show similar sensitivity to honey. Honey is also able to reverse antimicrobial resistance *in vitro* and reduce microbial pathogenicity. Moreover, no honey-resistant microbial strains have emerged to date and this may be unlikely due to the multifactorial nature of the antimicrobial properties of honey.

However, the medicinal properties and therapeutic benefits of honey in clinical practice have not been explored to their full potential. The main factors limiting a wider therapeutic application of honey as an antimicrobial agent are mainly related with lack of standardized antibacterial activity, due to the variation in chemical composition and physical properties of honeys from different sources, and insufficient knowledge of the mechanisms underlying the antimicrobial activity.

The mechanisms that account for the antimicrobial effects of honey, although incompletely understood, are diverse and often specific for certain microorganisms. The bactericidal activity of honey, its impact on biofilm formation, quorum sensing and the expression of virulence factors turn honey into an attractive antimicrobial strategy for prophylaxis or in the treatment of chronic wound infections not responding to conventional (antibiotic) therapy.

Medical-grade manuka honey, with a broad spectrum and potent antimicrobial activity, has been gaining clinical acceptance as a topical antimicrobial agent in wound treatment, and there is a continuous effort in the search for honeys from other sources with enhanced antimicrobial properties.

Further studies on the antimicrobial activity of honey, namely the effect on microbial growth *in vivo*, are required. It would also be very useful to identify the components within honey that are responsible for each type of action. In addition, innovative research for *in vivo* animal and human studies are needed in order to clarify the multiple antimicrobial activity of honey and to overcome problems associated with *in vivo* use, leading to the production of a highly valued antimicrobial honey in clinical practice.

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