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ARTICLE

Inhibition by Chestnut Honey of *N*-Acyl-L-homoserine Lactones and Biofilm Formation in *Erwinia carotovora*, *Yersinia enterocolitica*, and *Aeromonas hydrophila*

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Bacteria are able to communicate and coordinate certain processes using small secreted signaling molecules called autoinducers. This phenomenon, known as "quorum sensing" (QS), may be essential for the synchronization of virulence factors as well as biofilm development. The interruption of bacterial QS is acknowledged to attenuate virulence and considered to be a potential new therapy to treat infections caused by pathogenic bacteria. *N*-Acyl-L-homoserine lactones (AHLs) have been identified as the main bacterial signaling molecules in Gram-negative bacteria. This study evaluates the capacity of chestnut honey and its aqueous and methanolic extracts to inhibit bacterial AHL-controlled processes in *Erwinia carotovora*, *Yersinia enterocolitica*, and *Aeromonas hydrophila*. This study is the first in applying liquid chromatography coupled with tandem mass spectrometry to determine the QS inhibitory activity of honey against pathogenic bacteria. The tandem mass spectrometry analysis of culture supernatants confirmed the presence of three main AHLs: *N*-(3-oxohexanoyl)-L-homoserine lactone (3-oxo-C6-HSL) and *N*-hexanoyl-L-homoserine lactone (C6-HSL) in *E. carotovora* and *Y. enterocolitica* and *N*-butanoyl-L-homoserine lactone (C4-HSL) in *A. hydrophila*. The effect of chestnut honey and its aqueous and methanolic extracts (0.2 g/mL) on AHL concentration and biofilm formation in bacterial cultures was determined. The obtained results revealed their potential use as QS inhibitors or regulators of the degradation of QS signals, with the methanolic extract showing less inhibitory capacity. Thus, the QS inhibitory activity of chestnut honey seems to be related to the aqueous phase, suggesting that the carbohydrate fraction contains an antipathogenic substance responsible for the inhibitory activity.

KEYWORDS: Quorum sensing; cell−cell communication; natural compounds; anti-QS activity; inhibition of quorum sensing; quorum quenching; quorum sensing inhibitors; *Chromobacterium violaceum*; *Agrobacterium tumefaciens*; LC-MS/MS; pathogenic bacteria

INTRODUCTION

Many opportunistic pathogens possess the ability to monitor their own population and to regulate gene expression in response to changes in cell population density using diffusible signaling compounds, which accumulate throughout growth (*1*). This type of communication has been referred to as "quorum sensing" (QS). Most Gram-negative bacteria, including *Erwinia carotovora*, *Yersinia enterocolitica*, and *Aeromonas hydrophila*, use *N*-acyl-L-homoserine lactones (AHLs) as signal molecules to monitor their population density. Bacteria employ QS to control different physiological processes including secretion of virulence factors and biofilm formation (*2*−*4*). *E. carotovora* is a phytopathogen that causes soft rot on a broad range of hosts. Many *Erwinia* species, including *E. carotovora*, produce *N*-(3-oxohexanoyl)-L-homoserine lactone (3-oxo-C6-HSL) and *N*-hexanoyl-L-homoserine lactone (C6-HSL), which are signal molecules of a QS system consisting of an AHL synthase (ExpI) and an autoinducer receptor protein (ExpR) that control several virulence factors (*5*). *Y. enterocolotica* is a mammalian enteropathogen capable of causing adenitis, septicemia, and gastrointestinal syndromes, normally after the consumption of contaminated food or through direct inoculation following a blood transfusion (*6*). *Yersinia* spp. produce AHLs as QS signal molecules, but their contribution to virulence has not been completely clarified. Atkinson et al. (*7*) established that QS regulates motility, at least in part, in *Y. enterocolitica*. As mentioned above for *E. carotovora*, *Y. enterocolotica* produce 3-oxo-C6-HSL and C6-HSL (*8*). *Aeromonas* spp. are Gram-negative rods belonging to the family Vibrionaceae. *A. hydrophila* has been described as an opportunistic pathogen that causes gastrointestinal diseases (*9*). This bacterium produces *N*-butanoyl-L-homoserine lactone (C4-HSL) as the principal AHL (*10*). The contribution of AHL-dependent QS to the virulence of this pathogen has been associated with biofilm development and exoprotease production (*11*, *12*).

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The discovery that many pathogenic bacteria use QS to regulate their colonization and virulence factors makes it an attractive therapeutic target (*13*). The major advance of this novel strategy is that new antimicrobials aim to inhibit virulence rather than growth. The use of antipathogenic compounds avoids the problem of resistance associated with many bactericidal and bacteriostatic drugs as it specifically inhibits virulence factors that can cause direct harm to the host with little or no impact on its normal microbiota (*14*). Therefore, there is a major interest to find natural or synthetic compounds, active in small quantities, which are capable of interfering with QS in pathogenic bacteria in order to disrupt their pathogenicity/virulence factors (*5*). The selected antipathogenic compounds should not disrupt bacterial and host metabolism and should not cause harmful side effects. Ni et al. (*4*) have published an extended review about all of the compounds that have potential use as QS inhibitors, including synthetic AHL analogues, halogenated furanone, natural products, and plant-derived compounds. Recently, Truchado et al. (*15*) demonstrated the ability of different unifloral honeys as QS inhibitors against *Chromobacterium violaceum*. However, the QS inhibitory activity of honey was indirectly determined by the quantification of violacein production.

Several strategies can be used to determine the QS inhibitory activity of a natural product. This is the case of the bacterium *C. violaceum*, which is one of the AHL biosensors most commonly used (*22*, *23*). The AHL biosensors provide a simple, rapid, and sensitive means to detect QS inhibitors without requiring any sophisticated instrumentation. However, they are limited to the determination of a reduced range of AHL structures (*24*). Unequivocal chemical analysis and quantification of AHLs in bacterial culture supernatants rely on analytical tools and separation techniques including gas chromatography (GC), high-performance liquid chromatography (HPLC), ultrahigh-pressure liquid chromatography (UPLC), mass spectrometry (MS), capillary electrophoresis (CE), micellar electrokinetic chromatography, and the use of synthetic standards (*24*−*26*).

It is known that bacterial biofilms play an important role in the pathogenesis of many human and plant pathogens (*16*). Many authors have associated the biofilm formation with > 80% of all the microbial infections associated with the urinary tract, dental plaque, and gingivitis, among others (*17*, *18*). Additionally, biofilms are a concern in the food industry, as human pathogens form biofilms on food and food contact surfaces, enhancing their ability to survive in the food-processing environment (*19*). It is well established that AHLs play an important role in biofilm formation in foodborne pathogens (*20*, *21*). Thus, there is a great interest in preventing and controlling biofilm formation by using naturally occurring compounds such as honey.

In this study, the potential of chestnut honey as a QS inhibitor has been determined by quantifying the AHL inhibition or degradation in bacterial cultures of three pathogenic bacteria (*E. carotovora*, *Y. enterocolitica*, and *A. hydrophila*) using AHL biosensors and specific detection and quantification by LC-MS/MS spectrometry. The capacity of chestnut honey to inhibit bacterial AHL-controlled processes such as biofilm formation was also determined.

MATERIALS AND METHODS

Reagents. The AHLs used in this study, 3-oxo-C6-HSL, C6-HSL, and C4-HSL, were purchased from Sigma (St. Louis, MO). Formic acid, ethyl acetate, acetic acid, and ethanol were of analytical grade and methanol and trifluoroacetic acid (TFA) were of HPLC grade and supplied by Merck (Darmstadt, Germany). Milli-Q system (Millipore Corp., Bedford, MA) ultrapure water was used in this study.

Chestnut Honey and Its Aqueous and Methanolic Extracts. Chestnut honey (*Castanea sativa*) was obtained and certified by the Agricultural Research Council (CRA-API, Bologna, Italy). The botanical origin was certified by the traditional analysis methods including sensorial, pollen, and physicochemical analyses. Chestnut honey was diluted with distilled water to obtain a working solution of 2 g/mL. To determine the active compounds present in chestnut honey, the honey was divided into aqueous and methanolic extracts. For the fractionation, the working solution of 2 g/mL was adjusted to pH 3 with HCl and filtered through a Sep-Pak C_{18} cartridge, which was previously activated with methanol (10 mL) followed by water (10 mL). The polar compounds, which were not retained in the cartridge, were collected and termed as aqueous extract. Then, the cartridge was washed with 10 mL of water, and the nonpolar compounds that remained adsorbed in the cartridge were eluted with 2 mL of methanol. The collected nonpolar fraction was termed the methanolic extract. The pH values of the resulting solutions were 5.1 for the chestnut honey, 6.9 for the aqueous extract, and 7.1 for the methanolic extract.

Strains and Culture Conditions. Two different AHL biosensor systems were used to screen the inhibition of AHL production. *Chromobacterium violaceum* wild-type strain 026 (CV026) and *Agrobacterium tumefaciens* NT1 were provided by the Laboratory of Food Microbiology and Food Preservation (University of Ghent, Belgium). *C. violaceum* presents a LuXR homologue, CviR, which regulates the production of a purple pigment when induced by specific AHLs (*23*, *27*). *A. tumefaciens* carries the plasmid p ZLR4 and produces a blue color from the hydrolysis of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) by the β-galactosidase, in the presence of particular AHLs (*23*). Strains tested for AHL production were *E. carotovora* (CECT 225), *Y. enterocolitica* (CECT 4315), and *A. hydrophila* (CECT 389), all obtained from the Spanish Type Culture Collection (Valencia, Spain). Stocks of the strains were stored at −80 °C in Luria−Bertani broth (LB broth acc. to MILLER) (Scharlau Chemie, S.A., Barcelona, Spain) with 30% glycerol. When required, strains were routinely grown aerobically with shaking in LB broth and incubated at 30 °C for 24 h. LB broth, solidified with 1.2% agar (Scharlau Chemie, S.A.) when required, was supplemented with the appropriate antibiotics (*C. violaceum* CV026 kanamycin 20 μg/mL; *A. tumefaciens* gentamycin 50 μg/mL). The pH of the culture media was 7.1.

Detection of the QS Inhibitory Capacity of Chestnut Honey Using Bacteriological Monitoring Systems. The QS inhibitory capacity of chestnut honey and its aqueous and methanolic extracts was assayed by the agar-well diffusion test as previously described by McClean et al. (*27*) to detect molecules that antagonize AHL stimulation using the biosensor strains *C. violaceum* CV026 and *A. tumefaciens*. Agar plates were prepared by adding 10^5 colony-forming units (cfu)/mL of an overnight culture of the biosensor strain to the LB containing 1.2% (w/v) agar supplemented with 100 nM C6-HSL (Sigma). In the case of *C. violaceum* CV026, wells were filled with different dilutions of the chestnut honey and its aqueous and methanolic extracts (0.1, 0.2, 0.3, and 0.4 g/mL) made in distilled water. Plates were incubated at 30 °C for 24 h and then examined for the presence of inhibition zones around wells by contrast camera imaging (ProtoCOL, Synoptics, Cambridge, U.K.). Inhibition of the violacein synthesis was defined by the presence of colorless haloes in a purple background. Agar plates containing 10^5 cfu/mL of *A. tumefaciens* were used to determine the inhibitory capacity of chestnut honey and its aqueous and methanolic extracts against the AHL production of *E. carotovora*, *Y. enterocolitica*, and *A. hydrophila*. In this case, overnight cultures of each bacterium containing chestnut honey and chestnut honey extracts at a final concentration of 0.2 g/mL were extracted twice into an equal volume of acidified ethyl acetate (0.5% formic acid) and incorporated in the plate wells. Plates were incubated at 30 °C for 24 h and then examined for the presence of induction zones around wells by contrast camera imaging (ProtoCOL). The induction zones were defined by the presence of a blue halo.

Extraction, Detection, Identification, and Quantification of AHLs Produced by *E. carotovora*, *Y. enterocolitica*, and *A. hydrophila* by LC-MS/MS. The extraction of AHLs was performed as previously described (*23*), with some modifications. Extracts for analytical LC-MS/MS were prepared from 50 mL of bacterial stationary phase cultures in LB broth. After incubation at 30 °C for 48 h, bacterial cells were

removed by centrifugation at 9632*g* for 15 min at 4 °C, and supernatants were filtered through a Millex-HV_{13} 0.45 μm filter (Millipore Corp.). The cell-free supernatants were extracted twice into an equal volume of acidified ethyl acetate (0.5% formic acid). The solvent was removed under vacuum at 35 °C and dried extract reconstituted in 5 mL of methanol. The solvent was removed by evaporation under N_2. Then, the residue was redissolved in 400 μL of methanol, filtered through a Millex-HV_{13} 0.45 μm filter (Millipore Corp.), and stored at −20 °C until further analysis by LC-MS/MS.

The detection, identification, and quantification of AHLs were studied as previously described by Morin et al. (*28*) with some modifications. The AHLs were analyzed in a HPLC system equipped with an Agilent 1100 series diode array and a mass detector in series (Agilent Technologies, Waldbronn, Germany). The HPLC system consisted of a binary pump (G1312 A), an autosampler (G1313 A), a degasser (G1322 A), and a photodiode array detector (G1315 B) controlled by software (v. A08.03). The column used was a 250 mm × 4.6 mm i.d., 5 μm, Li-Chrospher 100 RP-18, with a 4 mm × 4 mm i.d. guard column of the same material (Merck, Darmstadt, Germany) with water (A) and methanol (B) as HPLC grade solvents (Merck). An isocratic profile began with 50% B in A for 10 min to reach 60% B in A at 10.1 min, 80% B in A at 15 min, and 90% B in A at 18 min, followed by a gradient isocratic until 30 min. The flow rate of 0.4 mL/min was split 1/20 using a microsplitter valve. The postcolumn additive, a mixture of 5 mM ammonium acetate and 0.03% trifluoroacetic acid in methanol−water, was added using a syringe pump and a syringe (500 μL) at a flow rate 150 μL/h.

The mass detector was an ion trap spectrometer (G2445A) equipped with an electrospray ionization (ESI) system and controlled by software (v. 4.1). The nebulizer gas was N_2; the pressure and the flow rate of the dryer gas were set at 15 psi and 7 L/min, respectively. The full scan mass covered the range from *m*/*z* 95 to 250. Collision-induced fragmentation experiments were performed in the ion trap using He as collision gas, with voltage ramping cycles from 0.3 to 2 V. The heated capillary and voltage were maintained at 350 °C and 4 kV, respectively. Some parameters have an important effect on sensitivity. Thus, different analytical conditions were applied to optimize the detection of the different AHLs.

The split was directly adjusted up to the maximum rate (1/20) to gain the highest MS sensitivity. For the optimization of ESI, source flow injection analyses (FIA) with each standard (20 μM) and different nebulizer and dryer gas flows as well as temperatures were tested. Optical MS and trap parameters were optimized by direct infusion to the ESI source. Automatic optimization option of the MS for each standard was used. The total chromatogram time was divided into segments to set the ideal specific conditions for each AHL. We also compared the different MS analyzing modes to reach the best sensitivity conditions. We tested the multiple reaction monitoring (MRM) and the selected ion monitoring (SIM) modes. MRM was tested on several MS/MS transitions of the corresponding ions. We concluded that SIM was a better mode for collecting higher signal intensities of each AHL. The common parameters for all lactones were as follows: octopole, 2.4 V; octopole Δ, 2.0 V; octopole RF, 120.0 V; lens 1, −5.0 V; and lens 2, −60.0 V. The detector voltages were multiplier, −1740 V, and dynode, −7.0 V. MS data were acquired in the positive mode. The identification and quantification of the peaks were based on MS/MS fragmentation products ions. The final LC-MS/MS analyses were performed the same day using the autosampler to avoid fluctuation in the ionization of the equipment. The area of the ion *m*/*z* 102 was selected to quantify each AHL because of its specificity and its better signal-to-noise-ratio (*28*). Authentic markers of AHLs were used for quantitation of individual lactones.

Quantification of the Inhibition by Chestnut Honey of AHLs in *E. carotovora*, *Y. enterocolitica*, and *A. hydrophila* Using LC-MS/MS. Cultures of *E. carotovora*, *Y. enterocolitica*, and *A. hydrophila* were grown in 50 mL of broth containing chestnut honey or its aqueous and methanolic extracts (0.2 g/mL) and incubated at 30 °C for 24 h. Bacterial cultures grown in LB broth without the addition of chestnut honey or chestnut honey extracts were used as a control. After incubation, AHLs present in the culture media were extracted with acidified ethyl acetate and subjected to analysis by LC-MS/MS as previously described. The potential degradation of AHLs by chestnut honey was determined by adding a known amount of synthetic standards (3-oxo-C6-HSL, C6-HSL, and C4-HSL) to 50 mL of LB broth untreated and treated with the honey extracts. The AHL concentration in LB broth was quantified after 2 h of incubation as previously described.

Table 1. Inhibition of Violacein in *C. violaceum* CV026 Cultures Untreated and Treated with Chestnut Honey and Its Aqueous and Methanolic Extracts at Different Concentrations[a]

	concentrations			
treatment	0.1 g/mL	0.2 g/mL	0.3 g/mL	0.4 g/mL
honey	5.7 ± 3.3a	21.6 ± 3.3a	27.7 ± 6.7a	31.9 ± 6.1a
aqueous extract	7.4 ± 0.5a	16.4 ± 0.9b	20.3 ± 0.5a	24.4 ± 0.6a
methanolic extract	0.0 ± 0.0b	0.0 ± 0.0c	0.0 ± 0.0b	0.0 ± 0.0b

[a] The results are the mean (n = 6) ± standard deviation. Values followed by different letters indicate significant difference at $P < 0.05$.

Inhibition of Biofilm Formation of *E. carotovora*, *Y. enterocolitica*, and *A. hydrophila* by Chestnut Honey. The effects of chestnut honey and its aqueous and methanolic extracts (0.2 g/mL) on biofilm formation by *E. carotovora*, *Y. enterocolitica*, and *A. hydrophila* were studied using the crystal violet assay, previously described by Girennavar et al. (*21*), with some modifications. Overnight cultures of the selected bacteria were diluted (1:100) in buffered peptone water (Scharlau Chemie, S.A.). Wells of the sterile round-bottom 96-well polystyrene plates (Nalge Nunc International, Rochester, NY) containing 160 μL of LB broth and 20 μL of stock solution (2 g/mL) of chestnut honey or its aqueous and methanolic extracts were inoculated with 20 μL of the overnight diluted culture. The control contained 160 μL of LB broth, 20 μL of sterile water, and 20 μL of the overnight diluted culture. The negative control contained sterile culture medium (160 μL) and distilled water (40 μL). After 24 h of incubation at 30 °C, the wells were emptied and washed three times with sterile water. The biofilm layer on the wall of the wells was fixed with 200 μL of acidified methanol (acetic acid 33%). The biofilms were dyed with 125 μL of crystal violet (0.3%) per well for 15 min, and the excess pigment was removed by washing the plates three times with distilled water. For quantification of attached cells the crystal violet was solubilized with 200 μL of 95% ethanol, and the absorbance was measured at 570 nm using a spectrophotometer (Synoptics, Cambridge, U.K.). Data were obtained as an average of eight replicate wells.

Inhibition of *E. carotovora*, *Y. enterocolitica*, and *A. hydrophila* Growth by Chestnut Honey. The antimicrobial activity of chestnut honey against *E. carotovora*, *Y. enterocolitica*, and *A. hydrophila* was evaluated. Ten milliliters of LB broth was inoculated with 10 μL of a working culture of each pathogenic bacteria and chestnut honey at a final concentration of 0.2 g/mL. Inoculated honey solutions were mixed followed by incubation at 30 °C for 20 h. Inoculated and incubated 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.). Microbial counts were expressed as log colony-forming units per milliliter.

Statistical Analysis. At least three replications of each experiment, separately in time, were carried out. In each experiment, two samples were evaluated per treatment and tested bacteria. ANOVA and Brown−Forsythe tests were used depending on the homogeneity of the variances. When significant differences were observed, Tukey or Dunnett T3 tests were performed using SPSS 17.0 for Windows. A significance level of $P \leq 0.05$ was selected to determine differences between samples.

RESULTS AND DISCUSSION

QS Inhibitory Capacity of Chestnut Honey Using Bacteriological Monitoring Systems. The capacity of chestnut honey and chestnut honey extracts (aqueous and methanolic) to inhibit or degrade AHL in *C. violaceum* cultures was, in most cases, concentration dependent (**Table 1**). Compared to control, chestnut honey and its aqueous extract showed a significant drop in violacein content, even at the lowest tested concentration (0.1 g/mL) (**Table 1**). However, the methanolic extract of chestnut honey did not show inhibitory activity when using the agar-well diffusion test (**Table 1**). The obtained results suggest that most of the active compounds, which inhibit or degrade AHLs, are

present in the aqueous extract. This is in agreement with previously published data reporting the ability of different unifloral honeys as QS inhibitors against the bacterium model *C. violaceum* (*15*). These authors also concluded that phenolic compounds, mainly present in the methanolic extract, did not significantly contribute to the inhibitory activity of honey. Weston et al. (*29*) also found that all of the antibacterial activity of the manuka honey seemed to be eluted with the carbohydrates, whereas no significant activity was observed for the phenolic compounds. Thus, the QS inhibitory activity, as well as the antimicrobial capacity of honey, seems to be related to water-soluble compounds, rather than phenolics mainly present in the methanolic extract (*15*). Because the monosaccharides have no antibacterial properties, it has been suggested that an antibacterial substance is being "carried" by the monosaccharides, which form the bulk of the mass of honey (*29*).

The capacity of chestnut honey and chestnut honey extracts to inhibit or degrade AHL in *E. carotovora*, *Y. enterocolitica*, and *A. hydrophila* cultures was determined using the biosensor strain *A. tumefaciens*. This biosensor is sensitive to most 3-oxo-AHLs, whereas *C. violaceum* mainly responds to short-chained unsubstituted AHLs (*27*). Because *E. carotovora* and *Y. enterocolotica* produce 3-oxo-C6-HSL, *A. tumefaciens* was more appropriate to detect these types of molecules. All of the sterile-filtered culture supernatants from outgrown *A. hydrophila*, *E. carotovora*, and *Y. enterocolitica* induced the AHL monitor bacteria *A. tumefaciens* in the well diffusion assay. **Table 2** shows the different induction zones generated by the AHL of the selected bacteria in culture media. As expected, the induction zones were larger in control plates, to which chestnut honey or chestnut honey extracts were not added. However, in the presence of chestnut honey and its aqueous and methanolic extracts, the induction zones were significantly reduced (**Table 2**). As previously observed using the biosensor *C. violaceum*, honey and its aqueous extract were the most active fractions, reducing AHL concentration in the bacterial cultures. In this assay, the methanolic extract also reduced the AHL concentration in the culture media of the three tested bacteria. This confirms the higher sensibility of *A. tumefaciens* as a biosensor strain when compared to *C. violaceum* (*27*). However, the inhibitory capacity of the methanolic extract was much lower than those recorded for honey and its aqueous extract (**Table 2**).

Table 2. Induction of Blue Color Production in *A. tumefaciens* NT1 by Supernatants of *E. carotovora*, *Y. enterocolitica*, and *A. hydrophila* Cultures Grown in LB Broth Untreated and Treated with 0.2 g/mL of Chestnut Honey and Its Aqueous and Methanolic Extracts[a]

	induction zones		
treatment	*E. carotovora*	*Y. enterocolitica*	*A. hydrophila*
control	38.8 ± 0.7a	36.9 ± 3.7a	39.2 ± 0.7a
honey	28.8 ± 0.4c	28.5 ± 0.8b	19.7 ± 1.3d
aqueous extract	25.8 ± 0.3d	25.2 ± 0.3b	25.6 ± 2.0c
methanolic extract	34.0 ± 2.3b	29.4 ± 2.6b	29.5 ± 4.1b

[a] The results are the mean (n = 6) ± standard deviation. Values followed by different letters indicate significant difference at $P < 0.05$.

Detection and Quantification of AHLs by LC-MS/MS. As previously mentioned, the method of analysis employed here is based on the online LC-MS/MS detection and quantification developed by Morin et al. (*28*). In the present study, to reduce interface contamination and mainly to improve the MS response, the flow rate of the eluent was reduced by 1/20 using a postcolumn split, which considerably increased the signal intensity final flow rate (0.02 mL/min). Additionally, the postcolumn additive, a mixture of 5 mM ammonium acetate and 0.03% trifluoroacetic acid in methanol−water (50:50, v/v) reduced the formation of the adduct ion $[M + Na]^+$, without a suppression effect of the $[M + H]^+$ signal or an increase in the background signal. The plot of specific ion $[M + H]^+$ chromatographs allowed a good identification of each AHL even if two or more AHLs coeluted in the same chromatographic segment, such as 3-oxo-C6-HSL and C4-HSL (**Figure 1**). The MS study of AHL standards showed $[M + H]^+$ ions at m/z 172, 214, and 200 and corresponding to 3-oxo-C6-HSL, C6-HSL, and C4-HSL, respectively. This study also revealed that all of the strains produced similar AHLs, which have in common the homoserine lactone ring structure ($C_4H_8O_2N$)

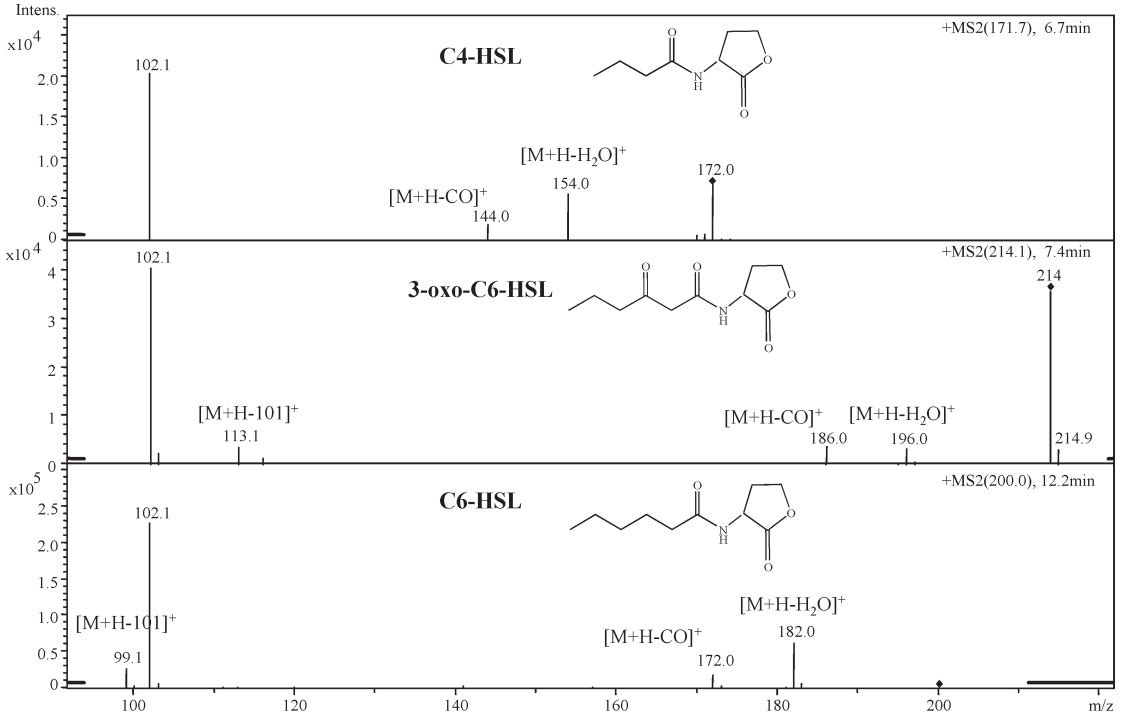

Figure 1. MS/MS spectra of C4-HSL, 3-oxo-C6-HSL, and C6-HSL.

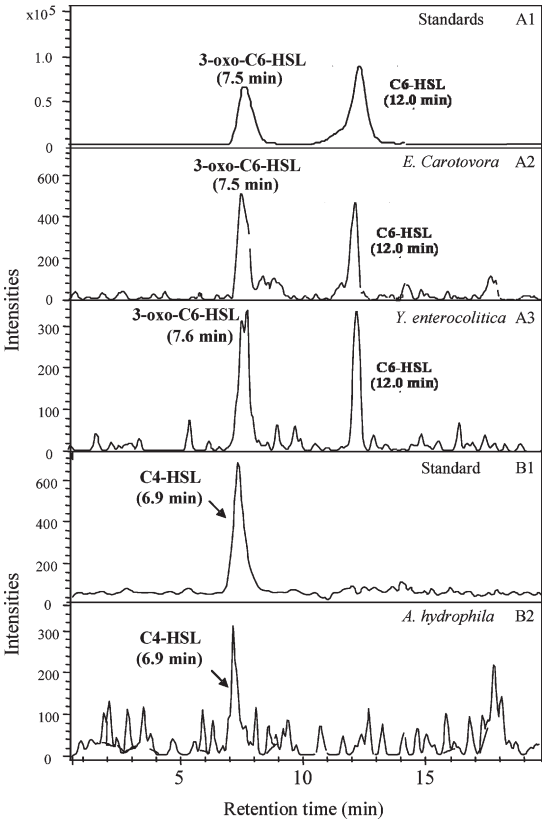

Figure 2. Selected *m*/*z* 102 chromatograms of 3-oxo-C6-HSL and C6-HSL from standards (**A1**) and supernatants of *E. carotovora* (**A2**) and *Y. enterocolitica* cultures (**A3**) and selected *m*/*z* 102 chromatograms of C4-HSL from standard (**B1**) and supernatant of *A. hydrophila* culture (**B2**).

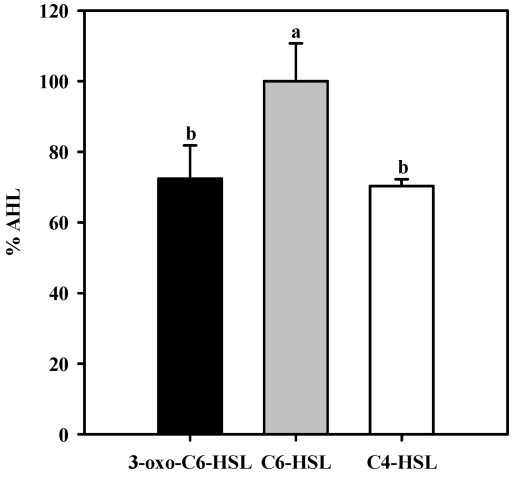

Figure 3. Percentage of AHL in uninoculated culture media treated with chestnut honey and chestnut honey extracts and using untreated samples as the reference value. Vertical bars represent the standard deviation ($n = 3$). Bars labeled with different letters indicate significant difference at $P < 0.05$.

and varied only in the acyl chain length and substitution at carbon 3. The mass spectra showed complex fragmentation patterns, but a predominant fragment ion appeared at *m*/*z* 102 by protonation of the homoserine lactone ring (**Figure 1**) (*28*). On the other hand, the molecular ion $[M + H - 101]^+$, which resulted from the loss of the homoserine lactone ring, was detected in relatively minor abundance (**Figure 1**). Thus, for the LC-MS/MS detection of AHLs, the molecular ion *m*/*z* 102 was chosen in preference to ion

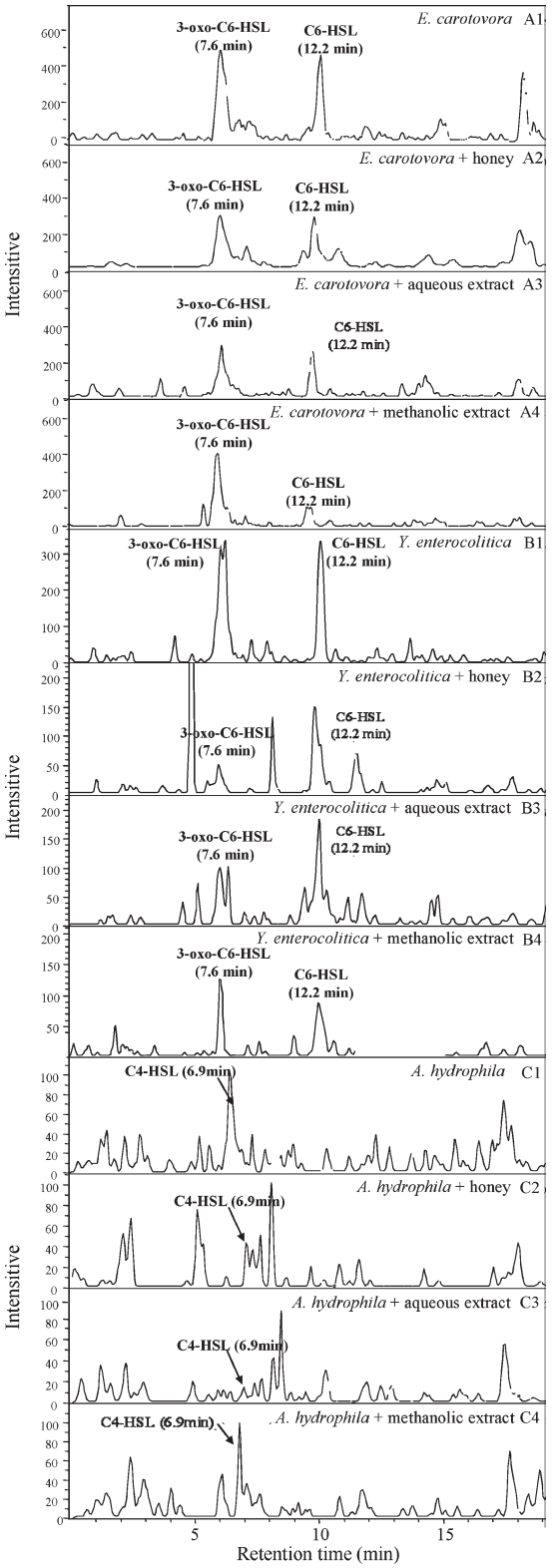

Figure 4. MS/MS chromatograms of 3-oxo-C6-HSL and C6-HSL from supernatant of *E. carotovora* culture (**A1**) and supernatants of *E. carotovora* culture supplemented with 0.2 g/mL of chestnut honey (**A2**) and its aqueous (**A3**) and methanolic extracts (**A4**), 3-oxo-C6-HSL and C6-HSL from supernatant of *Y. enterocolitica* culture (**B1**) and supernatants of *Y. enterocolitica* culture supplemented with 0.2 g/mL chestnut honey (**B2**) and its aqueous (**B3**) and methanolic extracts (**B4**), and C4-HSL from supernatant of *A. hydrophila* culture (**C1**) and supernatants of *A. hydrophila* culture supplemented with 0.2 g/mL chestnut honey (**C2**) and its aqueous (**C3**) and methanolic extracts (**C4**).

Table 3. Percentage of Inhibition of AHL Production of *E. carotovora*, *Y. enterocolitica*, and *A. hydrophila* by 0.2 g/mL of Chestnut Honey and Its Aqueous and Methanolic Extracts[a]

	E. carotovora		*Y. enterocolitica*		*A. hydrophila*
treatment	3-oxo-C6-HSL	C6-HSL	3-oxo-C6-HSL	C6-HSL	C4-HSL
honey	45.1 ± 7.5ab	69.7 ± 9.6a	84.9 ± 1.4a	54.7 ± 12.2a	76.2 ± 4.1a
aqueous extract	50.7 ± 4.3a	76.8 ± 3.0a	85.5 ± 1.6a	62.0 ± 5.3a	61.9 ± 3.1b
methanolic extracts	40.2 ± 2.3b	55.2 ± 8.1b	80.8 ± 5.0a	64.3 ± 4.8a	41.9 ± 2.0c

[a] The results are the mean ($n = 4$) ± standard deviation. Values followed by different letters indicate significant difference at $P < 0.05$.

$[M + H - 101]^+$ as the former gave more abundant ions for all of the AHLs (*30*). Other observed fragments were the pseudomolecular ions at $[M + H - H_2O]^+$ and $[M + H - CO]^+$, which were obtained from protonation of the carbonyl function of the acyl chain or the ethoxy function of the lactone ring, respectively (**Figure 1**).

The potential interference of the ingredients of the bacterial culture media (LB broth) was avoided by means of an ethyl acetate extraction of the LB broth followed by the LC-MS/MS analysis. The MS analysis showed that LB broth extracted with acidified ethyl acetate did not contain any peak corresponding to m/z 102 (data no shown). The specific identification of each of the AHLs extracted from the bacterial culture media was based on the detection of the fragment ions of each AHL and the retention times of the standards (**Figure 2**). The area of the ion m/z 102 was selected to quantify each AHL because of its specificity and its better signal-to-noise-ratio (*28*). **Figure 2** shows the selected ion m/z 102 chromatograms for the AHLs produced by *E. carotorova*, *Y. enterocolitica*, and *A. hydrophila*. The identical traces for extracts and the synthetic 3-oxo-C6-HSL and C6-HSL standards confirmed these AHLs as the major signal molecules present (*30*, *31*). Additionally, the major signal molecule synthesized by *A. hydrophila* was identified as C4-HSL by MS. This is in accordance with previous studies on the AHL production of *Aeromonas* spp. (*6*, *10*).

Inhibition by Chestnut Honey of AHLs and Biofilm Formation in *E. carotovora*, *Y. enterocolitica*, and *A. hydrophila*. The reduction of AHL concentrations in bacterial cultures treated with chestnut honey could be the result of either (i) inhibition of cell growth, (ii) degradation of AHLs, or (iii) inhibition of the AHL production. With regard to the first hypothesis, it was observed that microbial counts of bacterial cultures untreated and treated with chestnut honey were very similar, without significant differences after 20 h of incubation. Microbial loads were close to the maximum population density in both untreated and treated samples, with average values of 9.3 ± 0.1 log cfu/mL for *E. carotovora* and 9.1 ± 0.1 log cfu/mL for *Y. enterocolitica* and *A. hydrophyla*. The second hypothesis refers to the potential degradation of AHLs by chestnut honey. The quantification of AHLs in uninoculated broth untreated and treated with chestnut honey and its aqueous and methanolic extracts revealed that chestnut honey was able to degrade the 3-oxo-C6-HSL and C4-HSL present in the culture media. However, C6-HSL seemed to be unaffected by chestnut honey (**Figure 3**). Therefore, the anti-QS activity of chestnut honey was, at least partially, due to the degradation of the signal. To test the viability of the third hypothesis, the capacity of chestnut honey and its aqueous and methanolic extracts to inhibit the AHL production by *E. carotovora*, *Y. enterocolitica*, and *A. hydrophyla* was also evaluated. **Figure 4** shows the MS/MS profile of the AHL peaks at 102 produced by *E. carotovora*, *Y. enterocolitica*, and *A. hydrophila*, untreated and treated with chestnut honey or its aqueous and methanolic extracts. The MS/MS profiles showed the capacity of chestnut honey to reduce the

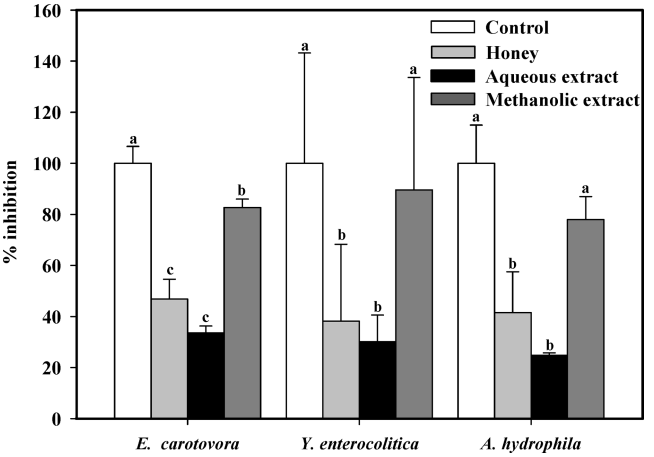

Figure 5. Percentage of inhibition of biofilm formation in *E. carotovora*, *Y. enterocolitica*, and *A. hydrophila* cultures grown in LB broth untreated and treated with chestnut honey and its aqueous and methanolic extracts. Vertical bars represent the standard deviation ($n = 8$). Bars labeled with different letters indicate significant difference at $P < 0.05$.

AHL production in the tested pathogenic strains. The highest inhibitory activity was observed against 3-oxo-C6-HSL and C4-HSL production by *Y. enterocolitica* and *A. hydrophila*, respectively. **Table 3** shows the percentage of inhibition of AHL calculated, taking as reference the AHL production of each bacterial strain in LB broth without the addition of chestnut honey or its aqueous and methanolic extracts. When it is taken into account that AHL degradation was always < 30%, the inhibition of QS by chestnut honey can also be attributed to its capacity in inhibiting AHL production.

As previously found using the biosensor strain, the MS/MS analysis confirmed the higher inhibitory activity of the aqueous extract and chestnut honey (**Table 3**). This corroborates a previous finding, which attributes the antimicrobial and QS inhibitory activities to the aqueous honey extract (*15*, *29*). It was expected that the methanolic extract would show the lowest inhibitory activity in all of the tested strains. However, in the case of *Y. enterocolitica*, no significant differences were observed between the chestnut honey and its aqueous and methanolic extracts. These results also agree with the data obtained using the biosensor strain *A. tumefaciens* (**Table 2**).

Many research studies have been focused on the prevention and control of biofilm formation by using natural compounds. In this study, the effect of chestnut honey and its aqueous and methanolic extracts on biofilm formation by *E. carotovora*, *Y. enterocolitica*, and *A. hydrophila* was evaluated and is summarized in **Figure 5**. Biofilm formation of all the tested strains was significantly reduced by chestnut honey and its aqueous extract. However, only slight reductions were observed when the methanolic extract was added to the culture media of the three tested bacteria. It was observed that *Y. enterocolitica* and *A. hydrophila*

showed a significant reduction in biofilm formation, ranging from 58.3 to 61.7% when honey was added to the culture media and from 69.7 to 75.0% when the aqueous extract of chestnut honey was added. *E. carotovora* was less affected, but it also showed significant reductions of 53.2 and 66.4% after the addition of honey and its aqueous extract. The methanolic extract also reduced ($P = 0.03$) biofilm formation in the tested strains, ranging from 10.4 to 20.5%. Previous studies have demonstrated the efficacy of manuka honey in reducing biofilm formation of *Pseudomonas aeruginosa* and dental plaque (*32*, *33*). However, most of the research papers found in the literature evaluated the effect of natural compounds extracted from plants on the biofilm formation of pathogenic bacteria (*4*, *21*). It is, therefore, reasonable to continue investigating the inhibition of biofilm formation by different types of honeys and to determine the potential therapeutic uses of this natural product.

In conclusion, the present study evidenced the capacity of chestnut honey and its aqueous extract as QS inhibitors in *E. carotovora*, *Y. enterocolitica*, and *A. hydrophila*. The QS inhibition was due to both the degradation of AHLs and the inhibition of AHL production by the bacterial strains. However, no growth inhibition was observed at the tested honey concentration. Additionally, it was observed that the interruption of the QS system caused an attenuation of bacterial AHL-controlled processes, such as the reduction in biofilm formation by the tested pathogenic bacteria. Among the tested honey extracts (0.2 g/mL), the aqueous extract showed the highest inhibitory activity against AHL and biofilm formation, suggesting that the QS inhibitory activity seems to be related to the aqueous fraction. Considering that QS inhibitors may help to attenuate virulence and reduce biofilm formation, they may be of interest in the development of novel natural microbial intervention strategies.

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