1 **Quorum sensing inhibitory and antimicrobial activities of honeys and**
2 **the relationship with individual phenolics**

3 P. Truchado, F. López-Gálvez, M. I. Gil, F. A. Tomás-Barberán and A. Allende*

4
5 *Research Group on Quality, Safety and Bioactivity of Plant Foods, CEBAS-CSIC, P.O.*
6 *Box 164, Espinardo, Murcia, 30100, Spain*

* Author for correspondence: Tel.: +34-968-396-275; Fax: +34-968-396-213; E-mail:

aallende@cebas.csic.es

Abstract

Recent studies have evidenced the role of cell to cell communication or quorum sensing in the food spoilage, indicating that bacterial food spoilage could be regulated by quorum sensing inhibitors (QSI). Owing to the importance of QS during bacterial spoilage, much research is being focus on searching QSI, mainly natural products. The beneficial properties of honey as antimicrobial and antioxidant agent have been widely reported. However, as far as we known, the role of different unifloral honeys as a QSI has not been jet reported. In this study, the inhibitory activity of 29 unifloral honeys was evaluated using the bacterial model *Cromobacterium violaceum*. It was observed that all the tested honeys were able to inhibit the production of acyl-homoserine lactones (AHLs) produced by the *C. violaceum*. However, chestnut and linden honey samples showed the highest anti-QS activity, while orange and rosemary were the less effective inhibiting the production of AHLs. When honey samples from the same floral origin obtained from different geographical regions were compared, they showed similar anti-QS activity. Thus, it could be concluded that one of the factors which influences the anti-QS activities, should be deriving from the floral origin and independent from the geographic region. It was observed that unifloral honey samples showed “non-peroxide” anti-QS activity, which was not linearly correlated with its total and individual phenolic compounds. The obtained results showed that honey could have a potential use in the food industry as food preservative due to its anti-QS activity, although further research is needed to determine the active fraction of the honey responsible for the QS quenching.

1. Introduction

Since ancient times, antimicrobial properties have been attributed to honey, usually related to both the physical properties of osmosis and the antibacterial properties of hydrogen peroxide and non-peroxide compounds (Taormina, Niemira, & Beuchat, 2001; Weston, 2000; Zumla & Lulat, 1989). Non-peroxide antibacterial activity of honey has been associated to high sugar concentration, antioxidant and proteinaceous compounds as well as other unidentified components (Lee, Churey & Worobo, 2008; Mundo, Padilla-Zakour, & Worobo, 2004). Previous studies have linked the antimicrobial activity of honey and propolis to flavonoids (including flavones, flavonols, flavanones and dihydroflavonols) and other phenolics (mainly substituted cinnamic acids and their esters) (Aljadi & Yusoff, 2003; Küçük, Kolayli, Karaoğlu, Ulusoy, Baltai & Cadan, 2007; Povona et al., 2007). Due to these antimicrobial properties honey has been generally considered as a natural food preservative (Mundo et al., 2004).

Actually, up till now, investigations were restricted to whether or not honey could kill or inhibit the growth of bacteria. However, as previously described for some plant extracts, the antimicrobial properties of honey might only represent one facet of its anti-infective potential (Adonizio, Downum, Bennett, & Mathee, 2006). The capacity of honey to inhibit the interaction between the bacterium and the food may be also of interest to avoid food spoilage. Gram, Ravn, Rasch, Bruhn, Christensen, & Givskov (2002) defined the spoilage potential of a microorganism as the ability of a pure culture to growth and produce the metabolites that are associated with the spoilage of a particular product. It has been demonstrated that many bacteria are able to regulate the expression of phenotypic characteristics as a function of cell density under the control of chemical signal molecules. These auto-inducer molecules have been identified as

oligopeptides in Gram-positive bacteria and acylated homoserine lactones (AHLs) in Gram-negative bacteria (Novick, Ross, Projan, Kornblum, Krieswirth, & Moghazeh, 1993; Miller & Bassler, 2001). The ability of bacteria to sense and response to population density has been termed as cell-to-cell communication or “quorum sensing” (QS). In fact, many bacterial physiological functions such as luminiscence, virulence, motility, sporulation, biofilm formation, etc., are regulated by QS systems (Gram et al., 2002; Lindum, Anthoni, Christophersen, Eberl, Molin, & Givskov, 1998; Winson et al., 1995). For this reason, Rach et al. (2005) hypothesized that if QS systems regulate bacterial mechanism in food spoilage then, inhibition of the communication underlying the QS systems could be a good strategy to reduce or prevent the spoilage reactions.

Knowing the importance of QS during bacterial pathogenesis and spoilage, research has been focused on inhibiting QS using bacterial biosensors and indicators (Choo, Rukayadi, & Hwang, 2006). Among all the possibilities to interrupt the QS system, the use of anti-quorum sensing (anti-QS) compounds can be of great interest to avoid bacterial infections (Adonizio et al., 2006; Rice, McDougald, Kumar, & Kjelleberg, 2005). Such “antipathogenic” compounds, in contrast to antibacterial compounds, do not kill bacteria or stop their growth and are assumed not to lead to the development of resistant strains (Otto, 2004). Except for the halogenated furanones from the red alga *Delisea pulchra*, most of the identified anti-QS compounds of non bacterial origin came from plant origin (Adonizio et al., 2006; Choo et al., 2006; Teplitski, Robinson & Bauer, 2000; Bauer, & Teplitski, 2001). However, up till now, only scarce information has been published regarding the QS-activity of honey (Rasmussen et al., 2005).

Screening for AHL production from bacterial strains has typically relied on bacteriological monitor systems, which consists of a phenotypic response, activate

through an AHL-receptor protein (Ravn, Christensen, Molin, Givskov, & Gram, 2001). This is the case of *Chromobacterium violaceum*, a Gram-negative water and soil bacterium highly sensitive to most short-chained unsubstituted AHLs (*N*-hexanoyl homoserine lactone, HHL), which phenotypic response is the production of violacein, a water-insoluble purple pigment with antibacterial activity (McClellan et al., 1997; Steindler & Venturi, 2006).

The purpose of this study was to investigate the anti-QS and antimicrobial properties of different unifloral honeys against *C. violaceum* to determine its possible use as novel QS inhibitor (QSI). Additionally, the relationship between the individual phenolic content of different unifloral honeys and its antimicrobial and anti-QS activities has been also evaluated.

2. Materials and methods

2.1. Honey samples

Twenty nine honey samples of 14 different floral origins and 15 different geographical locations were provided by the Agricultural Research Council (CRA-API, Bologna, Italy) and Institute of Molecular Biology (Slovak Academy of Sciences, Bratislava, Slovakia), which received samples from different raw honey beekeepers in Italy and Slovakia, respectively. A Spanish commercial honey (Quexigal, Avila, Spain) was also included in this study. **Table 1** lists the honey samples by floral source, geographical location and country. During the experiments, samples were kept at 5 °C in the dark in airtight containers. Prior to testing, a 66.6 % (w/v) working solution was prepared diluting 2 g of each honey sample with 1 ml of sterile distilled water.

2.2. *Strains and culture conditions*

Cromobacterium violaceum wild-type strain CECT 494, obtained from the Spanish Type Culture Collection (Valencia, Spain) was used to determine QS inhibitory and antimicrobial activities. This wild type of strain produces and responds to the cognate autoinducer molecules C6-AHL and C4-AHL, which makes this strain excellent for screening (Adonizio et al., 2006; McClean, Pierson & Fuqua, 2004). This bacteriological monitor system generates a phenotypic response by the production of a purple pigment (violacein) when induced by the presence of AHLs. The bacterium was routinely grown aerobically with shaking in Luria-Bertani broth (02-385, LB BROTH acc. to MILLER, Scharlau Chemie, S.A. Barcelona, Spain) supplemented with 0.5 % of NaCl and incubated at 30 °C for 24 h. Acylated homoserine lactone (AHL) plate assays were performed using LB agar (02-385, LB BROTH acc. to MILLER + 07-004, Agar Bacteriological, Scharlau Chemie, S.A.), using an agar concentration of 1.2 %. Antibacterial activity of honey was carried out using LB agar (02-385, LB BROTH acc. to MILLER + 07-004 Agar Bacteriological, Scharlau Chemie, S.A.) with a final concentration of 1 % of agar.

2.3. *Inhibition of AHL Production by Honey*

The inhibitory activity of honey samples was assayed by the agar-well diffusion test. Plates were made by adding approximately 10^5 cfu/ml of an overnight culture of *C. violaceum* to the LB agar (1.2 %) (Scharlau Chemie, S.A.). Wells were filled with 20 μ l of different concentrations (200, 400, 600 and 800 μ g/mL) of each honey. Plates were incubated for 18 to 24 h at 30 °C prior to the determination of inhibition zone sizes by contrast camera imaging (Synoptics, Cambridge, United Kingdom). The experiment was carried out twice and there were three replications per treatment.

Flask-incubation assays were carried out to quantify the inhibitory activity of honey samples. The bacterium was incubated for 18 h and inoculated to $OD_{600nm} = 0.1$ in Erlenmeyer flasks containing LB broth (Scharlau Chemie, S.A.) and LB supplemented with different honey samples at different concentrations (200, 400, 600 and 800 $\mu\text{g/ml}$). The flasks were incubated at 30 °C in a shaking incubator. The quantification of the violacein production was carried out following the protocol described by [Choo et al. \(2006\)](#), where 1 ml culture from each flask was centrifuged at 13,000 rpm for 10 min to precipitate the insoluble violacein. Then, the culture supernatant was discarded and the pellet was solubilized in 1 ml of DMSO, vortexed until the violacein was homogenized, and centrifuged at 13,000 rpm for 10 min to remove the cells. Absorbance of each violacein-containing supernatant was measured at 585 nm in a UV-vis spectrophotometer (Hewlett Packard 8453). This experiment was carried out four times and there were three replications per treatment.

2.4. Antibacterial Activity of Honeys

Antimicrobial activity of chestnut, linden, tilia, orange and rosemary honeys against *C. violaceum* was evaluated. Ten millilitres of LB broth (Scharlau Chemie, S.A.) was inoculated with 10 μl of a working culture of *C. violaceum* and the amount of honey needed for each selected concentrations (200, 400, 600 and 800 $\mu\text{g/ml}$). Inoculated honey solutions were mixed followed by incubation at 30 °C for 24 h. Inoculated honey samples (1 mL) were diluted in 1 % sterile buffered peptone water (BPW) (AES Laboratoire, Combourg, France) (1:10 dilution). Appropriate dilutions were then spread onto LB agar (1 %) (Scharlau Chemie, S.A.). Colonies were counted after 24 h incubation at 30 °C. Microbial counts were expressed as log cfu per milliliter.

2.5. *Measurement of Non-Peroxide Anti-QS and Antibacterial Activities of Honey*

Selected honey samples (chestnut, linden, tilia, orange and rosemary) were diluted at 66.6 %, with 0.1 M potassium phosphate buffer (pH 7) treated and untreated with catalase from bovine liver (13,500 units mg solid⁻¹, Sigma-Aldrich St Louis, MO) at ratio of 2,700 units mg solid⁻¹ per 1 g of honey. The anti-QS and antimicrobial activities of the selected honeys treated and untreated with catalase were carried out as above described.

2.6. *Phenolic Composition of Honeys*

For the extraction of phenolic compounds, honey samples (10 g) were dissolved with five parts of water (adjusted to pH 2 with HCl) until completely fluid. This solution was flushed through an activated Sep-Pak C₁₈ cartridge (Waters, Milford, MA), previously activated with methanol (10 mL) followed by water (10 mL). Then, the phenolic compounds were eluted with methanol (2 mL). The methanol extracts were filtered through a 0.45µm filter (Millex-HV13, Millipore Corp., USA) and stored at -20 °C until further analysis by HPLC-DAD.

Samples of 50 µl were analyzed by HPLC (Agilent 1100 Series, Agilent Technologies, Waldbronn, Germany) equipped with a binary pump (G1312 A), a degasser (G1322 A), a photodiode array diode array detector (G1315 B) and a mass detector in series (Agilent Technologies). The samples were injected by a model (G1313 A) autosampler. The mass detector was an ion trap spectrometer (G2445A) equipped with an electrospray ionization (ESI) system. The nebulizer gas was nitrogen, the pressure and the flow rate of the dryer gas were set at 65 psi and 11 L min⁻¹, respectively. The full scan mass covered the range from m/z 100-1000. Collision-induced fragmentation experiments were performed in the ion trap using helium as

collision gas, with voltage ramping cycles from 0.3 up to 2 V. The heated capillary and voltage were maintained at 350 °C and 4 kV, respectively. Mass spectrometry data were acquired in the negative mode. Chromatographic separations were carried out a C₁₈ LiChroCART column (Merck, Darmstadt, Germany) (RP-18, 250 x 4 mm; 5 µm particle size) protected with a 4 x 4 mm C₁₈ LiChroCART guard column, with 1% formic acid (A) and methanol (B) as solvents (99.9%, HPLC grade; Merck, Darmstadt, Germany). Elution was performed with a gradient starting with 10 % B in A to reach 30 % B in A at 20 min, 45 % B in A at 30 min, 60% B in A at 40 min, 70% B in A at 45 min, 90% B in A at 60 min and then became isocratic for 5 min. The flow rate was of 1 mL min⁻¹ and chromatograms were recorded at 290, 320, 340 and 360 nm.

The phenolic compounds were identified according to their UV spectra, molecular weights, retention time and their MS-MS fragments and wherever possible, with commercially available standards. Hydroxycinnamic acid derivatives were quantified as chlorogenic acid at 320 nm, flavonols, and flavones as quercetin and chrysin, respectively, at 340 nm. Flavanones were quantified as hesperetin at 290 nm. All these markers were purchased from Sigma (St. Louis MO), except chrysin from Carl Roth OGH (Karlsruhe, Germany). The results were expressed as micrograms per 100 grams of honey.

3. Results and discussion

3.1. Inhibition of AHL production by honey

Fifteen unifloral honeys, which are identified in **Table 1** with the symbol (*), were tested for its capacity to inhibit the production of AHLs produced by *C. violaceum*. It was observed that in most of the cases, honeys at 400 µg/mL exerted inhibitory activity

as estimated by the size of the inhibition zone (Table 2). However, at the lowest applied concentration (200 µg/mL), only chestnut and linden honeys showed inhibition zones due to the inhibition of AHLs production (Table 2). Figure 1 shows the observed inhibition zones caused after the addition of different concentrations (200, 400, 600 and 800 µg/mL) of a selection of honeys (chestnut, linden, orange, rosemary and tilia) by using the agar-well diffusion method. Taking into account that the agar-well diffusion method is effective for the detection of AHL inhibition but it cannot reliably quantify the relative amount of AHL present in a given culture, the flask-incubation assay was carried out to quantify the inhibitory activity of honey samples (Blosser & Gray, 2000). Thus, the anti-QS activity of different unifloral honeys was evaluated as their ability to inhibit the violacein production by *C. violaceum* (Cho et al., 2006). As previously observed in the agar-well diffusion method, honey samples showed concentration-dependent inhibitory activity, and compared to control, all unifloral honeys showed a significant drop in violacein production, even at the lowest tested concentration (200 µg/mL) (Fig. 2). On the other hand, chestnut and linden honeys showed the highest anti-QS activity while orange and rosemary were the honeys which showed the lowest inhibitory activity (Fig. 2). When the honey concentration was increased, the observed differences among the anti-QS activity of the tested unifloral honeys were even more marked. In fact, when 800 µg/mL of each honey sample were applied, *C. violaceum* was only able to produce violacein in the presence of orange honey.

Additionally, differences in the anti-QS activity of honeys from the same floral origin but obtained from different geographical origins were evaluated (Fig. 3). In this experiment, and based on the previously obtained results, 5 unifloral honeys from up to 8 different geographical places, were selected (Table 1). Thus, chestnut and linden honeys were selected as the best QSI and orange and rosemary honeys were selected

1 because they showed the lowest anti-QS activity. Tilia honey was included in the assay
2 due to its close relationship with the floral origin of linden honey. It was observed that
3 honeys from the same floral origin showed similar anti-QS activity regardless the
4 geographical origin (Fig. 3). The obtained results confirmed that the chestnut honey
5 showed the highest anti-QS activity despite the geographical origins.

6 It is well known that honey contains hydrogen peroxide, which present high
7 antimicrobial activity, so this compound could be also responsible for the anti-QS
8 activity observed in the unifloral honeys. To determine if the inhibitory activity of QS
9 was due to the presence of hydrogen peroxide, the experiment was repeated with the
10 addition of the enzyme catalase (Weston, Mitchell & Allen, 1999). It was observed that,
11 in general, the inhibitory activity was lower when the hydrogen peroxide present in the
12 honey was destroyed with the catalase, mainly at 400 µg/mL and higher honey
13 concentrations (Fig. 4). However, when compared to control, all the honeys remained
14 active even at the lowest concentration (200 µg/mL).

16 3.2. Antibacterial Activity of Honeys

17 The inhibition zones produced on lawns of the indicator strain and the inhibition
18 of violacein production could be the result of either (i) quenching of QS signals or (ii)
19 inhibition of cell growth, although growth inhibition would produce a clear halo versus
20 a turbid halo for the QS inhibition (Adonizio et al., 2006). To determine the main origin
21 of the anti-QS activity, the antimicrobial activity of chestnut, linden, orange, rosemary
22 and tilia honeys was tested at different concentrations 200, 400, 600 and 800 µg/mL. It
23 was observed that none of the tested honeys showed antimicrobial activity when applied
24 at 200 µg/mL (data not shown). However, the same honey concentration was enough to
25 significantly reduce the production of violacein when compared to control (Figs. 3 and

4). Table 3 shows the growth inhibition of *C. violaceum* after overnight incubation in LB broth supplemented with 400, 600 and 800 µg/mL of honey. The observed antimicrobial activity was concentration-dependent. In fact, the tested unifloral honeys showed moderate inhibition against *C. violaceum* when applied at 400µg/mL, but it significantly increased when the applied concentration was 800 µg/mL (Table 3). There were also differences among the antibacterial activity of different unifloral honeys. In general, when honey was applied at a concentration of 800 µg/mL, chestnut, orange, rosemary and tilia showed similar antimicrobial activity reducing *C. violaceum* growth by approximately 1 or 2 log units. However, linden reduced bacterial counts by 8.5 log units, showing the highest growth inhibition. Thus, only high honey concentrations (600 and 800 µg/mL) were effective inhibiting growth of *C. violaceum*. When the same experiment was repeated with the addition of catalase to eliminate the antimicrobial activity of hydrogen peroxide, the antimicrobial activity of the honey samples was reduced (Table 3).

3.3. Relationship between the Antipathogenic Activity and the Content of Individual Phenolic Compounds of Honeys.

To determine the relationship between the antipathogenic activity and the phenolic compounds of different unifloral honeys, the total phenolic content as well as the content of individual phenolic compounds (hydroxycinnamic acids, flavonols and flavonoids) were evaluated (Fig. 5). It was observed that all honey samples showed similar but quantitatively different phenolic profiles (Fig. 5A). When the individual phenolic compounds were evaluated, it was observed that the honeys which presented the highest antipathogenic activity also showed a low phenolic content (Fig. 5B, C and D). In fact, chestnut honey showed one of the lowest contents of hydroxycinnamic

acids, flavonols and flavanones (Fig. 5B, C and D). Tilia honey, which showed high antipathogenic activity also showed one of the highest phenolic content (Fig. 5A). On the other hand, some of the honeys with lower anti-QS activity, such as canola honey, showed the highest phenolic content (Fig. 5A). Thus, in general, there was not a linear relationship between the content of individual phenolic compounds and the antipathogenic activity of the tested honey samples.

4. Discussion

The antimicrobial activity of honey as well as the variability in antimicrobial activity of honeys from different floral sources have been widely reported (Baltrušaityte, Venskutonis, & Čeksterytė, 2007; Lee et al., 2008a; Mundo et al., 2004; Taormina et al., 2001). However, honey might have other antipathogenic activities, such as the inhibition of QS. There is available data that provide evidence of the role of QS in the food spoilage, suggesting that one way to prevent spoilage is the control of QS (Ammor, michaelidis & Nychas, 2008). Several compounds have been identified as QSI, although the most well-known examples of QSI compounds are the halogenated furanones (Rasch et al., 2007). Unfortunately, these QS inhibitors are unsuitable for human use as they are too reactive and may be too toxic to be used in medicine, agriculture and industry (Bosgelmez-Tinaz, Ulusoy, Ugur & Ceylan, 2007). Hence, there is a current need for the identification of new, nontoxic QSI compounds. Recent studies have demonstrated the potential use of different plant extracts as QSI (garlic, vanilla, pea seedlings, *Scorzonera sandrasica* and L-canavanine obtained from *Medicago sativa*) (Bosgelmez-Tinaz et al., 2007; Choo et al., 2006; Teplitskiet al., 2000; Persson, Hansen, Rasmussen, Skindersø, Givskov & Nielsen, 2005). However, as far as we know, the ability of different unifloral honeys as QSI has not been reported.

1 In this study we observed that all the 29 honey samples inhibited the AHL
2 production when compared to control, even at the lowest tested concentration (200
3 $\mu\text{g/mL}$). The anti-QS activity was concentration-dependent as the inhibition activity
4 increased with increasing honey concentration. Among all, chestnut and linden honeys
5 seemed to be the most efficient QSI. Therefore, significant differences were observed
6 among honey samples from different floral origin. On the other hand, when honeys
7 from the same floral origin but obtained from different geographical locations were
8 compared they showed similar anti-QS activity. Thus, it could be concluded that one of
9 the factors which influences both, the antimicrobial and anti-QS activities, should be
10 originating from the floral origin and independent from the geographic region.
11 Additionally, the antimicrobial activity of five different honey samples were evaluated
12 to determine if the inhibition in AHL production was due to quenching of QS signals or
13 inhibition of cell growth ([Adonizio et al., 2006](#)). It was observed that low
14 concentrations (200 and 400 $\mu\text{g/mL}$) of honey samples did not significantly inhibit
15 growth of *C. violaceum*. Thus, when low honey concentrations were applied, the
16 reduction in AHL production could be mainly attributed to quenching of QS signals.

17 The variability in antimicrobial activity of honeys from different floral origins has
18 been attributed to the botanical and geographical origins of honey and bee-origin
19 metabolism products, such as bacteriocin-like compounds and peptide antibiotics
20 ([Baltrušaityte et al. 2007](#); [Lee et al., 2008a, b](#); [Mundo et al., 2004](#); [Weston 2000](#)). In like
21 manner and based in our results, the anti-QS properties observed in the tested honey
22 samples could be attributed to the same compounds. Furthermore, different honey
23 constituents have been characterized as responsible for the antimicrobial activity, such
24 as sugars, volatiles, beeswax, nectar, pollen and propolis ([Weston, 2000](#); [Mundo et al.,](#)
25 [2004](#)). Previous studies reported that a significant part of the antimicrobial activity

observed in honey samples is due to their hydrogen peroxide content ([Lee et al., 2008](#); [Taormina et al., 2001](#)). However, they found that catalase-treated honeys also showed inhibitory activity, which may be attributable to non-peroxide related factors. In the present study, catalase-treated honeys showed antimicrobial and anti-QS activities, although this antipathogenic activity was lower when compared to untreated samples. Therefore, it could be suggested that other factors of plant origin might be responsible for the “non-peroxide” antipathogenic activity of honeys ([Weston, 2000](#)).

On the other hand, [Popova et al., \(2007\)](#) found a significant negative correlation between the concentration of total phenolics in the propolis and the minimum inhibitory concentration (MIC), which can be use as an evidence of the important role of phenolic compounds in the antimicrobial properties of honey. What is more, high phenolic content of honey samples has been also associated with high antimicrobial activity ([Küçük et al., 2007](#)). Therefore, in the present study we evaluate the relationship between the phenolic content of different unifloral honeys and its antimicrobial and anti-QS activities. However, we did not found any relationship between the total and individual phenolic contents of honey and their bioactivity. According with us, [Weston, Brocklebank & Lu \(2000\)](#) determined that the phenolic components of the New Zealand manuka honey were not responsible for the presence or absence of antibacterial activity. Consequently, it could be concluded that the phenolic products from nectar, pollen or propolis are only partly responsible for the residual non-peroxide antibacterial activity of honey ([Weston et al., 1999](#)). However, other non-phenolic compounds associated with their floral origin, such as different floral markers, could be responsible of the high anti-QS activity of specific honey samples like chestnut and linden honeys. Therefore, one might conclude that phenolic compounds contribute to the “non-peroxide” anti-QS

activity but the contribution of these components is relatively small as no linear relationship was observed between the phenolic content and the inhibitory activity.

5. Conclusions

Few studies have been conducted to investigate the potential of different compounds to reduce food spoilage by inhibiting bacteria cell-to-cell communication and consequently the spoilage mechanisms. In the present work, the ability of different unifloral honeys as QSI has been proven against the bacterium model *C. violaceum*. The potential use of honey or specific honey components, responsible of the QS inhibitory activity, as QSI is relevant to food industry as it might be used as a mild preservation technique. Unifloral honey samples showed “non-peroxide” anti-QS activity and it was not linearly correlated with the total and individual phenolic compounds. Thus, it is clear that further research have to be carrying out to clarify which honey constituents are responsible for the “non-peroxide” anti-QS activity.

Acknowledgements

Support of the EU-Funded Project “Bees in Europe and Sustainable Honey Production” (“BEE SHOP”, Ref: FOOD 2006-022568-BEESHOP) is gratefully acknowledged. Authors thank the Agricultural Research Council (CRA-API, Bologna, Italy) and the Institute of Molecular Biology (Slovak Academy of Sciences, Bratislava, Slovakia) for supplying honey samples from Italy and Slovakia. A. Allende and F. López-Gálvez hold grants founded by CSIC (JaeDoc-2008 and I3P contracts) and P. Truchado holds a PhD grant from the Seneca Foundation (Murcia, Spain).

References

1 Adonizio, A. L., Downum, K., Bennett, B. C., & Mathee, K. (2006). Anti-quorum
2 sensing activity of medicinal plants in southern Florida. *J. Ethnopharmacol.*, 105,
3 427–435.

4 Aljadi A.M. & Yusoff K.M. (2003). Isolation and Identification of phenolic acids in
5 Malaysian Honey with antibacterial properties. *Turk J Med Sci*, 33, 229-236.

6 Ammor, M.S., Michaelidis, C., & Nychas, G-J., E. (2008). Insights into the role of
7 quorum sensing in food spoilage. *J. Food Protect.*, 71, 1510-1525.

8 Baltrušaityte,V., Venskutonis, P.R., & Čekstertytė, V. (2007). Antibacterial activity of
9 honey and beebread of different origin against *S. aureus* and *S. epidermis*. *Food*
10 *Technol. Biotechnol.*, 45, 201-208.

11 Bauer, W. D., & Teplitski, M. (2001). Can plants manipulate bacterial quorum sensing?
12 *Aust. J. Plant Physiol.*, 28, 913–921.

13 Blosser, R. S., & Gray, K. M. (2000). Extraction of violacein from *Chromobacterium*
14 *violaceum* provides a new quantitative bioassay for *N*-acyl homoserine lactone
15 autoinducers. *J. Microbiol. Meth.*, 40, 47–55.

16 Bosgelmez-Tinaz, G., Ulusoy, S., Ugur, A., & Ceylan, O. (2007). Inhibition of quorum
17 sensing-regulated behaviors by *Scorzonera sandrasica*. *Current Microbiol.*, 55,
18 114-118.

19 Choo, J. H., Rukayadi, Y., & Hwang, J. -K. (2006). Inhibition of bacterial quorum
20 sensing by vanilla extract. *Lett. Appl. Microbiol.*, 42, 637–641.

21 Gram, L., Ravn, L., Rasch, M., Bruhn, J. B., Christensen, A. B., & Givskov, M. (2002).
22 Food spoilage—interactions between food spoilage bacteria. *Int. J. Food*
23 *Microbiol.*, 78, 79– 97.

- 1 Küçük, M. Kolaylı, S. Karaoğlu, S. Ulusoy, E., Baltacı, & Candan, F. (2007). Biological
2 activities and chemical composition of three honeys of different types from
3 Anatolia. *Food Chemistry*, 100, 526-534.
- 4 Lee, H., Churey, J. J., & Worobo, R. W. (2008). Antimicrobial activity of bacterial
5 isolates from different floral sources of honey. *Int. J. Food Microbil.*, doi:
6 10.1016/j.ijfoodmicro.2008.04.030.
- 7 Lee, H., Churey, J. J., Worobo, R. W. 2008. Purification and structural characterization
8 of bacillomycin F produced by a bacterial honey isolate active against
9 *Byssoschlamys fulva* H25. *J. Appl. Microbiol.*, doi:10.1111/j.1365-
10 2672.2008.03797.x.
- 11 Lindum, P. W., Anthoni, U., Christophersen, C., Eberl, L., Molin, S., & Givskov, M.
12 (1998). N-acyl-L-homoserine lactone autoinducers control production of an
13 extracellular lipopeptide biosurfactant required for swarming motility of *Serratia*
14 *liquefaciens* MG1. *J. Bacteriol.*, 180, 6384–6388.
- 15 McClean, K. H., Winson, M. K., Fish, L., Taylor, A., Chhabra, S. R., Camara, M.,
16 Daykin, M., Lamb, J. H., Swift, S., Bycroft, B. W., Steward, G. S. A. B., &
17 Williams, P. (1997). Quorum sensing and *Chromobacterium violaceum*:
18 exploitation of violacein production and inhibition for the detection of N-
19 acylhomoserine lactones. *Microbiology*, 143, 3703–3711.
- 20 McClean, R.J.C., Pierson III, L.S. & Fuqua, C. (2004). A simple screening protocol for
21 the identification of quorum signal antagonists. *J. Microbiol. Methods*, 58, 351–
22 360.
- 23 Miller, B. M., & Bassler, B. L. (2001). Quorum sensing in bacteria. *Annu. Rev.*
24 *Microbiol.*, 55, 165–199.

- Mundo, M. A., Padilla-Zakour, O. I., & Worobo, R. W. (2004). Growth inhibition of foodborne pathogens and food spoilage organisms by select raw honeys. *Int. J. Food Microbiol.*, 97, 1 – 8.
- Novick, R. P., Ross, H. F., Projan, S. J., Kornblum, J., Krieswirth, B. N. & Moghazeh, S. (1993). Synthesis of staphylococcal virulence factors is controlled by a regulatory RNA molecule. *EMBO J.* 12, 3967–75.
- Otto, M. (2004). Quorum-sensing control in *Staphylococci* -- a target for antimicrobial drug therapy? *FEMS Microbiol. Lett.*, 241, 135–141.
- Persson, T., Hansen, T.H., Rasmussen, T.B., Skindersø, M.E., Givskov, M., & Nielsen J. (2005). Rational design and synthesis of new quorum-sensing inhibitors derived from acylated homoserine lactones and natural products from garlic. *Org. biomol. Chem.*, 3, 253-262.
- Popova, M. P., Bankova, V. S., Bogdanov, S., Tsevetkova, I., Naydenski, C., Marcuzzan, G. L. & Sabatini, A. G. (2007). Chemical characteristics of poplar type propolis of different geographic origin. *Apidologie*, 38, 306-311.
- Rasch, M., Andersen, J. B., Nielsen, K. F., Flodgaard, L. R., Christensen, H., Givskov, M. & Gram, L. (2005). Involvement of bacterial quorum-sensing signals in spoilage of bean sprouts. *Appl. Environm. Microbiol.*, 71, 3321–3330.
- Rasch, M., Rasmussen, T. B., Andersen, J. B., Persson, T., Nielsen, J., Givskov, M., & Gram, L. (2007). Well-known quorum sensing inhibitors do not affect bacterial quorum sensing-regulated bean sprout spoilage. *J. Appl. Microbiol.*, 102, 826–837.
- Rasmussen, T. B., Bjarnsholt, T., Skindersø, M. E., Hentzer, M., Kristoffersen, P., Kôte, M., Nielsen, J., Eberl, L., & Givskov, M. (2005). Screening for quorum-sensing inhibitors (QSI) by use of a novel genetic system, the QSI selector.

- 1 Ravn, L., Christensen, A. B., Molin, S., Givskov, M., & Gram, L. (2001). Methods for
2 acylated homoserine lactones produced by Gram-negative bacteria and their
3 application in studies of AHL-production kinetics. *J. Microbiol. Methods*, 44,
4 239–251.
- 5 Rice, S. A., McDougald, D., Kumar, N., & Kjelleberg, S. (2005). The use of quorum-
6 sensing blockers as therapeutic agents for the control of biofilm-associated
7 infections. *Curr. Opin. Investigat. Drugs*, 6, 178–184.
- 8 Steindler, L., & Venturi, V. (2007). Detection of quorum-sensing N-acyl homoserine
9 lactone signal molecules by bacterial biosensors. *FEMS Microbiology Letters*,
10 266, 1–9.
- 11 Taormina, P. J., Niemira, B. A., & Beuchat, L. R. (2001). Inhibitory activity of honey
12 against foodborne pathogens as influenced by the presence of hydrogen peroxide
13 and level of antioxidant power. *Int. J. Food Microbiol.*, 69, 217–225.
- 14 Teplitski, M., Robinson, J. B., & Bauer, W. D. (2000). Plants secrete substances that
15 mimic bacterial N-acyl homoserine lactone signal activities and affect population
16 density-dependent behaviors in associated bacteria. *Mol. Plant Microb. Interact.*,
17 13, 637–648.
- 18 Weston, R. J., Mitchell, K. R., & Allen, K. L. (1999). Antibacterial phenolic
19 components of New Zealand manuka honey. *Food Chemistry*, 64, 295–301.
- 20 Weston, R. J. (2000). The contribution of catalase and other natural products to the
21 antibacterial activity of honey: a review. *Food Chemistry*, 71, 235–239.
- 22 Weston, R. J., Brocklebank, L. K., & Lu, Y. (2000). Identification and quantitative
23 levels of antibacterial components of some New Zealand honeys. *Food Chemistry*,
24 70, 427–435.

- 1 Winson, M. K., Camara, M., Latifi, A., Foglino, M., Chhabra, S. R., Daykin, M., Bally,
2 M., Chapon, V., Salmond, G. P. C., Bycroft, B., Lazdunski, A., Stewart, G. S. A.
3 B., & William, P. (1995). Multiple N-Acyl-L-homoserine lactone signal
4 molecules regulate production of virulence determinants and secondary
5 metabolites in *Pseudomonas aeruginosa*. Proceedings of the National Academy of
6 Sciences of the United States of America 92, pp. 9427–9431.
- 7 Zumla, A., & Lulat, A. (1989). Honey- a remedy rediscovered. J. R. Soc. Med. 82, 384–
8 385.

1 **Table 1.** Floral origin, country and geographical location of 29 honey samples.

Honey samples	Floral origin	Geographical location	Country
Acacia (*)	<i>Robinia pseudacacia</i>	Bologna	Italy
Canola (*)	<i>Brassica napus</i>	Bologna	Italy
Cherry blossom (*)	<i>Prunus avium</i>	Frossaco	Italy
Chesnut-1 (*)	<i>Castanea sativa</i>	Bologna	Italy
Chesnut-2	<i>Castanea sativa</i>	Bologna	Italy
Chesnut-3	<i>Castanea sativa</i>	Siena	Italy
Chesnut-4	<i>Castanea sativa</i>	Arezzo	Italy
Chesnut-5	<i>Castanea sativa</i>	Pistoia	Italy
Chesnut-6	<i>Castanea sativa</i>	Torino	Italy
Chesnut-7	<i>Castanea sativa</i>	Arezzo	Italy
Chesnut-8	<i>Castanea sativa</i>	Bologna	Italy
Eucalyptus (*)	<i>Eucalyptus spp</i>	Commercial	Spain
Lavander (*)	<i>Lavandula ssp</i>	Puimoisson	France
Linden-1 (*)	<i>Tilia argentea</i>	Sebecheleby	Slovakia
Linden-2	<i>Tilia argentea</i>	Bratislava	Slovakia
Linden-3	<i>Tilia argentea</i>	Banská Štiavnica	Slovakia
Lucerne (*)	<i>Mendicago sativa</i>	Bologna	Italy
Orange-1 (*)	<i>Citrus spp.</i>	Tornareccio-CH	Italy
Orange-2	<i>Citrus spp.</i>	Manfredonia-FG	Italy
Rape (*)	<i>Rapessed (Brassica campestris)</i>	Sebechleby	Slovakia
Rosemary-1 (*)	<i>Rosmarinus officinalis</i>	Manfredonia-FG	Italy
Rosemary-2	<i>Rosmarinus officinalis</i>	Tornareccio-CH	Italy
Sunflower (*)	<i>Helianthus annus</i>	Žembovce	Slovakia
Taraxacum (*)	<i>Taraxacum officinalis</i>	Bologna	Italy
Tilia-1 (*)	<i>Tilia ssp</i>	Bologna	Italy
Tilia-2	<i>Tilia ssp</i>	Minerbio-BO	Italy
Tilia-3	<i>Tilia ssp</i>	Torino	Italy
Tilia-4	<i>Tilia ssp</i>	Bologna	Italy
Tilia-5	<i>Tilia ssp</i>	Bologna	Italy

Table 2. Inhibition of AHL production of different concentrations (200, 400, 600 and 800 µg/mL) of 15 unifloral honeys assayed by the agar-well diffusion test and expressed as the diameter (mm) of the inhibition zone obtained. The results are the mean of five replicates ± standard deviation.

UNIFLORAL HONEYS	HONEY'S CONCENTRATIONS			
	200 µg/mL	400 µg/mL	600 µg/mL	800 µg/mL
Acacia	0.0±0.0	82.7±11.0	89.3±13.3	134.0±2.0
Cherry Blossom	13.3±11.7	80.0±4.0	97.3±6.1	116.0±4.0
Chestnut	25.7±1.8	71.3±12.7	98.7±5.8	108.7±2.3
Canola	0.0±0.0	40.7±21.9	79.3±9.2	133.3±21.9
Eucalyptus	0.0±0.0	74.7±1.2	93.3±8.1	122.0±17.8
Lavander	6.0±10.4	63.3±4.2	93.3±6.4	124.7±3.1
Linden	15.1±8.2	54.0±17.4	85.3±6.1	124.7±2.3
Lucerne	0.0±0.0	67.3±1.2	97.3±1.2	112.7±5.0
Orange	0.0±0.0	80.7±5.0	103.3±4.2	128.0±3.5
Rape	25.3±1.2	75.3±2.3	105.3±2.3	107.3±2.3
Rhododendron	24.0±3.5	87.3±5.0	99.3±5.8	114.7±1.2
Rosemary	0.0±0.0	32.7±7.0	76.0±7.2	151.3±4.2
Sunflower	0.0±0.0	60.7±7.0	100.7±1.2	104.7±3.1
Taraxacum	0.0±0.0	78.7±7.6	102.0±4.0	112.0±10.4
Tilia	8.2±5.4	46.7±6.1	80.0±2.0	144.7±2.3

Table 3. Growth inhibition of *C. violaceum* after overnight growth in LB broth supplemented with different unifloral honeys at 400, 600 and 800 µg/mL and with and without the addition of catalase. Values represent cfu log₁₀ (N₀/N) ± standard deviation.

Honeys	Honey's concentrations	Without catalase	With catalase
Chestnut	400 µg/mL	0.23±0.18	0.29±0.10
Linden		0.53±0.49	0.32±0.06
Orange		0.07±0.0	0.20±0.17
Rosemary		0.11±0.06	0.06±0.07
Tilia		0.06±0.00	0.18±0.08
Chestnut	600 µg/mL	0.95±0.01	0.38±0.10
Linden		3.21±0.49	0.36±0.08
Orange		0.27±0.15	0.22±0.04
Rosemary		1.03±0.63	0.01±0.09
Tilia		1.29±0.56	0.11±0.08
Chestnut	800 µg/mL	1.51±0.09	0.93±0.12
Linden		8.54±0.08	8.54±0.08
Orange		1.09±0.14	0.86±0.11
Rosemary		1.83±0.04	0.94±0.06
Tilia		0.99±0.03	0.89±0.07

FIGURE CAPTIONS

Figure 1. Anti-QS activity using *Cromobacterium violaceum*. Anti-QS activity was tested using 20 μ L of different concentrations (200, 400, 600 and 800 μ g/ml) of unifloral honeys of chestnut, tilia, orange, rosemary and linden.

Figure 2. Inhibition of violacein production by unifloral honeys (acacia, cherry blossom, chestnut, canola, eucalyptus, lavender, linden, lucerne, orange, rape, rhododendron, rosemary, sunflower, taraxacum and tilia) at different concentration (200, 400, 600 and 800 μ g/mL). Vertical bars represent means of three replications $\pm$ standard deviation.

Figure 3. Inhibition of violacein production by unifloral honeys (chestnut, linden, orange, rosemary and tilia) originating from different geographical regions at different concentration (200 and 400 μ g/mL). Vertical bars represent means of three replications $\pm$ standard deviation.

Figure 4. Inhibition of violacein production by unifloral honeys (chestnut, linden, orange, rosemary and tilia) at different concentration (200, 400, 600 and 800 μ g/mL) with and without the addition of catalase to destroyed the honey content of hydrogen peroxide. Vertical bars represent means of three replications $\pm$ standard deviation.

Figure 5. Total phenolic, hydroxycinnamic acid, flavonol and flavanone content of unifloral honeys (chestnut, linden, orange, rosemary and tilia). Vertical bars represent means of three replications $\pm$ standard deviation.

1 **Figure 1.**

2

3

4

1 **Figure 2.**

2

3

4

5

6

1 **Figure 3.**

2

3

1 **Figure 4.**

1 **Figure 5.**

2
3