Antimicrobial activity of fifteen Italian honeys against Paenibacillus larvae ATCC 9545

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ABSTRACT. Currently, American Foulbrood (AFB) represents one of the most important problems for beekeepers, due to economic losses and to the absence of an effective therapeutic treatment. The aim of this work was to characterize fifteen Italian honeys in order to assess their inhibitory activity against *Paenibacillus larvae* ATCC 9545. Each honey was analyzed for the activity of the following enzymes: glucose oxidase and catalase. Moreover, melissopalynological analysis and other biochemical parameters, namely gluconic acid, total phenolic and total flavonoid contents were determined. For each honey, the Minimum Inhibitory Concentration (M.I.C.) and the Minimum Bactericidal Concentration (M.B.C.) against *P. larvae* were determined.

All tested honey samples had an inhibitory activity on *P. larvae*. In particular, the lowest M.I.C. and M.B.C. values (53.8 mg/mL and 107.5 mg/mL, respectively) were recorded for an *Arbutus* honey sample. *Arbutus* honeys also had the highest gluconic acid and total phenolic contents (12.6 ± 1.7 g/kg and 243.2 ± 25.1 mg/kg, respectively) and the highest glucose oxidase activity (13.0 ± 1.9 nM H2O2/min). Dark honeys, including *Arbutus*, seem to have a higher gluconic acid content and a higher antimicrobial activity. Thus, honey characterization, including colour and physico-chemical characteristics (e.g. gluconic acid concentration, total phenolic and total flavonoid contents, glucose oxidase activity), could be crucial for the assessment of its employment against *P. larvae*.

Keywords: American Foulbrood; antimicrobial activity; honey; *Paenibacillus larvae*, *Arbutus* honey

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1. INTRODUCTION

Honeybees gather the nectar or the secretions from plants or the excretions of insects and enrich them with their own substances producing honey (Libonatti et al., 2014). Honey is composed of 80-85% carbohydrates, 15-17% water, 0.3% proteins, 0.2% ashes, and low quantities of aminoacids and vitamins, as well as other minor components (Cantarelli et al., 2008). Honey contains several organic acids (Suárez-Luque et al., 2002; Cherchi et al., 1994; Nelson and Mottern, 1931), among which gluconic acid is the most represented, although in variable percentage among different honeys (Cherchi et al., 1994; Stinson et al., 1960). Furthermore, honey contains some enzymes, mainly glucose oxidase, diastase, invertase and catalase (White and Doner, 1980).

Honey is known to be effective against bacteria, which cause life-threatening infections in humans (Mandal and Mandal, 2011). The high sugar concentration, the presence of hydrogen peroxide and the low pH value represent honey's main antibacterial factors, although other antibacterial compounds, such as methylglyoxal and Defensin 1, have been identified in some honey types (Kwakman and Zaat, 2012). Two important classes of non-peroxide antimicrobial compounds present in honey are flavonoids (e.g. pinocembrin, pinobanksin and chrysin) and phenolic acids (e.g. caffeic acid and ferulic acid) (Wahdan, 1998).

Fidaleo et al. (2011) testing Italian honey against human pathogenic bacteria, showed that Gram-positive bacteria were more susceptible to the inhibitory action of honey. The same Authors showed that many Italian honeys have an antimicrobial activity against meticillin-resistant *Staphylococcus aureus* and *Staphylococcus epidermidis,* two of the most common pathogens associated with nosocomial infections. Taormina et al. (2001) showed that dark-colored honeys generally have a higher inhibitory activity against foodborne pathogens compared to light-colored honeys and also have a higher antioxidant activity.

Regarding honeybees (*Apis mellifera* L.) pathogens, *Paenibacillus larvae* is the etiological agent of American foulbrood disease (AFB), the most devastating bacterial disease affecting honeybees worldwide (De Graaf et al., 2006). *P. larvae* is a Gram-positive, spore-forming, facultative anaerobic, catalase-negative microorganism (Peters et al., 2006).

P. larvae is transmitted by spores-containing honey and royal jelly and it is infectious only for larvae (Genersch, 2010; Spivak and Reuter, 2001). Spore transmission is possible in daughter swarms (Fries et al., 2006) and among apiaries (Lindström et al., 2008). Colonies selected for their hygienic behaviours showed a higher resistance to AFB (Spivak and Reuter, 2001). Currently, an effective treatment against this pathology is not available and the authorities consider the destruction of infected colonies with fire as the only effective control measure (Williams, 2000). In order to identify alternative methods to contrast *P. larvae*, Audisio et al. (2011) isolated lactic acid bacteria from *A. mellifera* bee gut and tested their inhibitory activity against this pathogen. Nafea et al. (2014), testing four Egyptian honeys, showed that honey has inhibitory properties against *P. larvae*. Furthermore, Erler et al. (2014), using three types of honey stores against some bacterial pathogens, including *P. larvae*, associated the honey inhibitory property to the colony ability of being highly adaptive in its "social immunity" against different pathogens.

This work aimed to characterize some Italian honeys and investigate their inhibitory properties against the vegetative form of *P. larvae* ATCC 9545, in order to evaluate if the inhibitory capacity varied for each honey and if some compound of honey could be associated to the inhibition.

2. MATERIAL AND METHODS

2.1 Honey samples

Fifteen different Italian honeys, identified with letters from A to O, were used in this study (Table 1). Among these, thirteen were produced in Tuscany, one in Sicily (G) and one in Sardinia (J). Honey C was the only one from honeydew. Honey B was crystallized in the jar, while all other honeys were liquid. When the analyses were performed, all honey samples were about 2 years old, assuming that beekeepers would preferably use the oldest products

Table 1. Identification of honey samples, their commercial name and division in honey colour.

Samples	Commercial name of honey samples	Honey colour	Characteristic pollen grain percentage
A	*Robinia* honey	Light (20 mm Pfund)	*Robinia* 18%
B	Multifloral honey	Light (70 mm Pfund)	Compositae H 32%
C	Honey from honeydew of *Metcalfa pruinosa*	Dark (> 100 mm Pfund)	Prevalence of honeydew elements
D	Multifloral honey	Dark (90 mm Pfund)	*Tamarix* 29%
E	*Arbutus* honey*	Dark (85 mm Pfund)	*Castanea* 63%
F	*Eucalyptus* honey	Dark (85 mm Pfund)	*Eucalyptus* 94%
G	*Thymus* honey*	Light (70 mm Pfund)	*Lotus* 50%
H	*Arbutus* honey	Dark (> 100 mm Pfund, < 114 mm Pfund)	*Arbutus* 8%
I	*Hedysarum* honey	Light (50 mm Pfund)	*Hedysarum* 49%
J	*Arbutus* honey	Dark (> 100 mm Pfund, < 114 mm Pfund)	*Arbutus* 8%
K	*Castanea* honey	Dark (100 mm Pfund)	*Castanea* 95%
L	Multifloral honey	Light (70 mm Pfund)	*Rubus* f 16%
M	Multifloral honey	Dark (90 mm Pfund)	*Tamarix* 29%
N	Multifloral honey	Light (75 mm Pfund)	*Lotus* 78%
O	Multifloral honey	Dark (95 mm Pfund)	*Trifolium pratense* gr 29%

Identified as multifloral honey by melissopalinolgical analysis

to treat the hives and keep fresh products for the sale. Colour of each honey was measured with a Lovibond comparator and expressed in mm Pfund (Devillers et al., 2004). Based on colour, honeys were grouped (Table 1) in medium-light honeys (lower than 80 mm Pfund) and dark honeys (equal or higher to 80 mm Pfund) in according to Persano Oddo et al. (2000).

2.2 Melissopalynological analyses

The microscopic analysis of each honey and the identification of its palynological components were carried out according to melissopalynological method (Louveaux et al., 1978). A reference collection from Pisa University and different pollen morphology guides were used for the identification of the pollen types. The obtained melissopalynological profiles were then compared with the guidelines by Colombo et al. (2007), in order to confirm honey samples botanical origin.

2.3 Biochemical analyses

Total flavonoid and total phenolic contents were measured according to Özkök et al. (2010) and expressed as mg of quercetin equivalents (QE)/kg and mg of gallic acid equivalents (GAE)/kg of honey, respectively.

Glucose oxidase activity was determined using the protocol of Cohen (1973), with some modifications. In particular, each honey sample was diluted with distilled water at a final concentration of 430 mg/mL. The total protein concentration was measured with Qubit 2.0 Fluorometer (Invitrogen, Thermo Fisher Scientific). Fifteen μg of total protein in 50 μL of each sample were placed in a cuvette with 1.5 mL of 100 mM Hepes buffer (pH 7), 3,3′-diaminobenzidine tetrahydrochloride (0.18 mg/mL), and peroxidase from horseradish (0.02 mg/mL). The solution was analysed using a Ultrospec 2100 UV pro spectrophotometer (Amersham Biosciences, United Kingdom). Absorbance values were obtained at 352 nm at 0 and 120 minutes. Data were expressed in nM H_2O_2 produced/min.

Gluconic acid content was measured using a D-gluconic acid kit UV-method (Boehringer Mannheim, R-Biopharm, Germany, kit cod. 10428191035) and expressed as g/kg of honey.

Catalase activity was measured according to Huidobro et al. (2005) and expressed as Kf (catalase activity (min-1) per gram of honey).

2.4 Determination of Minimum Inhibitory Concentration (M.I.C.) and Minimum Bactericidal Concentration (M.B.C.) values

Paenibacillus larvae subsp. *larvae* type strain ATCC9545 was obtained from the BCCM/LMG Bacteria Collection (Ghent, Belgium). It was grown in Brain and Heart Infusion broth (BHI) (Thermo Scientific Oxoid, Milan, Italy) at 37°C for 24 h in aerobic condition and Tryptone Soy Agar (TSA) (Thermo Scientific Oxoid, Milan, Italy) at 37°C for 48-72 h in aerobic condition.

Resistance of *P. larvae* ATCC 9545 vegetative form against different honeys was evaluated with a broth microdilution method. M.I.C. values were determined for each honey according to Flesar et al. (2010), with modifications. Each honey sample was dissolved in BHI broth, sterilized by filtration with 0.20 μm membrane, and used to fill microtiter plates with twofold serial dilutions ranging from 820 ng/mL to 430 mg/mL. Each well was then inoculated with 5 μL of a *P. larvae* suspension at a concentration of 6·108 CFU/mL. Inoculated and sterile BHI broths were used as positive and negative control, respectively. In order to assess the inhibitory activity of sugar against *P. larvae*, the M.I.C. value of a sugar solution ranging from 1.6 μg/mL to 850 mg/mL was determined. Sugar solution was composed of 15% sucrose, 32% glucose and 38% fructose. After 24 h of incubation at 37°C in aerobic conditions and in a humid chamber, the M.I.C. value for each honey was determined as the lowest concentration at which a clearly visible reduction in growth was observed compared to the positive control. The experiment was replicated three times.

In order to obtain the M.B.C. values, a drop from the microplate wells corresponding to honey concentrations equal and higher to the M.I.C. value , was streaked onto TSA plates and incubated at 37°C for 48 h M.B.C. value was determined as the lowest honey concentration

Table 2. Biochemical profiles of honeys analyzed.

	Total flavonoids (QE mg/kg)	Total phenols (GAE mg/kg)	Glucose oxidase activity (nM H_2O_2 produced/min)	Gluconic acid (g/kg)	Catalase activity (K_f)
A	25.7	114.5	7.7	1.8	62.2
B	24.4	143.9	6.1	11.9	112.9
C	32.3	248.8	6.2	8.6	128.7
D	29.5	141.9	14.5	9.8	124.3
E	36.1	224.5	14.7	9.0	100.8
F	36.6	202.9	7.4	9.7	78.5
G	28.2	184.1	10.3	7.9	93.6
H	29.5	307.6	15.6	14.4	52.9
I	23.4	152.7	7.2	3.8	35.9
J	25.3	197.7	8.1	14.5	41.1
K	32.7	168.6	5.1	11.9	34.6
L	17.7	150.3	3.5	9.2	43.9
M	24.1	140.0	6.5	11.6	141.6
N	29.7	132.0	5.8	6.5	6.3
O	23.6	115.7	6.3	9.3	18.3
Mean	27.9	175.0	8.3	9.3	71.7
SD	5.2	53.7	3.7	3.5	43.0
Max	36.6	307.6	15.6	14.5	141.6
Min	17.7	114.5	3.5	1.8	6.3

GAE: gallic acid equivalents; QE: quercetin equivalents

which allowed no colonies growth on agar plates. The experiment was replicated three times.

M.I.C. and M.B.C. values calculated as the mode value of the three replicates.

2.5 Statistical analysis

Statistical analysis was performed by JMP software (SAS, 2008). The presence of a correlation among biochemical parameters and M.I.C. and M.B.C. values was tested with Spearman correlation Test. The differences in honey's biochemical analysis results between dark and light honeys were analyzed by non-parametric Wilcoxon (Rank Sums) Test. Differences were considered significant if associated with a p value < 0.05.

3. RESULTS

3.1 Melissopalynological analyses

Results from melissopalynological analyses confirmed the botanical origin indicated on the commercial labels, except in two cases: honeys E and G, sold as *Arbutus* and *Thymus* honeys, respectively, were instead multifloral honeys (Table 1).

3.2 Biochemical analyses

In Table 2 are reported the results of the biochemical analyses of the different honey samples.

Total flavonoid content ranged between 17.7 and 36.6 QE mg/kg, which corresponded to honey L (multifloral honey) and F (*Eucalyptus* honey), respectively.

All samples showed a total phenolic content higher than 100 GAE mg/kg, and the lowest content was found in honey A (*Robinia* honey) (114.5 GAE mg/kg) and the highest in honey H (*Arbutus* honey) (307.6 GAE mg/kg).

Table 2 also shows honeys' glucose oxidase activities. The highest value of glucose oxidase activity was found in honey H (*Arbutus* honey) (15.6 nM H_2O_2 produced/min) and the lowest in honey L (multifloral honey) (3.5 nM H_2O_2 produced/min).

Gluconic acid values varied among different honey samples. Honeys H and J (*Arbutus* honeys) showed the highest contents of gluconic acid (14.4 and 14.5 g/kg, respectively), while honey A (*Robinia* honey) had the lowest gluconic acid content (1.8 g/kg). From the correlation analysis, the colour of honey (in mm Pfund) was significantly correlated with gluconic acid

Table 3. M.I.C. and M.B.C. values (mg/mL) calculated as the mode value of three replicates for each honey.

	A	B	C	D	E	F	G	H
M.I.C. (mg/mL)	107.5	215.0	215.0	215.0	107.5	215.0	107.5	107.5
M.B.C. (mg/mL)	NB	430.0	430.0	430.0	430.0	430.0	430.0	215.0
	I	J	K	L	M	N	O	Sugar
M.I.C. (mg/mL)	215.0	53.6	215.0	215.0	215.0	107.5	107.5	212.5
M.B.C. (mg/mL)	430.0	107.5	NB	430.0	430.0	430.0	215.0	NB

NB: not bactericidal

Table 4. Biochemical profiles of honeys divided into dark honeys and light honeys.

	Total phenols (GAE mg/kg)	Total flavonoids (QE mg/kg)	Glucose oxidase activity (nM H_2O_2 produced/min)	Gluconic acid (g/kg)	Catalase activity (K_f)	M.I.C. (mg/mL)	M.B.C. (mg/mL)
Dark honeys	193.1[A]±18.3	29.1[A]±1.8	9.7[A]±1.33	11.1[A]±1.0	80.3[A]±15.4	53.8-215.0[A]	107.5-NB[A]
Light honeys	154.3[A]±16.4	26.5[A]±2.0	6.7[A]±1.25	7.3[B]±1.1	61.9[A]±16.7	107.5-215.0[A]	430.0-NB[A]

Different uppercase letters in the same column show a statistically significant difference between dark and light honeys (GAE: gallic acid equivalents; QE: quercetin equivalents; NB: not bactericidal).

content ($p<0.001$). Indeed, grouping based on colour, the examined dark honeys had a significantly higher gluconic acid content than light honeys (Table 4). No other significant correlations were observed between the other physico-chemical parameters (minimum p-value=0.062 for flavonoids and phenols contents and maximum p-value=0.878 for colour and catalase activity).

The catalase activity showed a remarkable variability, with a Kf ranging from 6.3 (N, multifloral) to 141.6 (M, multifloral).

3.3 Determination of M.I.C. and M.B.C. values

Table 3 shows M.I.C. and M.B.C. values of tested honey samples against *P. larvae* ATCC 9545. All the honey samples had an inhibitory activity on *P. larvae*.

Honey J (*Arbutus* honey) showed the highest antimicrobial activity, with M.I.C. and M.B.C. values of 53.8 and 107.5 mg/mL, respectively. Honeys A (*Robinia* honey) and K (*Castanea* honey) had no bactericidal activity at the concentrations tested. Sugar solution, which contained 85% of sugars, also showed an inhibitory activity, but no bactericidal effect. M.B.C. values showed a correlation with honey colour ($p < 0.05$), with a lower bactericidal concentrations for higher mm Pfund values, and thus darker honeys. It was not possible to highlight any other correlation between M.I.C. and M.B.C. values and the other analyzed parameters (minimum

p-value=0.081 for M.I.C. and glucose oxidase activity and maximum p-value=0.960 for M.I.C. and phenols content).

4. DISCUSSION

The first aim of the present study, to evaluate if the inhibitory capacity against *P. larvae* vegetative form could be different for each honey, has been achieved. In fact, results showed that honeys H, J (*Arbutus* honeys) and O (multifloral honey) were the most effective against *P. larvae*. Honey J had the lowest M.I.C. and M.B.C. values (53.8 mg/mL and 107.5 mg/mL, respectively), and it was followed by honeys H and O, which showed the same M.I.C. and M.B.C. values (107.5 mg/mL and 215.0 mg/mL, respectively). Thus, based on these results, it would be interesting to study a way to employ honeys against *P. larvae*. For this purpose the use of old and no longer marketable honeys could be economical, keeping in mind that a high HMF content (>150 ppm) could cause bee mortality (Brodschneider and Crailsheim, 2010).

A different gluconic acid content in different honey samples was indeed detected in accordance with several authors (Persano Oddo et al., 2008; Mato et al., 2006; Pulcini et al., 2004; Mato et al., 1997). In particular, honeys H and J (*Arbutus* honeys) had the highest gluconic acid contents (14.4 and 14.5 g/kg of honey, respectively). Similar values of gluconic

acid contents were reported for *Arbutus* honey by Pulcini et al. (2004). Honey O (multifloral honey) also showed a high gluconic acid content (9.3 g/kg). However, similar values were detected for other honeys, which did not show the same inhibitory activity (e.g. honey D, a multifloral honey). On the other hand, honey A (*Robinia* honey), which showed the lowest gluconic acid content (1.8 g/kg) was not bactericidal (Table 2 and Table 3).

Total phenolic and total flavonoid contents obtained in the present study are in accordance with those reported by Perna et al. (2012) for some Italian honeys. Concerning the total phenolic content, honey H (*Arbutus* honey) showed the highest value (307.6 GAE mg/kg). The antioxidant activity of phenolic acids is well known (Özkök et al., 2010) and phenolic antioxidants are able to inhibit a wide range of Gram-negative and Gram-positive bacteria (Taormina et al., 2001). Despite a moderate total phenolic content (197.7 GAE mg/kg), honey J (*Arbutus* honey) resulted the most effective in inhibiting *P. larvae*.

Dark honeys inhibitory activity against pathogens has been reported by Molan (1992), as well as their higher antioxidant properties (Taormina et al., 2001; Frankel et al., 1998). Our results, including correlation analysis, suggest that dark honeys have a higher antimicrobial activity and higher gluconic acid content than light honeys (table 4). Sadly, we did not find any significant correlations between honey colour and any other physico-chemical parameters (minimum p-value=0.078 for phenols content), although other Authors observed a correlation between colour and phenolic or flavones and flavonols contents (Kuś et al., 2014; Pontis et al., 2014; Bertoncelj et al., 2007). In fact, a different colour of honey reflects different phisico-chemical characteristics. Among dark honeys, the higher antimicrobial activity was recorded for *Arbutus* honey and honey J in particular. Our study included only 2 *Arbutus* honey samples so no statistical correlation of antimicrobial activity to this particular type of honey was possible.

It is known that *Arbutus* honey is characterized by the presence of glucosides (arbutin and methylarbutin) and a high content of phenols and gluconic and homogentisic acids (Floris et al., 2007). An investigation carried out on Sardinian honeys, reported that *Arbutus* honey showed a variable range of inhibition depending on bacterial strain and honey composition (Alamanni et al., 1990). Therefore, also considering our data, further studies on a higher number of different *Arbutus* honey samples might highlight a peculiar antimicrobial activity of this type of honey and its mechanisms.

In terms of disinfection capacity, catalase and glucose oxidase activities have an antagonistic effect. In fact, while, in presence of water, glucose oxidase converts D-glucose in gluconic acid and hydrogen peroxyde (Mahmoud and Owayss, 2006), catalase, on the other hand, converts hydrogen peroxide to oxygen and water. Honey catalase derives from pollen and nectar of plants (Molan, 1992).

Honey H (*Arbutus* honey) showed a high glucose oxidase activity and a low catalase activity, while honeys J (*Arbutus* honey) and O (multifloral honey) presented low levels of both enzymes activities. Antibacterial effectiveness of honeys J and O could be partly explained by the fact that even if hydrogen peroxide was produced in low concentration, it was also slowly converted to oxygen and water, exerting its antimicrobial activity in the honey samples for a longer time.

A preliminary study showed that glucose oxidase and gluconic acid seem to have an inhibitory activity against *P. larvae* vegetative form *in vitro* (Sagona et al., 2015). Indeed, Bucekova et al. (2014) suggest the idea that breeding novel honeybee lines expressing higher amounts of glucose oxidase, could help to increase the antibacterial efficacy of the hypopharyngeal gland secretion, with a positive influence on the resistance of colonies against bacterial pathogens.

5. CONCLUSIONS

In conclusion, different factors such as gluconic acid, glucose oxidase activity, catalase activity and phenolic acid content seem to play a role in honey inhibitory activity against *P. larvae*. Since honey is a complex substrate characterized by many compounds, its properties, including antibacterial

activity, can be affected by all of them, either alone or in an additive, synergistic or antagonistic way. From our results, the social immune system components, such as glucose oxidase and gluconic acid, appear to be important factors for *P. larvae* inhibition. Botanical origin, as indicated by the different pollens, since it influences honey composition, could also play a key role against *P. larvae.*

COMPETING INTERESTS

We have no competing interests.

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