Antimicrobial activity of a standardized medical honey on bacterial isolates from infected skin lesions of non-traditional companion animals

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Wound.

Summary
In recent years, due to the growing phenomenon of antimicrobial resistance, the search for alternative strategies to antibiotic treatments is increasing and a considerable interest for the use of medical honey in clinical practice has emerged. Honey has been used for the treatment of skin lesions, in both humans and animals. However, knowledge concerning the use of medical honey in non-traditional companion animals is scarce. The aim of this study was to assess the antibacterial activity of a standardized medical honey (Revamil, BFactory) against bacterial strains isolated from skin lesions of non-traditional companion animals. The minimum bactericidal concentration (MBC) of Revamil honey against seventeen clinical isolates and three reference strains was established. The medical honey showed antimicrobial activity against both Gram-positive and Gram-negative bacteria. Growth was inhibited for all the strains at concentrations of medical honey ranging from 10 to 40%. *Pseudomonas oryzihabitans* and *Alcaligenes faecalis* showed the lowest MBC (10%). The reference strain *Staphylococcus aureus* ATCC25923 showed a higher sensitivity to 20% honey compare to the corresponding clinical isolate (P = 0.001). The observed results suggest that Revamil could represent an effective therapeutic aid, useful for the reduction of antibiotic use, in case of pathological skin infections in non-traditional companion animals.

Introduction

The loss of integrity of the skin barrier, caused by mechanical, thermal or chemical injuries, facilitates bacterial contamination of the underlying tissues, which can lead to wound colonization or, at worst, to invasive infection. Complications related to bacterial contamination of the wound and bacterial interactions with the damaged tissues can cause impaired wound healing. Non-healing wounds frequently show pathologic inflammation and even suppurative discharge (Guo and DiPietro 2010, Rosique *et al.* 2015). This what normally occurs in all vertebrate classes including reptiles. The methods used to house captive reptiles generally predispose these animals to a variety of opportunistic microbial pathogens and reptile wounds are frequently contaminated with both Gram-positive and Gram-negative bacteria (Mitchell *et al.* 2004). Therefore, broad-spectrum antibiotic therapy may be required to control microbial populations contaminating the wounds (Bowler *et al.* 2001). However, antibiotic resistance is considered one of the most serious public health problems of our century and the growing antibiotic resistance in veterinary medicine is a current threat to human health (Prestinaci *et al.* 2015, Tang *et al.* 2017). According to the international guidelines on the prudent use of antimicrobials in veterinary medicine[1], honey and other alternative therapies were used for the treatment of skin lesions, in both humans and animals (Bowler *et al.* 2001, Carnwath *et al.* 2014, Di Ianni *et al.* 2015b, Olofsson *et al.* 2016, Pelizzone *et al.* 2014, Subrahmanyam 1991). However, knowledge concerning the use of medical honey in non-traditional companion animals is lacking. Honey is produced by honey bees using the nectar of flowers or honeydew and is mostly composed

[1] Commission Notice, Guidelines for the prudent use of antimicrobials in veterinary medicine (2015/C299/04), Official Journal of the European Union, 11/09/2015 C299, S. 7.

of glucose and fructose. It also contains vitamins, minerals, amino acids, enzymes, organic acids and other compounds. The beneficial properties of honey were known since ancient times (Molan and Rhodes 2015) and its therapeutic use remained popular until the advent of antibiotics (Langemo *et al.* 2009). The antibacterial activity of honey was reported in numerous studies (Basualdo *et al.* 2007, Mandal and Mandal 2011, Subrahmanyam 1991, Vandamme *et al.* 2013). Honey exerts bacteriostatic and bactericidal activities (Vandamme *et al.* 2013). Many enzymes are present in an internal pouch of the bee called "crop" and are added to honey. The glucose oxidase catalyzed the glucose oxidation to form gluconic acid and hydrogen peroxide. Gluconic acid lowers the pH and the hydrogen peroxide boosts the bactericidal action (Minden-Birkenmaier and Bowlin 2018, Molan and Rhodes 2015). The lowering of pH at 3.5-4 causes a series of events essential to the process of tissue repairing: reduction in protease activity in the wound site, increasing of oxygen release from hemoglobin and stimulation of fibroblast and macrophage activity. Furthermore, the hydrogen peroxide stimulates the production of the vascular endothelial growth factor (VEGF) and sterilize the wound site (Minden-Birkenmaier and Bowlin 2018, Molan and Rhodes 2015). In addition to glucose oxidase, the invertase produced by the bee increases the strength of the osmotic potential of the honey dividing sucrose into fructose and glucose (Minden-Birkenmaier and Bowlin 2018, Molan and Rhodes 2015). Fluids into the wound are drawn out of damaged tissues leading to drying of cellular tissues and bacterial death (Molan and Rhodes 2015). In addition, phenolic compounds, organic acids, vitamins and flavonoids exert antioxidant activities and boost the antimicrobial effect of the honey. Flavonoids neutralize free radicals produced by the hydrogen peroxide (Minden-Birkenmaier and Bowlin 2018, Molan and Rhodes 2015). However, despite the increase of studies on the use of honey for the wound healing of either traumatic or surgical origin, only a few studies on its use on infected wounds were done. Some authors analyzed the effect of the honey on the growth of selected intestinal bacteria (Shin and Ustunol 2005) and against pathologic bacteria frequently isolated from skin wounds of mammals, including humans (Basualdo *et al.* 2007).

The number of pet reptiles or other non-traditional companion animals is steadily increasing, leading to greater scientific interest in the medical and reproductive aspects of these animals (Bertocchi *et al.* 2018, Di Ianni *et al.* 2014, Di Ianni *et al.* 2015a, Taddei *et al.* 2010). In reptiles, bacteria can cause skin diseases, secondary to traumatic wounds or management errors (Mitchell *et al.* 2004) and infected wounds that are not promptly treated may rapidly evolve causing sepsis or septic shock. The systemic and multi-organ involvement may result in death (Harkewicz 2001). The aim of this study was to assess the antibacterial activity of a standardized medical honey (Revamil, BFactory) against bacterial strains isolated from skin lesions of pet reptiles and other non-traditional companion animals.

Materials and methods

Tested product, bacterial strains and reagents

The tested product was a standardized medical honey in gel formulation (Revamil, BFactory), consisting of glucose oxidase (GOX) positive 100% pure honey.

Samples for bacterial isolation were collected by swabs from infected skin lesions of 17 captive animals, brought to the Veterinary Teaching Hospital of the Department of Veterinary Science of the University of Parma to be treated for different injuries. The animals were kept as pets and sample collection was part of the normal diagnostic process. The swabs were immediately plated onto tryptose agar (Oxoid) containing 5% of bovine erythrocytes and MacConkey agar (Difco) and incubated aerobically for 24 hours at 37 °C. Identification of bacterial isolates was based on their growth and colony characteristics, Gram staining, cellular morphology, catalase and oxidase reactions. Species identification was carried out using API biochemical test systems (bioMérieux), as well as conventional biochemical tests (Quinn *et al.* 1994). Clinical bacterial strains are reported in Table I.

Three bacterial reference strains, *Staphylococcus aureus* ATCC25923, *Escherichia coli* ATCC25922 and *Pseudomonas aeruginosa* ATCC27853, were also evaluated.

The following reagents were used for the antimicrobial activity evaluation: Bacto Agar (Becton Dickinson, Sparks, USA), Bacto Brain Heart Infusion (BHI) broth (Becton Dickinson, Sparks, USA), Mueller Hinton (MH) broth (Becton Dickinson, Sparks, USA), Phosphate Buffer (PB). Agarized medium for colony-forming unit (CFU) counts was prepared by the addition of 1.5% of Bacto Agar (w/v) to MH broth. Sterility control was performed for all the prepared media by incubation for 24 hours at 37 °C in air.

Evaluation of the minimum bactericidal concentration (MBC)

The standardized medical honey was dissolved in PB at a 50% (v/v) concentration by stirring with a magnetic stir bar at room temperature. Eighty microliters of the 50% emulsion were serially

Table I. *Clinical bacterial isolates.*

Sample	Origin	Species	Lesion	Bacterial isolate	API® identification
S1	Turtle	*Trachemis scripta*	Skin wound	*Morganella morganii*	API 20 E 0174000
S2	Turtle	*Testudo hermanni*	Skin wound	*Klebsiella oxytoca*	API 20 E 5245773
S3	Turtle	*Testudo hermanni*	Skin wound	*Pseudomonas oryzihabitans*	API 20 E 0200000
S6	Rat	*Rattus norvegicus*	Skin abscess	*Staphylococcus aureus*	API Staph 6736353
S7	Turtle	*Trachemis scripta*	Skin wound	*Staphylococcus xylosus*	API Staph 6736452
S8	Snake	*Python regius*	Infected skin burns	*Micrococcus* spp.	API Staph 0006000
S9a	Snake	*Python regius*	Stomatitis	*Pseudomonas aeruginosa*	API 20 NE 1154575
S9b	Snake	*Python regius*	Stomatitis	*Klebsiella oxytoca*	API 20 E 5255773
S10	Snake	*Python regius*	Necrotic stomatitis	*Stenotrophomonas maltophilia*	API 20 E 5202000
S11	Turtle	*Testudo hermanni*	Skin wound	*Staphylococcus auricularis*	API Staph 6300000
S12/1	Duck	*Anas platyrhynchos*	Skin wound	*Pseudomonas aeruginosa*	API 20 NE 0154575
S12/2	Duck	*Anas platyrhynchos*	Skin wound	*Escherichia coli*	API 20 E 5144572
S13/2	Snake	*Etherodon nasicus*	Skin abscess	*Alcaligenes faecalis*	API 20 NE 0000057
S14/1	Snake	*Epicrates cenchria*	Skin wound	*Citrobacter braakii*	API 20 E 3644553
S14/2	Snake	*Epicrates cenchria*	Skin wound	*Pseudomonas aeruginosa*	API 20 NE 0154575
S15	Snake	*Epicrates cenchria*	Skin wound	*Pseudomonas aeruginosa*	API 20 NE 0554575
S16	Snake	*Python regius*	Skin wound	*Pseudomonas aeruginosa*	API 20 NE 0554575

diluted in a microtiter plate to obtain the following concentrations of standardized medical honey in PB: 50%, 25%, 12.5%, 6.25% and 3.125%.

For each bacterial strain, three to five colonies from fresh agar plates were inoculated into tubes containing BHI broth. Tubes were briefly vortexed using a vortex mixer and incubated at 37 °C in a shaker at 225 revolution per minute (r.p.m.) for 3-4 hours to reach the log-growing phase. Bacterial suspension was centrifuged at 1,000 g for 20 min and gently resuspended in PB. Bacterial suspension was adjusted spectrophotometrically at 600 nm with 1 cm path length to an optical density value in the range 0.08-0.13, containing approximately 10^8 CFU/ml in PB. The bacterial suspension was further diluted to reach a bacterial concentration of $2.5x10^6$ CFU/ml. Twenty microliters of this bacterial suspension were inoculated into wells containing 80 µl of PB at increasing concentrations of standardized medical honey and into control wells. Final concentrations of standardized medical honey were therefore as follow: 40%, 20%, 10%, 5% and 2.5%. Final bacterial concentration was 5×10^5 CFU/ml. Only for Gram-positive strains, 2% of MH broth was present in the final suspension to allow bacterial growth. Conversely, for Gram-negative strains no addition was required. Inoculated wells were incubated in air at 37 °C for 24 hours. After incubation, 20 microliters from each tube were serially diluted and plated onto agarized Mueller Hinton, to perform the CFU count. For each strain and for each standardized medical honey concentration, three independent experiments, each with three replicates, were performed. Growth controls were performed by testing the bacterial strains with the same procedure as above, but in absence of standardized medical honey. Agarized Mueller Hinton plates were read by counting the number of CFU after 24 hours of incubation in air at 37 °C. The test was considered valid when no contaminant growth was present in sterility controls and growth (CFU count of at least 400 CFU/ml) was visible onto growth control Mueller Hinton plates. The MBC was defined as the lowest concentration of standardized medical honey at which there is no growth of the organism.

Statistical analysis

Differences between treatments were analyzed by heteroscedastic one-way ANOVA. Homogeneity of variances was assessed by Levene's test. Multiple comparisons were performed by Games-Howell test. Comparisons between the different strains of the same bacterial species at each concentration of honey were performed by T test. Differences at $P < 0.05$ were considered statistically significant. Statistical analysis was performed by SPSS version 26 software (IBM).

Results

All replicates of each bacterial strain showed reproducibility of results. MBC values were expressed as percent concentration of standardized medical honey and are reported in Table II.

For each bacterial strain, logarithmic (Log) reduction of CFU/ml as a function of medical honey concentration, compared to growth in the absence of honey, was evaluated and reported in Figures 1 and 2. At honey concentrations ranging from 10 to 40%, depending on the bacterial strain, growth was inhibited for all the strains (Figures 1 and 2). Among the tested bacterial strains, those which showed the lowest MBC (10%) were the clinical isolates *Pseudomonas oryzihabitans* and *Alcaligenes faecalis* (Figure 2B and 2H, Table II). Reference strains showed a pattern of sensitivity to the presence of medical honey similar to those of clinical isolates of the same bacterial species, especially at low concentrations (Figures 1A and 2A, C, D). However, *S. aureus* ATCC25923 and *E. coli* ATCC25922 showed a lower MBC compared to the corresponding clinical isolates (Figures 1A and 2C, Table II), the difference was statistically highly significant for *S. aureus* at 20% Revamil (P=0.001). Moreover, several statistically significant differences between the different *P. aeruginosa* strains for all the concentrations of honey in the range 0-10% were found (with P values of significant differences ranging from < 0.001 to 0.049). Therefore, the considered standardized medical honey was able to completely inhibit bacterial growth of all the tested strains at the concentration of 40%. Some strains were completely inhibited also in presence of a lower concentration of medical honey (20%), notably *S. aureus* ATCC25923, *Micrococcus* spp., *Staphylococcus auricularis, E. coli* ATCC25922, all the *P. aeruginosa* tested strains, *Morganella morganii* and *Citrobacter braakii*. Finally, *P. oryzihabitans* and *A. faecalis* were even more sensitive to honey, since their growth was completely inhibited by the concentration of 10%.

Table II. *Minimum bactericidal concentration results.*

		MBC value
Reference strains	*Staphylococcus aureus* ATCC25923	20%
	Escherichia coli ATCC25922	20%
	Pseudomonas aeruginosa ATCC27853	20%
Gram-positive isolates	S6 - *Staphylococcus aureus*	40%
	S7 - *Staphylococcus xylosus*	40%
	S8 - *Micrococcus* spp.	20%
	S11 - *Staphylococcus auricularis*	20%
Gram-negative isolates	S1 - *Morganella morganii*	20%
	S2 - *Klebsiella oxytoca*	40%
	S3 - *Pseudomonas oryzihabitans*	10%
	S9a - *Pseudomonas aeruginosa*	20%
	S9b - *Klebsiella oxytoca*	40%
	S10 - *Stenotrophomonas maltophilia*	40%
	S12/1 - *Pseudomonas aeruginosa*	20%
	S12/2 - *Escherichia coli*	40%
	S13/2 - *Alcaligenes faecalis*	10%
	S14/1 - *Citrobacter braakii*	20%
	S14/2 - *Pseudomonas aeruginosa*	20%
	S15 - *Pseudomonas aeruginosa*	20%
	S16 - *Pseudomonas aeruginosa*	20%

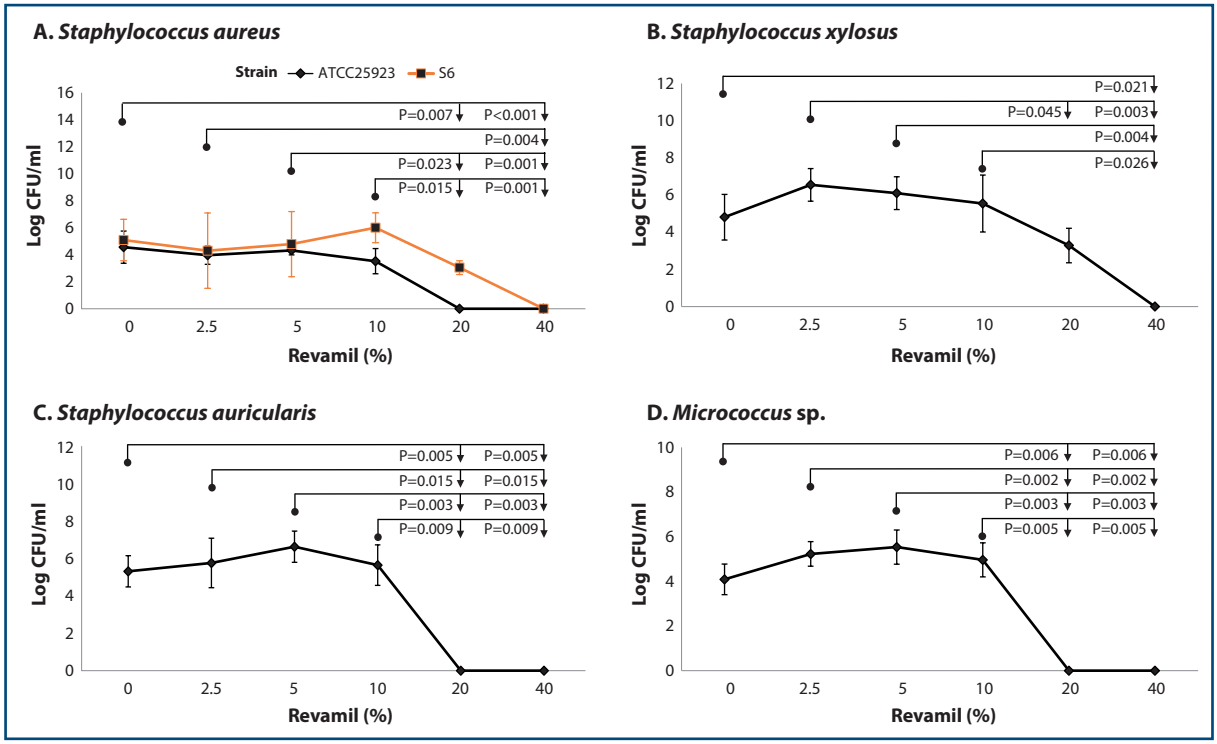

Figure 1. *Concentration-dependent inhibition of Gram-positive bacteria by medical honey.* The experiments were performed at least in triplicate and the error bars indicate ± 1 standard deviation. Statistically significant differences between treatments and P values are showed on graph.

Discussion

The development of antibiotic resistance in bacteria is a global emergency and infections caused by resistant bacteria are increasingly common in many different animal species (Sørum and Sunde 2001, Szmolka and Nagy 2013). Together with the search for more effective antimicrobials, increasing efforts to develop alternative therapies could help in reducing the use of antibiotics and limiting the spread of antibiotic resistance. Alternative therapies may find useful application especially in mild infections. The antibacterial properties of honey have long been known (Vandamme *et al.* 2013). Honey is widely used in human medicine for the management of acute, chronic, traumatic and post-surgical wounds (Ahmed *et al.* 2003), but

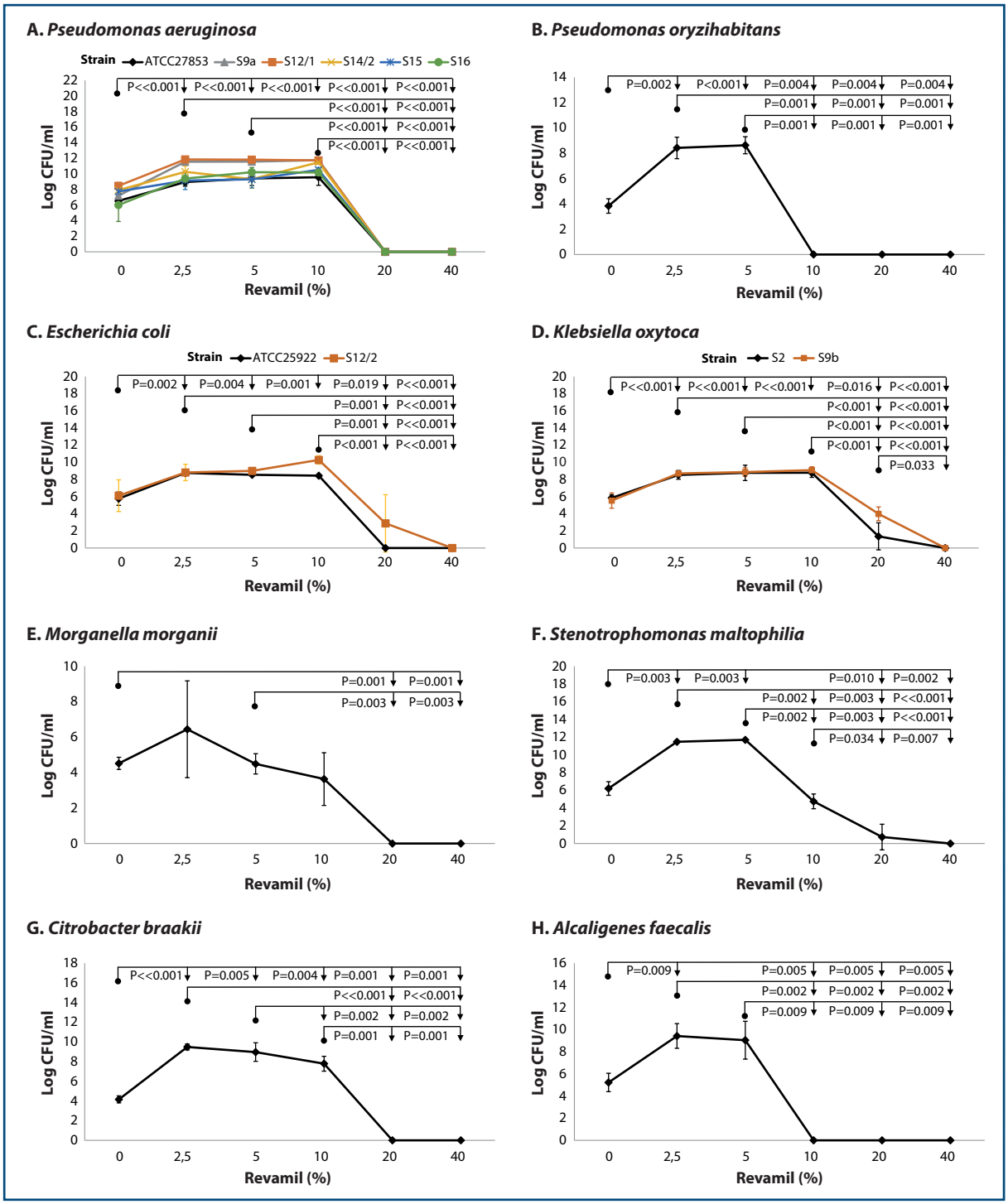

Figure 2. *Concentration-dependent inhibition of Gram-negative bacteria by medical honey.* The experiments were performed at least in triplicate and the error bars indicate ± 1 standard deviation. Statistically significant differences between treatments and P values are showed on graph.

also for ulcers, burns, eye diseases, skin diseases, oral mucosa problems, necrotic areas (Al-Waili 2004, Bardy *et al.* 2008, Biswal *et al.* 2003, Molan and Rhodes 2015, Subrahmanyam 1991). Moreover, cases of positive therapeutic response to honey in patients unresponsive to traditional treatments were reported (Bardy *et al.* 2008, Dunford and Hanano 2004, Efem 1988, Schumacher 2004). Regarding veterinary medicine, the effectiveness of different types of honey in the treatment of equine infected wounds was reported (Carnwath *et al.* 2014). However, to our knowledge no data are available on regarding non-traditional pets. With this study, we assessed the antimicrobial activity of a standardized medical honey against bacteria isolated from non-traditional companion animals, mostly reptiles. Moreover, three reference bacterial strains, belonging to the most representative species among the isolates, were tested. All bacterial strains were completely inhibited at honey concentrations between 10% and 40%, depending on the strain (Figure 1 and 2). Considering the *S. aureus* strains, our results agree with the literature (Almasaudi *et al.* 2017, Cooper *et al.* 1999, Cooper *et al.* 2002, Lu *et al.* 2014). Lu and colleagues (Lu *et al.* 2014) have showed an important inhibition by honey on the formation of *S. aureus* biofilm. Other authors have found a growth inhibition of both methicillin-sensitive *S. aureus* (MSSA) and methicillin-resistant *S. aureus* (MRSA) (Almasaudi *et al.* 2017, Cooper *et al.* 1999, Cooper *et al.* 2002). Moreover, the bactericidal activity of medical honey against some important resistant bacteria, such as MRSA, can be increased by the addition of a synthetic bactericidal peptide (Kwakman *et al.* 2011). Furthermore, some authors showed an inhibitory activity sustained by honey against *Streptococcus pyogenes, Streptococcus mutans, Proteus mirabilis, P. aeruginosa, Enterococcus faecium* and *Enterobacter cloacae* (Kwakman *et al.* 2008, Kwakman *et al.* 2010, Majtan *et al.* 2014). In general, as reported by Almasaudi and colleagues (Almasaudi *et al.* 2017), the antibacterial activity of medical honey was found both against Gram-positive (*S. aureus, Bacillus subtilis, Bacillus cereus, Enterococcus faecalis, Micrococcus luteus*) and Gram-negative (*E. coli, P. aeruginosa*, and *Salmonella* Typhi) bacteria (Gupta *et al.* 1993, Jeddar *et al.* 1985, Mohapatra *et al.* 2011). This study confirmed what found by other authors. A similar pattern of sensitivity to low honey concentrations was found between reference strains and clinical isolates of the same species. Significant differences , however, were observed between *P. aeruginosa* strains in absence of honey or at low honey concentrations. In particular, statistically significant differences between *P. aeruginosa* ATCC27853 and some of the other strains were found at concentration 0% and 2.5% of Revamil only. This could be due to differences in the bacterial concentration of the inoculum. Anyway, the bactericidal activity of Revamil was similar for all the strains of *P. aeruginosa* (MBC = 20%). The MBCs showed by the reference strains of *S. aureus* and *E. coli* were lower than those of the corresponding clinical isolates, although only for *S. aureus* the difference was statistically significant. This is in agreement with what reported by Voidarou and colleagues (Voidarou *et al.* 2011), who found a higher resistance of clinical isolates of *S. aureus, E. coli, Salmonella* Typhimurium, *Streptococcus pyogenes, Bacillus cereus* and *Bacillus subtilis* compared to their corresponding reference strains. In general, medical honey acts primarily as a hyperosmolar medium, but it also represents an important physical barrier because of its considerable viscosity. Its immunomodulatory effects together with the anti-inflammatory and antioxidant properties of its components improve wound healing (Majtan 2014). Moreover, the high content of nutrients promotes epithelialization and angiogenesis (Molan 2001). In particular, an important source of nutrients for the tissues is represented by the presence of carbohydrates, mostly glucose and fructose, with maltose, sucrose and isomaltose in smaller quantities. Carbohydrates represent about 80% of the honey components (Carnwath *et al.* 2014, Cavanagh *et al.* 1970, Cooper *et al.* 2002, Minden-Birkenmaier and Bowlin 2018). The rapid bactericidal activity of Revamil honey is primarily linked to the presence of Bee defensin-1 and the GOX enzima. This enzyme turns the honey sugar into gluconic acid and 3‰ hydrogen peroxide, effective against bacteria but not harmful to tissues (Kwakman *et al.* 2010). In conclusion, our result regarding antimicrobial activity of Revamil honey suggest that it could represent an effective therapeutic aid, useful for the reduction of antibiotic use, in case of pathological skin infections in non-traditional companion animals.

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