

Evaluation of antimicrobial activity of Italian honey for wound healing application in veterinary medicine

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Abstract

Honey as a topical treatment for infected wounds dates back to ancient times. However, few studies have been reported concerning the medical properties of Italian honey. In this study, the microbial contamination, the antimicrobial activity and the antibiotic residues of 6 different varieties of Piedmont honeys were evaluated. The antimicrobial activity of honeys was tested by agar well diffusion method and 1 honey for each variety has been selected and tested by broth micro-dilution test to determine Minimum Inhibitory Concentrations (MICs) and evaluated by Minimum Bactericidal Concentrations (MBCs). The honeys with a high level of antibacterial activity were analyzed for the presence of tetracyclines, sulfonamides and macrolide residues. The agar well diffusion method showed the greatest antimicrobial activity for honeydew, chestnut and lime tree honeys. The MICs and MBCs identified the close similarity to the medical manuka honey of honeydew, polyfloral and chestnut honey. The levels of antibiotic residues on these honeys were below the limit of quantification. Based on our results the Italian variety of honeydew showed the best antimicrobial activity and can be considered for the treatment of infected wounds in animals.

Keywords: antibacterial activity, Italian honey, manuka honey, MCB, MIC

Untersuchung zur antimikrobiellen Aktivität von italienischem Honig für die Wundheilung in der Veterinärmedizin

Die Anwendung von Honig zur Behandlung infizierter Wunden reicht weit zurück ins Altertum. In vorliegender Arbeit wurden die mikrobielle Verunreinigung, die antimikrobielle Aktivität und die Antibiotikarückstände von 6 verschiedenen Honigarten aus dem Piemont untersucht. Die antimikrobielle Aktivität wurde mit der Agar-Diffusionsmethode bestimmt und je eine Probe jeder Honigart mit dem Mikro-Verdünnungstest auf ihre minimale Hemmkonzentration (MIC) sowie minimale Bakterizidkonzentration (MBC) untersucht. Die Honigproben mit hoher antibakterieller Aktivität wurden auf das Vorhandensein von Tetracyklin, Sulfonamide und Makrolide analysiert. Mittels Agar-Diffusion zeigten Blatthonig sowie Honig von Kastanie und Linde die grösste antimikrobielle Aktivität. Die MIC und MBC liessen eine grosse Aehnlichkeit von Blatt-, Blüten- und Lindenhonig zum medizinisch verwendeten Manuka-Honig erkennen. Antibiotische Rückstände dieser Honigproben waren unterhalb der Bestimmungsgrenze. Aufgrund unserer Ergebnisse zeigten die verschiedenen italienischen Blatthonige die grösste antibakterielle Aktivität und können zur Behandlung infizierter Wunden bei Tieren eingesetzt werden.

Schlüsselwörter: Antimikrobielle Aktivität, Italienischer Honig, Manuka-Honig, MCB, MIC

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Introduction

The development of bacterial resistance to antibiotic therapy is understood to be a natural occurrence. Since the use of antibiotics became widespread over 50 years ago, bacteria have progressively developed resistance (Basualdo et al., 2007) and in particular multidrug-resistant strains such as *Staphylococcus aureus* methicillin-resistant (MRSA), *E. coli* extended-spectrum β -lactamase (ESBL) and *Pseudomonas aeruginosa* (Chong et al., 2011; Abdel-moein et al., 2012; Wang et al., 2012). In recent years the rise of multi-resistant bacteria has led to alternative antimicrobial strategies and the development of new compounds to be used instead of conventional antibiotic therapy. Therefore, substances such as honey, propolis, aloe vera and chitosan have been used to promote optimal healing wounds (Ammayappanand Moses 2009; Vandamme et al., 2013; Carnwath et al., 2014). In practice the use of honey-based products (e.g. solid membranes) applied directly on surgical wounds are preferred because they provide a physical barrier in the immediate post-operative period (Ward and Panitch, 2011). Therefore, the production of honey-based materials might represent a new therapeutic option.

The inhibitory effect of honey against numerous species of Gram-positive and Gram-negative bacteria, including *Staphylococcus aureus* and *Pseudomonas aeruginosa*, has been recognized since ancient times (Wang et al., 2012) as previously reported (Basualdo et al., 2007; Alandejani et al., 2009; Mohapatra et al., 2011). Moreover, honey is capable of killing bacteria even in their highly resistant biofilm state, proving to be more effective than any single commonly used antibiotic (e.g. rifampicin, cefazolin, oxacillin, gentamicin, etc.) (Wang et al., 2012). In addition, there is no evidence of bacterial resistance to honey although chemical antibacterial drugs induce microbial resistance and may have a detrimental effect on wound tissue (Cooper et al., 1991; Wilson et al., 2005). The antimicrobial activity of honey is attributed largely to osmolarity, acidity, hydrogen peroxide production, nectar, pollen and the presence of methylglyoxal (MGO) (Basualdo et al., 2007; Voidarou et al., 2011). However, the possible presence of antibiotic residues may also be taken into account as antibiotics are often used in bee-keeping as a preventive or therapeutic treatment in the protection of apiaries. Notwithstanding this, the use of antibiotics in Italy is absolutely prohibited (U.E., Reg. n. 37/2010).

The chemical composition and the related antibacterial effect of honeys vary depending on the plant source, season and production methods. The manuka honey produced in New Zealand from the manuka tree is well known for its therapeutic effect (Molan, 2002) and different studies have been performed worldwide on this

product (Lusby et al., 2005; George and Cutting, 2007). Carnwath et al. (2014) demonstrated that the use of honey for the treatment of skin wounds in animals and human is beneficial and promotes tissue healing. Interestingly, the manuka honey has been recently described as an important tool for wounds healing (Molan, and Rhodes, 2015).

Few studies have been conducted on antibacterial activity and wound-healing properties of honey produced in Europe and the Mediterranean area (Voidarou et al., 2011; Vica et al., 2014) such as Italy (Fidaleo et al., 2011; Tenore et al., 2012; Coniglio et al., 2013). In Piedmont (North West Italy), the geomorphology and the complexity of the flora and climate allow the production of a wide variety of honeys e.g. chestnut, dandelion, honeydews, lime tree and polyfloral. Thus, the aim of the present study was to characterize *in vitro* the antimicrobial activity and to evaluate the antibiotic residues of 6 different varieties of honeys produced in Piedmont in order to be applied in veterinary surgical procedures.

Materials and methods

Honey sample collection

The study was carried out on honey samples produced in Piedmont (North West Italy). The selected honeys were guaranteed as to flower-specificity and quality by experts of the Piedmont Honey Producers Association by palynological evaluation. In addition a preliminary selection on the beekeepers was done by this association that led to collect 26 unpasteurized honeys (5 chestnut honeys, 5 dandelion honeys, 5 honeydews, 4 lime tree honeys, 4 polyfloral mountain honeys and 3 rhododendron honey) derived from 9 Piedmont beekeepers. A medical grade manuka honey (Medihoney™) was used as a standard to compare our local honeys. Each sample was collected in a sterile container and kept in the dark at 4°C until analysis.

Bacterial strains and culture

Antibacterial properties of the honey samples were tested against the following strains isolated from canine wound infections: *Staphylococcus aureus* methicillin-resistant (MRSA), *S. aureus* methicillin-susceptible (MSSA), *Enterococcus faecalis*, *Escherichia coli*, *E. coli* producing extended-spectrum β -lactamase (ESBL), *Proteus mirabilis* and *Pseudomonas aeruginosa*. The honey samples were examined for the presence of bacterial contamination by culture on 5% sheep blood agar (Oxoid) overnight at 37°C.

Assessment of antibacterial activity

Undiluted honey samples were screened for their antimicrobial activity by the agar well diffusion method

(Sherlock et al., 2012). Bacterial strains were grown overnight in Trypticase Soy Broth (TSB) (Oxoid) at 37°C and adjusted to 0.5 McFarland standard. Each culture was inoculated on the surface of Petri plates. Subsequently, wells 6 mm diameter were bored into the surface of the agar. Each well was consequently filled with about 80 µl of honey sample. Plates were incubated at 37°C and after 24 h the inhibition zones were measured. Each assay was carried out in triplicate. Furthermore, a laboratory synthesized honey, which was prepared by dissolving in 10 ml of distilled water, 40% fructose, 30% glucose and 2% sucrose (Merck Millipore) was used in each experiment (Sherlock et al., 2012).

Subsequently, one sample for each variety of honey, showing the higher antimicrobial activity, was selected and tested by Minimum Inhibitory Concentrations (MICs) and Minimum Bactericidal Concentrations (MBCs) assays. MIC were determined by micro-method (Sherlock et al., 2012) and the final concentrations of honey samples used were 50%, 25%, 12.5%; 6.3%, 3.1%, 1.6% (v/v). Briefly, 10 µl of 0.5 Mac Farland standardized culture was added to 190 µl of tested diluted honey. MICs were determined by spectrophotometric assay to 600 nm. Control wells contained broth (negative or sterility control) and broth with bacteria (positive control). Plates were incubated in the dark at 37°C shaking at 150 rpm for 24 h. After 20 h of incubation at 37°C, bacterial growth inhibition was determined by monitoring the optical density (OD) which was read just prior to incubation (T0) and 24 h (T24) later at 600 nm. The OD for each replicate at T0 was subtracted from the OD for each replicate at T24. Then a value of 100% growth was assigned to the adjusted OD of the positive control. The percentage inhibition of growth was determined using the formula: $= 1 - (\text{OD test well} / \text{OD of corresponding control well}) \times 100$ (Patton et al. 2006). The MIC was reported as the lowest concentration of test material that inhibited bacterial growth. From the wells showing no visible sign of growth/turbidity in MIC determi-

nation, test bacteria were inoculated onto Trypticase Soy Agar (TSA) plates by streak plate method. The plates were then incubated at 37°C for 24 h. The smallest concentration showing no growth of test organisms was considered as the MBC. Each test was performed in triplicate.

Antibiotic residues analysis

The honeys with a high level of antibacterial activity (honeydew, polyfloral and chestnut) were analyzed by LC-MS/MS technique to detect the residue presence of the following classes of antibiotics: tetracyclines, sulfonamides and macrolide (tylosin) (Giannetti et al., 2010; Huq and Kallury, 2006).

Results

A preliminary selection on the beekeepers has been done by Agripiemonte Agripiemonte Miele that has permitted to select the 26 honeys introduced in our study.

Honey bacterial analysis

All 26 honeys were tested to evaluate the microbial contamination. Eighteen honeys resulted negative, 6 were contaminated by *Bacillus spp.* (1 chestnut honey, 2 dandelion, 1 honeydew, 1 lime tree honey, 1 polyfloral mountain honey) and 2 by coliforms (1 chestnut honey and 1 honeydew) and were further excluded.

Antibacterial activity of honey samples

The 18 honeys without bacterial contamination were screened by agar well diffusion method and the results are reported in Table 1. Greater antimicrobial activity was observed for honeydew, chestnut and lime tree honeys. In particular, honeydew produced a clearing zone comparable to manuka honey while dandelion and rododendrum honeys showed slow antibacterial activity.

Table 1: Antimicrobial activity by agar well diffusion method: diameters of zones of inhibition (including 6 mm well diameter) of 18 honey samples, manuka and a laboratory synthesized honey (LSH) against clinical strains.

Strains	Honey samples (mm of inhibition zone)																			
	Chestnut samples n.			Dandelion samples n.			Honeydews samples n.			Lime tree samples n.			Polyfloral samples n.			Rhododendron samples n.			Manuka	LSH
	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	1
<i>S. aureus</i> MSSA	25	21	20	–	10	10	31	21	20	17	28	19	22	17	21	13	13	12	28	11
<i>S. aureus</i> MRSA	20	19	20	–	–	8	20	21	20	14	21	14	15	14	15	12	11	10	20	–
<i>E. fecalis</i>	12	8	10	–	–	–	13	13	13	–	8	–	–	–	8	8	–	–	13	–
<i>E. coli</i>	12	11	13	–	10	9	16	14	14	14	13	13	12	13	16	10	9	10	13	8
<i>E. coli</i> ESBL	10	–	8	–	–	8	12	12	12	8	10	–	–	8	–	9	–	9	12	–
<i>P. mirabilis</i>	13	15	14	–	–	–	16	14	16	13	15	10	14	10	14	–	–	–	17	–
<i>P. aeruginosa</i>	15	11	–	–	–	–	8	10	8	–	–	–	10	–	14	–	–	–	14	–

Where no value is given (–), no zone of inhibition was observed

Table 2: Antimicrobial activity by micromethod assay: Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) assay of 6 honeys, manuka and laboratory synthesized honey (LSH) against clinical strains.

Strains	Honey samples (% of inhibition)															
	Chestnut n. 1		Dandelium n. 3		Honeydews n. 1		Lime tree n. 2		Polyfloral n. 3		Rhododendron n. 1		Manuka		LSH	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
<i>S. aureus</i> MSSA	12.5	12.5	25	50	3.1	3.1	25	25	12.5	12.5	25	50	3.1	3.1	50	50
<i>S. aureus</i> MRSA	25	25	25	25	25	25	25	25	12.5	25	25	25	12.5	12.5	50	50
<i>E. fecalis</i>	25	25	50	50	25	25	50	50	25	25	25	50	25	25	50	50
<i>E. coli</i>	25	25	25	50	12.5	12.5	25	25	25	25	25	50	12.5	12.5	50	50
<i>E. coli</i> ESBL	25	25	25	50	25	25	25	25	25	25	25	50	12.5	12.5	50	50
<i>P. mirabilis</i>	25	25	50	50	25	25	25	25	25	25	50	50	25	25	50	50
<i>P. aeruginosa</i>	25	50	50	50	25	50	50	50	25	50	50	50	25	25	50	50

One honey for each of the 6 different varieties included in this study was submitted to MICs and MBCs analysis and the results were showed in Table 2. Honeydew, polyfloral and chestnut honeys maintained antibacterial activity at a concentration of 25% and below. In particular, honeydew and polyfloral had a greater antimicrobial activity, more similar to manuka honey. In general, lime tree, rhododendron and dandelion produced a less antibacterial effect compared to the other tested honeys. The laboratory synthesized honey showed low antimicrobial activity in both considered assays.

Antibiotic residues

No antibiotic residues were detected in any of the investigated honeys (honeydew, polyfloral and chestnut honeys). Indeed, the results were below the limit of quantification (LOQ) corresponding to 0.005 mg/kg.

Discussion

Several studies all over the world have reported the emergence of multi-resistant bacteria in human and veterinary medicine. For this reason, honey was repurposed for wound care since it offers a good alternative to conventional antimicrobial drugs (Basualdo et al., 2007; George and Cutting, 2007; Mohapatra et al., 2011; Vica et al., 2014). In this study, 26 Italian honeys of 6 different varieties were characterized to evaluate the microbial contamination, the antimicrobial properties and the levels of antibiotic residues. A medical honey should be free from microbial contamination. Despite a strict beekeepers selection was done, 8 out of 26 tested honeys were contaminated by bacteria. Nevertheless the results may be considered encouraging since good antimicrobial activity against bacteria isolated from dog wounds was found in honeydew, chestnut and polyfloral honeys. Great variability in the antimicrobial activity of tested honeys was observed, probably due to the different bacterial susceptibility and to the flower source of honeys.

The honeydew, chestnut and polyfloral honeys presented a high bacteriostatic and bactericidal activity on Gram-positive bacteria, comparable to manuka honey. Moreover a interesting antimicrobial activity against *S. aureus* was demonstrated as reported by other authors (Basualdo et al., 2007; Vica et al., 2014), and good results were also obtained against the MRSA strain. These data are important, since various strains have already developed multi-resistance and are the major pathogens in surgical wound infections (Cruz et al., 2005). In the light of this problem, new therapeutic strategies for the treatment of infected wounds are necessary. The use of honey against bacterial infections will allow greater cost saving and greater product availability without toxic effects. Moreover, chestnut, honeydew and polyfloral honeys showed the same antimicrobial activity of manuka honey against *E. fecalis*, which is in contrast to recent studies reporting that *E. fecalis* is not inhibited by honey (Basualdo et al., 2007; Carnwath et al., 2014).

As to Gram-negative bacteria, all honey samples demonstrated good capability to inhibit the growth of both *E. coli* strains in accordance with earlier researches (Mohapatra et al., 2011; Carnwath et al. 2014). In particular honeydew showed the highest antimicrobial activity against *E. coli* ESBL. These data are in accordance with a recent Italian study (Coniglio et al., 2013). The antimicrobial activities of tested honeys were less effective against *P. aeruginosa* and *P. mirabilis* than *E. coli*. In particular, the antibacterial efficacies of honeys against *P. aeruginosa* were mainly bacteriostatic as reported previously (Basualdo et al., 2007; George and Cutting, 2007).

Among the 26 tested honeys the honeydew showed the highest microbiological activity, quite similar to the medical manuka honey results. Honeydew is a particular type of honey produced by Honeybees (*Apis mellifera*) from byproducts of *Metcalfa pruinosa* (Alma, 2000; Wilson and Lucchi, 1997 and 2000; Lucchi and Mazzoni, 2004). This characteristic, together with the large diversity of geobotanical conditions and floral species

present in Italy, contributed to produce the particular composition of honeydew (Marcazzan and Sabatini, 2008), which explains its antibacterial activity compared to other tested honeys. Our data are supported by a recent report (Majtan et al., 2011) that documented the exceptional antibacterial activity of honeydew against multi-resistant *Stenotrophomonas maltophilia*.

Interestingly, this is the first study that has measured the antibiotic residue in the tested honeys. The data are interesting since the levels of antibiotic residues were below LOQ, supporting the good beekeeping production and the effective antibacterial activity of the Piedmont honeys.

In conclusion, we demonstrated that honeydew and other local honeys of North West Italy, possess a high antibacterial activity, comparable to manuka honey without the presence of antibiotic residues. Therefore, they may be considered for the treatment of infected wounds to reduce the actual problems of antibiotics resistance. Further experiments will be necessary to ana-

lyze the chemical composition and to assess the application of honeydew honey and honeydew-based materials in surgical wounds.

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Conflict of interest

None of the authors have any financial or personal relationships that could inappropriately influence or bias the content of the paper.

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Etude de l’activité antimicrobienne de miels italiens sur la guérison des plaies en médecine vétérinaire

L’utilisation du miel pour le traitement des plaies infectées remonte à loin dans l’antiquité. Dans le présent travail, on étudie les contaminations microbiennes, l’activité antimicrobienne et les résidus d’antibiotiques dans 6 sortes de miels différentes provenant du Piémont. L’activité antimicrobienne a été mesurée au moyen d’une méthode de diffusion sur gel d’agar et un échantillon de chaque sorte de miel a été examiné quant à sa concentration minimale inhibitrice (CMI) et sa concentration minimale bactéricide (CMB) au moyen d’un test de micro-dilution. Les échantillons présentant une haute activité antibactérienne ont été analysés quant à la présence de tétracycline, de sulfamidés et de macrolides. Au test de diffusion sur agar, le miel de miellat ainsi que ceux de châtaignier et de tilleul ont démontré la plus grande activité antimicrobienne. Les CIM et CBM permettent de reconnaître une grande similitude entre les miels de miellat, de nectar et de tilleul avec le miel de Manuka utilisé à des fins thérapeutiques. Les résidus d’antibiotiques de ces échantillons se situaient en dessous des limites de détection. Sur la base de ces constatations, les divers miels de miellat italiens présentent la plus grande activité antimicrobienne et peuvent être utilisés pour le traitement de plaies infectées chez les animaux.

Studio sull’attività antimicrobica del miele italiano nella guarigione delle ferite in medicina veterinaria

L’uso del miele per il trattamento di ferite infette era già conosciuto nell’antichità. In questo lavoro sono stati analizzate la contaminazione microbica, l’attività antimicrobica e i residui di antibiotici da 6 diversi tipi di miele piemontese. L’attività antimicrobica è stata studiata con il metodo di diffusione su agar e ogni campione di tipo di miele con il test di micro-diluizione sulla loro minima concentrazione inibente (MIC) e sulla loro concentrazione minima battericida (MBC). I campioni di miele con elevata attività antibatterica sono stati analizzati sulla presenza di tetraciclina, sulfamidici e macrolidi. Il miele di castagno e quello di tiglio hanno dimostrato la maggiore attività antimicrobica, grazie al metodo di diffusione su agar. La MIC e la MBC hanno riconosciuto una grande somiglianza del miele di foglie, fiori e di tiglio con il miele di Manuka usato in medicina. I residui di antibiotici di questi campioni di miele erano al di sotto del limite di rilevazione. Sulla base dei nostri risultati, i vari miels di foglie provenienti dall’Italia hanno dimostrato la maggiore attività antibatterica e possono essere usati per trattare ferite infette negli animali.

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Evaluation of antimicrobial activity of Italian honey for wound healing application in veterinary medicine

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