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Inhibition of Quorum Sensing (QS) in *Yersinia enterocolitica* by an Orange Extract Rich in Glycosylated Flavanones

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ABSTRACT: Flavanones, flavonoids abundant in *Citrus*, have been shown to interfere with quorum sensing (QS) and affect related physiological processes. We have investigated the QS-inhibitory effects of an orange extract enriched in *O*-glycosylated flavanones (mainly naringin, neohesperidin, and hesperidin). The QS-inhibitory capacity of this extract and its main flavanone components was first screened using the bacteriological monitoring system *Chromobacterium violaceum*. We next examined the ability of the orange extract and of some of the flavanones to (i) reduce the levels of the QS mediators produced by *Y. enterocolitica* using HPLC-MS/MS, (ii) inhibit biofilm formation, and (iii) inhibit swimming and swarming motility. Additionally, we evaluated changes in the expression of specific genes involved in the synthesis of the lactones (*yenI*, *yenR*) and in the flagellar regulon (*flhDC*, *fleB*, *fliA*) by RT-PCR. The results showed that the orange extract and its main flavanone components inhibited QS in *C. violaceum*, diminished the levels of lactones secreted by *Y. enterocolitica* to the media, and decreased QS-associated biofilm maturation without affecting bacterial growth. Among the tested compounds, naringin was found to inhibit swimming motility. Exposure to the orange extract and (or) to naringin was also found to be associated with induction of the transcription levels of *yenR*, *flhDC*, and *fliA*. This work shows the in vitro QS-inhibitory effects of an orange extract enriched in flavanones against a human enteropathogen at doses that can be achieved through the diet and suggests that consumption of these natural extracts may have a beneficial antipathogenic effect.

KEYWORDS: *cell−cell communication, Citrus polyphenols, flavonoids anti-QS activity, quorum quenching, LC-MS/MS spectrometry, swimming, swarming, pathogenic bacteria, gene expression*

INTRODUCTION

Phenolic compounds extracted from medicinal and dietary plants are believed to have a range of beneficial effects against human chronic diseases such as cardiovascular diseases and inflammation.[1] A representative group of phenolic compounds is the flavonoids, consisting of different subgroups of compounds such as flavones, flavonols, flavanones, flavan-3-ols, and isoflavones. Citrus fruits are the main sources of flavanones in the diet, where they are present principally in the glycoside form. The more abundant flavanones glycosides in citrus fruits are hesperidin (hesperetin-7-*O*-rutinoside) and naringin (naringenin-7-*O*-neohesperidoside) with concentrations ranging from 200 to 590 mg/L and from 16 to 84 mg/L in orange juice, respectively.[2] Hesperidin and naringin are poorly absorbed because the glycoside group can only be hydrolyzed in the distal part of the intestine.[3] Therefore, these flavanone glycosides can reach the colon in their intact molecular form and may exert some effects on the microorganisms present in the distal part of the intestine.[4,5] Recently, several studies have demonstrated that certain flavonoids can act as inhibitors of the virulence of pathogenic bacteria by interfering with quorum sensing (QS) mechanisms.[6,7] QS is a regulatory mechanism that enables bacteria to make collective decisions with respect to the expression of a specific set of genes. It has been demonstrated that QS mechanisms amplify bacterial virulence by stimulating the expression of disease-causing attributes, such as motility, biofilm formation, and secretion of virulence factors.[8]

The genus *Yersinia* comprises human-pathogenic species such as the enteropathogenic *Yersinia enterocolitica*. This pathogenic bacterium is a γ-proteobacterium that colonizes the small intestine and can cause gastrointestinal distress as well as septicemia in immunocompromised patients.[9] *Y. enterocolitica* uses *N*-acylhomoserine lactones (AHLs) as QS signal molecules to coordinate the expression of a battery of genes.[10] The synthesis of AHLs by *Y. enterocolitica* is regulated by a gene encoding an AHL synthase (*yenI*) and a gene encoding a transcriptional activator named *yenR*.[11] The QS systems of *Y. enterocolitica* are also involved in the regulation of three major flagellar gene classes that control swimming and swarming motility.[12] Swarming motility is a flagellum-dependent behavior that facilitates bacterial migration over solid surfaces and is distinct from swimming motility, which occurs in fluid environments.[13] It is well established that bacterial biofilms play an important role in the pathogenesis of many human infections and increase bacterial resistance to antimicrobial agents.[14] Many studies have suggested that QS can play an

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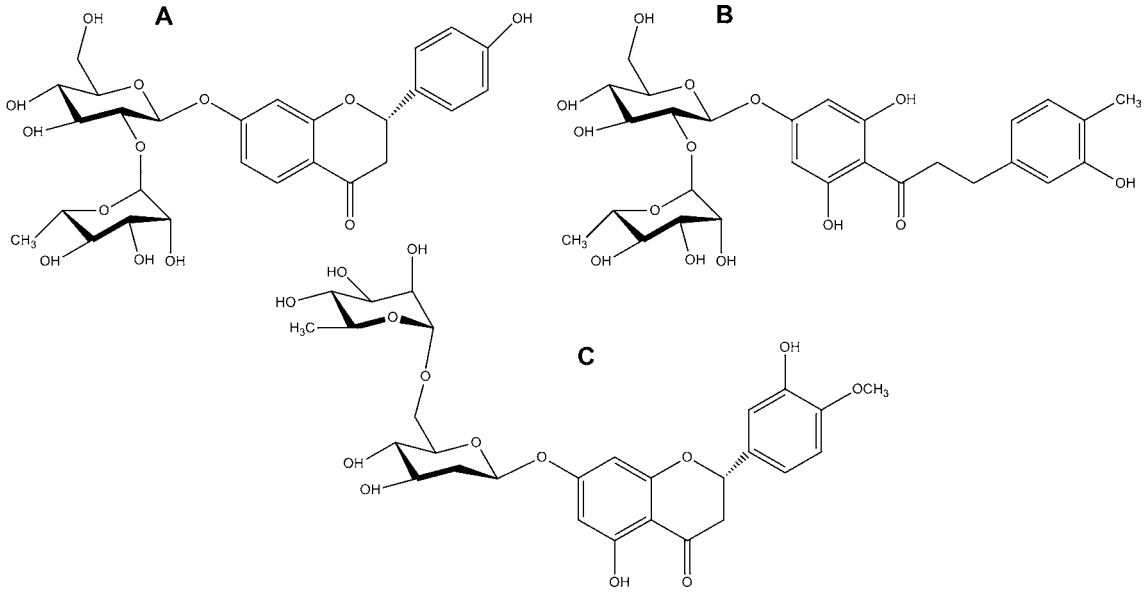

Figure 1. Chemical structures of the major flavanones present in the orange extract: (A) naringin; (B) neohesperidin; (C) hesperidin.

important role in the formation of fully developed mature biofilms in several bacteria.[15,16]

In view of the important role of the QS mechanisms in the regulation of different virulence factors such as motility and biofilm formation, the inhibition of QS systems is considered to be a potential way to reduce the virulence of pathogenic bacteria.[7,17,18] In previous studies, citrus flavonoids have been reported to efficiently inhibit QS mechanisms, cell−cell signaling, and biofilm formation in *Vibrio harveyi* and *Escherichia coli* O157:H7.[19] These authors also demonstrated that some flavonoids altered the expression of genes encoding the type 3 secretion system in *V. harveyi*. In agreement with these results, Vandeputte et al.[20] also showed that naringenin, a citrus flavonoid, dramatically reduced the production of AHLs *N*-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C12-HSL) and *N*-butanoyl-L-homoserine lactone (C4-HSL) as well as the expression of several QS-related genes (i.e., *lasI*, *lasR*, *rhlI*, *rhlR*, *lasA*, *lasB*, *phzA1*, and *rhlA*) in *P. aeruginosa* PAO1.[20] In the search for natural products that may act as QS inhibitors, we investigated the capacity of an orange extract rich in flavanones to inhibit the production of AHLs, as well as QS-controlled biofilm formation and motility, in the pathogenic bacteria *Y. enterocolitica*. Additionally, some of the molecular changes associated with the exposure of *Y. enterocolitica* to the orange extract or naringin were also evaluated.

MATERIALS AND METHODS

Materials. Naringin (naringenin-7-*O*-neohesperidoside), neohesperidin (hesperetin-7-neohesperidoside), hesperidin (hesperetin-7-*O*-rutinoside), and a soluble extract from bitter orange were all kindly provided by Zoster S.A. (Murcia, Spain). Naringenin (4′,5,7-trihydroxyflavanone) was from Sigma-Aldrich (Sigma Chemical, St. Louis, MO, USA) and cinnamaldehyde from Extrasynthèse (Genay, France). The extract was composed of approximately 42.1% soluble flavanones (% of the powder extract) with one most abundant compound, naringin (24.5 ± 2.6%), followed by neohesperidin (11.4 ± 0.8%), hesperidin (1.7 ± 0.4%), naringenin (0.4 ± 0.1%), hesperetin (0.4 ± 0.1%), and isosakuranetin (0.2 ± 0.1%).[5] The structures of the three main flavanone constituents of the orange extract are shown in Figure 1. The extract (a hygroscopic brown powder, 2.1% humidity) was kept in a tightly closed container within a desiccator at room temperature, and fresh stock solutions were prepared by dissolving 0.1 g of the powder extract in 50 mL of water. Dilutions of the stock solution were added to the cultures to obtain the final experimental concentrations stated for each assay. Controls were always treated with the equivalent amount of water. Stock solutions of the individual flavanones and cinnamaldehyde were prepared in DMSO, diluted, and added to the cultures to obtain the final experimental concentrations stated for each assay. Controls were always treated with the equivalent amount of DMSO (<0.1%). Pure commercially available *N*-hexanoyl-DL-homoserine lactone (C6-HSL, ≥97%, HPLC) and *N*-(3-oxohexanoyl)-DL-homoserine lactone (3-oxo-C6-HSL, ≥97%, HPLC) were obtained from Sigma Chemical and dissolved in water. All other chemicals were of analytical/HPLC grade. Ultrapure Millipore water (Millipore Corp., Bedford, MA, USA) was used for all solutions.

Bacterial Strains, Culture Conditions, and Growth. *Y. enterocolitica* subsp. *enterocolitica* (CECT 4315) and *Chromobacterium violaceum* (CECT 494) were obtained from the Spanish Type Culture Collection (CECT, Valencia, Spain). Stocks of the strains were stored at −80 °C in Luria−Bertani broth (LB broth Miller) (Scharlau Chemie S.A., Barcelona, Spain) supplemented with 30% glycerol. Unless otherwise stated, working cultures of both strains were routinely grown in LB broth at 30 °C under aerobic conditions with shaking for 24 h. The pH of the cultures was 6.92−6.99 and was monitored using a calibrated Crison MicropH 2000 pH-meter (Crison Instruments S.A., Barcelona, Spain).

For bacterial growth analyses, diluted cultures of *Y. enterocolitica* were grown in sterile 96-well microtiter plates as previously described.[21] The culture conditions were as indicated above with a final volume of 200 μL per well. Plates were incubated at 30 or 25 °C in the absence or presence of the test compounds with intermittent shaking, and the OD_{600} was measured hourly for 48 h using an Infinite M200 microplate reader (Tecan, Grödig, Austria).

***C. violaceum* Assay.** To evaluate the QS -inhibitory capacity of the orange extract and of its main flavanone components (naringin, neohesperidin, and hesperidin), we first measured the inhibition of the production of violacein using the biosensor strain *C. violaceum* as previously described.[22] Working solutions of the orange extract and of each individual flavanone were filtered through a sterile Millex-GP 0.22 μm filter (Millipore Corp.) and added to the culture medium at the final concentrations of 100, 200, and 400 μg/mL (as confirmed by LC-MS/MS analysis). Ten milliliters of LB broth was inoculated with 10 μL of a working culture of *C. violaceum* and incubated with each of the concentrations of the extract or the flavanones for 24 h. Then, 1 mL of culture was centrifuged at 19314*g* for 10 min to precipitate the insoluble violacein. The culture supernatant was discarded, and 1 mL

Table 1. Primers and Probes Used in the Study of Gene Expression in *Yersinia enterocolitica*

gene	product	sequence accession	primers	reporter (FAM)
yenI	*N*-acylhomoserine lactone synthase	YP_001005892.1	forward primer: GATGTGAGCCTACCTATTGATGGTT reverse primer: CCCACCATATCTCTTGCTAATGCTT	CCAGCCGATTCTTTG
yenR	quorum-sensing transcriptional activator	AM286415.1	forward primer: CCGGTTATATTGACGGCGAAAGATA reverse primer: CGGCGGAATCCGTCGAT	CTGGGACGATAATTCC
fliA	RNA polymerase sigma factor for flagellar operon	AM286415.1	forward primer: AACAACGGCTCGGACGT reverse primer: CTGGCGGTACTCAGTCAAATCAATA	AAGTCGCACAAAATC
fleB	thermoregulated motility protein	L33468.1	forward primer: GGCGGTCGAAAGATAATGAACAAAT reverse primer: AGATTCCAGCCAGAACTTGCA	CCAATCGCCAATGCTG
flhDC	motility master regulatory operon	AF081587.1	forward primer: GCGCGCTTGCAGATGTT reverse primer: GCTGCCTCTCAGCTCTTTATAGA reverse primer: GTGGACTACCAGGGTATCTAATCCT	TTGCGTCTCGCTTTCT
16S	16R rRNA	AM286415.1	forward primer: GCCCCCTGGACAAAGACT	TCCCCACGCTTTCG

of DMSO was added to the pellet. The solution was vortexed vigorously for 30 s to completely solubilize violacein and centrifuged at 19314*g* for 10 min to remove the cells. The violacein was quantified at OD_{585} using a UV-1603 spectrophotometer (Shimadzu, Tokyo, Japan).

Analysis of the AHLs Produced by *Y. enterocolitica* Using LC-MS/MS. To assess the specific inhibitory effect of the orange extract or of the flavanones on the levels of each of the two main AHLs produced by *Y. enterocolitica*, 50 mL of LB was inoculated with 100 μL of a bacteria working culture in the absence and presence of the test compounds (at 200 μg/mL) and incubated at 30 °C for 48 h. We additionally tested a combination of naringin and neohesperidin (49 and 22.8 μg/mL, respectively) to investigate a possible synergistic effect between these two most abundant flavanones in the extract. After incubation, bacterial cells were removed by centrifugation at 9632*g* at 4 °C for 15 min, and the supernatants were filtered through a Millex-GP 0.22 μm filter (Millipore Corp.). The cell-free culture supernatants were extracted (two times) with 50 mL of acidified ethyl acetate (0.5% formic acid). The organic phase was evaporated under reduced pressure at 35 °C and the residue reconstituted in 2 mL of methanol. Methanol was removed in a Speedvac SPD 121P concentrator (Thermo Savant, Waltham, MA, USA) and the residue redissolved in 400 μL of methanol, filtered through a Millex-HV_{13} 0.45 μm filter (Millipore Corp.), and stored at −20 °C prior to analysis by LC-MS/MS.

To further investigate a putative direct chemical interaction between the orange extract or each of the flavanones with the AHLs, we also measured the levels of C6-HSL and 3-oxo-C6-HSL in the culture media after incubation for 48 h of known concentrations of the AHLs (50 nM, 10 ng/mL) with the orange extract (200 μg/mL) equivalent to 49 μg/mL of naringin (84.5 μM), 22.8 μg/mL of neohesperidin (37.0 μM), and 3.4 μg/mL of hesperidin (5.6 μM) or each single flavanone (at the same concentration present in the extract) in the absence of *Y. enterocolitica*. Culture medium was extracted and filtered as above prior to LC-MS/MS analysis.

The levels of the AHLs were measured using LC-MS/MS following a method previously described [23] and further optimized for the specific analysis and relative quantification of C6-HSL and 3-oxo-C6-HSL in the culture media of several bacteria including *Y. enterocolitica*.[22] Briefly, the peaks corresponding to C6-HSL and 3-oxo-C6-HSL were identified on the basis of their MS/MS fragmentation ion products and on the retention times of commercial standards for each lactone. We selected the area of the characteristic single ion m/z 102 to quantitate each AHL because of its specificity and better signal-to-noise ratio. The specific use of the extracted ion chromatograms (EIC) for area calculation and quantification reduces the possibility of misinterpreting overlapping peaks. Samples were injected and analyzed on the same day and run under the same conditions to avoid the influence of the ionization fluctuation of the equipment. This method allows for relative quantification without the need for a standard curve. Results are presented as the percent of inhibition with respect to the quantity of AHLs in the control samples (without test compounds).

Biofilm Formation. The effects of the orange extract and its main flavanone components on the formation of biofilm by *Y. enterocolitica* were evaluated using the crystal violet assay[15] with some modifications.[22] A number of wells of a sterile round-bottom 96-well polystyrene plate (Nalge Nunc International, Rochester, NY, USA) containing 160 μL of LB broth and 20 μL of the orange extract or each of the flavanones (200 μg/mL final concentration) were inoculated with 20 μL of a working culture of *Y. enterocolitica* diluted (1:100) in buffered peptone water (Scharlau Chemie S.A.). Control wells (without the test compounds) contained 160 μL of LB broth, 20 μL of sterile water or an equivalent amount of DMSO (<0.1%), and 20 μL of a working culture of *Y. enterocolitica*. After 24 h of incubation, the wells were emptied and washed with sterile water (three times). The biofilm layer formed on the wall of the wells was fixed with 200 μL of acidified methanol (33% acetic acid), stained with crystal violet (0.1%) for 30 min, washed (four times) to eliminate unbound dye, and quantitated after solubilization of the crystal violet with 200 μL of 95% ethanol by measuring the OD_{570} using a spectrophotometer (Synoptics, Cambridge, U.K.). Background absorbance was determined in wells containing only sterile medium (160 μL) and distilled water (40 μL). Naringenin and cinnamaldehyde were used as positive controls.[19,24]

Motility. The bacterial motility was determined at 25 °C following a previously published method[13] with some modifications. Swimming and swarming motility were tested using the same growth medium containing 1% tryptone and 10 mM glucose supplemented with 0.30% Bacto agar (Difco, Detroit, MI, USA) for swimming and 0.35% Bacto agar for swarming. For the swarming assays, agar plates were point-inoculated in the center of each plate without penetrating the agar surface, whereas in the case of the swimming tests, the inoculum was stabbed into the medium with 1 μL of a working culture of *Y. enterocolitica* and incubated in the absence and presence of the soluble orange extract or each flavanone at 25 °C for 24 h. At the end of the incubation period, the swimming and swarming migration distances were calculated using a contrast camera imaging system (Synoptics). Cinnamaldehyde was included as a positive control.[24]

Bacterial RNA Extraction. For the analysis of gene expression changes associated with AHLs synthesis, bacteria were grown at 30 °C. For the analysis of gene expression changes in members of the flagellar regulon, bacterial cells were grown at 25 °C. Samples from control and treated cultures (at the concentrations stated) were taken at three different time points of the bacterial growth curve (6, 14, and 24 h).

Table 2. Percentage of Inhibition of the Violacein Produced by *C. violaceum* 494 Grown in LB Broth Supplemented with Three Different Concentrations of the Orange Extract or Each Individual Flavanone for 24 h[a]

	inhibition %			
concentration (μg/mL)	orange extract	naringin	neohesperidin	hesperidin
100	12.85 ± 6.43[a]	6.74 ± 1.83	10.50 ± 0.59	5.04 ± 4.14
200	22.79 ± 3.69	20.11 ± 8.49	13.20 ± 0.66	8.79 ± 3.83
400	29.70 ± 6.83	19.95 ± 6.43	18.95 ± 2.64	9.34 ± 9.92

[a]Results are all the mean ± SD of three independent experiments (n = 2 replicates per experiment). Values are all significantly different from untreated controls ($P < 0.05$).

Total RNA was extracted using the MICROBexpress kit (Applied Biosystem, Foster City, CA, USA) according to the manufacturer's instructions. The purity and concentration of the samples were checked measuring the absorbance at 260 and 280 nm using the Nanoquant plate in an Infinite M200 microplate reader (Tecan). Only RNA samples with an Abs_{260}/Abs_{280} ratio between 2 and 2.2 were used for gene expression analyses.

Gene Expression Analyses. Two genes expressed in *Y. enterocolitica* and reported to be involved in the synthesis of AHLs (*yenI*, *yenR*) and three members of the flagellar regulon system (*fliA*, *fleB*, *flhDC*) were selected for the study.[12,13,25] Primers and probes were designed on the basis of their coding sequences using the Custom Assay Design Tool (Taqman sytem) (Applied Biosystems, Foster City, CA, USA) (Table 1). Relative expression was measured by one-step quantitative RT-PCR Taqman system (Applied Biosystems, Madrid, Spain) run on the ABI 7500 system following the manufacturer's conditions, using a total reaction volume of 25 μL in a MicroAmp Optical 96-well plate covered by optical adhesive covers and using Taqman Universal Master Mix (Applied Biosystems, Madrid, Spain). The expression levels of each gene were normalized to the levels of the housekeeping gene *16S rRNA* utilizing a standard curve method for relative quantification.

Statistical Analysis. Results of the phenotypic bacterial responses are presented as the mean value ± SD of three independent experiments with at least two replicates per experiment. Gene expression analyses were carried out in at least four biological replicates, and RT-PCR assays consisted of three technical replicates run under identical conditions. Statistical analyses were performed using PASW Statistics 18 for Windows (SPSS Inc., Chicago, IL, USA). The Kolmogorov–Smirnov test showed agreement of the empirical distribution of the data with normality assumption. Levene's test was used to assess the equality of variances, and statistical differences between experimental groups were determined by the unpaired Student's t test, using two-tailed and $P < 0.05$ or $P < 0.01$ as the level of significance.

RESULTS

QS Inhibition by the Orange Extract and Its Main Flavanone Components Using a Biosensor Strain. The capacity of the orange extract and of naringin, neohesperidin, and hesperidin to reduce the production of violacein by *C. violaceum* is presented in Table 2. Results show that the orange extract and each of the individual flavanones caused a modest but significant inhibition of the production of violacein at the three concentrations tested. With this bacterial model, no significant differences were detected between the inhibition caused by the extract and that caused by the tested flavanones except for hesperidin, which exhibited a slightly lower inhibitory activity at the two highest concentrations tested (200 and 400 μg/mL).

Effects of the Orange Extract and Its Main Flavanone Components on the Levels of AHLs Produced by *Y. enterocolitica* Using LC-MS/MS. The putative anti-QS activity of the orange extract, naringin, neohesperidin, and hesperidin against *Y. enterocolitica* was first examined by quantitating the effects of these compounds on the levels of AHLs produced by these bacteria. LC-MS/MS analysis of the AHLs present in the bacterial culture supernatants confirmed that the two major AHLs produced by *Y. enterocolitica* were 3-oxo-C6HSL and C6HSL and that exposure to 200 μg/mL of the orange extract or of each of the flavanones for 48 h caused a decrease in both peaks as shown in the chromatograms depicted in Figure 2. Results expressed as percent of inhibition

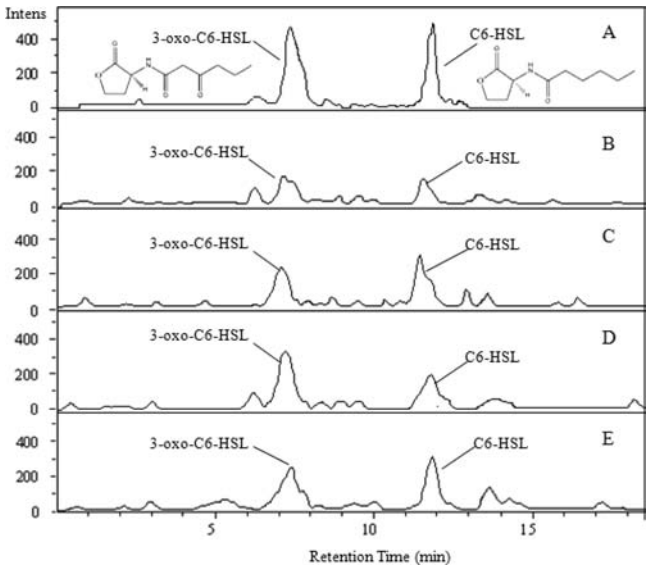

Figure 2. LC-MS/MS chromatograms of 3-oxo-C6-HSL and C6-HSL present in the supernatant from *Y. enterocolitica* control cultures (A) and from cultures supplemented with orange extract (B), naringin (C), neohesperidin (D), or hesperidin (E).

with respect to the quantity of AHLs in the control samples (without test compounds) are presented in Figure 3. These data corroborated the QS-inhibitory effects detected with the *Chromobacterium* assay but, unlike this method, a significant difference was observed between the inhibitory effect of the complete extract (68.5 and 64.4% for 3-oxo-C6HSL and C6HSL, respectively) and that of the individual flavanones (values between 24.2 and 36.5%). Exposure of *Y. enterocolitica* to a mixture of naringin and neohesperidin at the concentrations present in the extract also showed that the mixture of these two flavanones did not inhibit the production of 3-oxo-C6HSL (17.0%) or C6HSL (20.4%) as much as the complete orange extract and controverted a synergistic effect between these two main components of the extract. We cannot exclude that other components of the extract may contribute to the observed inhibitory effect.

To exclude the possibility that the decrease in the levels of AHLs might have been caused by some degradation and (or)

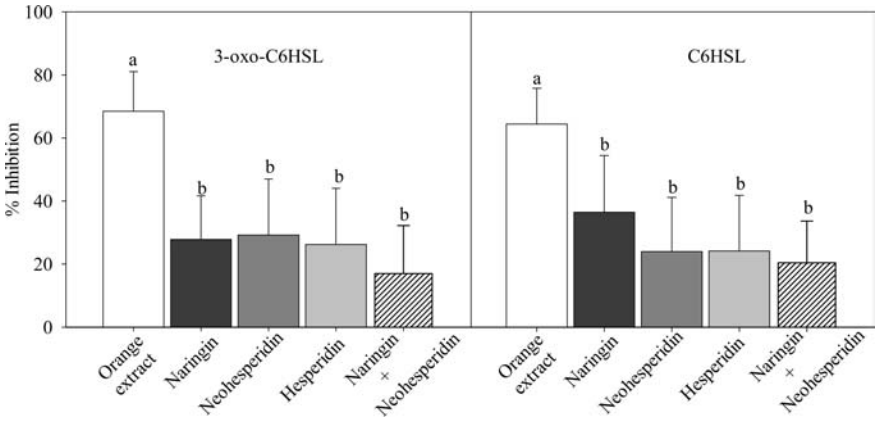

Figure 3. Percentage of inhibition of the production of 3-oxo-C6-HSL and C6-HSL by *Y. enterocolitica* grown in LB broth supplemented with the orange extract, naringin, neohesperidin, or hesperidin. Values are indicated as the percent of inhibition with respect to the quantity of AHLs present in the control untreated cultures and are the mean ± SD (displayed as error bars) of three independent experiments (n = 2 replicates per experiment). Bars labeled with different letters indicate significant differences at $P < 0.05$. Values are all significantly different from untreated controls ($P < 0.05$).

transformation of the AHLs as a consequence of a direct interaction of these molecules with the orange extract or the flavanones, we incubated known concentrations of 3-oxo-C6HSL and C6HSL in uninoculated broth supplemented or not with the test compounds. Our results revealed that the orange extract, as well as the flavanones, were able to slightly decrease the levels of 3-oxo-C6-HSL and C6-HSL in the absence of *Y. enterocolitica*. Inhibition values were all in the range from 2 to 10% of the total inhibition observed in the presence of *Y. enterocolitica*. Overall, these data indicate that the anti-QS activity of the orange extract and the flavanones against *Y. enterocolitica* may be, at least, partially caused by an interference with the production of the autoinducer signals by the bacterium.

Effect of the Orange Extract and Naringin on the Growth Kinetic Parameters of *Y. enterocolitica*. To further ascertain that the observed anti-QS effects were not due to an inhibitory effect on the bacterial growth, we next determined the growth kinetic parameters of *Y. enterocolitica* following incubation with the orange extract or its main flavanone component, naringin. The results (Table 3) showed that no significant changes were observed in the maximum specific growth (μ_{max}) at the three concentrations tested. Moreover, there were no significant differences in the maximum absorbance values attained at the stationary phase (A) between the bacteria grown in culture media supplemented with the extract or naringin and the control cultures. Equally, the lag phase duration was not significantly affected by the addition of the orange extract or naringin. These results indicate that at the three concentrations tested, the growth rate of *Y. enterocolitica* is not affected by the orange extract or naringin and that, therefore, the decrease observed in the concentration of the AHLs cannot be attributed to any bactericidal or bacteriostatic effect on *Y. enterocolitica*. On the basis of these results a 200 μg/mL intermediate concentration was selected for further studies.

Table 3. Growth Kinetic Parameters of *Yersinia enterocolitica* Grown at 30 °C in the Absence (Control) and Presence of the Orange Extract or Naringin[a]

	maximum specific growth rate (h^{-1})			
	A^b	$\mu_{max}{}^c$	λ^d (h)	R^{2e}
control	0.30 ± 0.04	0.02 ± 0.02	12.08 ± 2.67	0.99
orange extract				
100 μg/mL	0.33 ± 0.06	0.02 ± 0.01	12.20 ± 2.41	0.98
200 μg/mL	0.34 ± 0.07	0.02 ± 0.00	11.27 ± 4.08	0.98
400 μg/mL	0.29 ± 0.04	0.02 ± 0.00	14.29 ± 0.27	1.00
naringin				
100 μg/mL	0.36 ± 0.02	0.02 ± 0.01	10.16 ± 0.47	0.98
200 μg/mL	0.36 ± 0.00	0.02 ± 0.01	11.69 ± 2.37	0.99
400 μg/mL	0.32 ± 0.04	0.02 ± 0.01	11.82 ± 2.15	0.98

[a]Values are presented as the mean ± SD (n = 3). [b]Maximum absorbance value at the stationary phase. [c]Maximum specific growth. [d]Lag phase duration in hours. [e]Coefficient of determination.

Effect of the Orange Extract on *yenI* and *yenR* Expression. In an attempt to identify some of the putative molecular changes that may be associated with the decrease in the concentration of AHLs produced by *Y. enterocolitica*, we compared the expression levels of specific genes involved in the synthesis (*yenI* and *yenR*) before and after exposure of the bacteria to the orange extract (200 μg/mL) for 6, 14, and 24 h (Figure 4). Batch-to-batch variability in the expression of these genes (expression noise) has been reported to range from 0.7 to 1.3[21] and, therefore, only values above 1.3 or below 0.7 (shown in Figure 4 by dashed lines) were considered as indicators of up-regulation or down-regulation by the treatment. RT-PCR analyses from at least four biological independent replicates revealed that the expression of *yenI* was not altered following exposure of the bacterial cells to the extract. However, treatment of the cells with the orange extract was associated with a small increase in the expression of *yenR* more noticeable at 24 h (relative expression treated/control = 1.8 ± 1.4). This increase was significant when compared to gene expression ratios at 6 h ($P < 0.05$).

Inhibition of Biofilm Formation in *Y. enterocolitica* by the Orange Extract and Its Main Flavanones. The inhibitory effects of the orange extract and its main flavanones (200 μg/mL) on the formation of biofilm by *Y. enterocolitica* are presented in Figure 5. Naringenin (95 μg/mL) and cinnamaldehyde (50 μg/mL) were included as positive controls. Under the conditions of our assay, these two compounds inhibited the formation of biofilm by 68.1 ± 6.7 and 60.0 ± 10.7%, respectively. The formation of biofilm was also significantly reduced by the extract and all of the tested

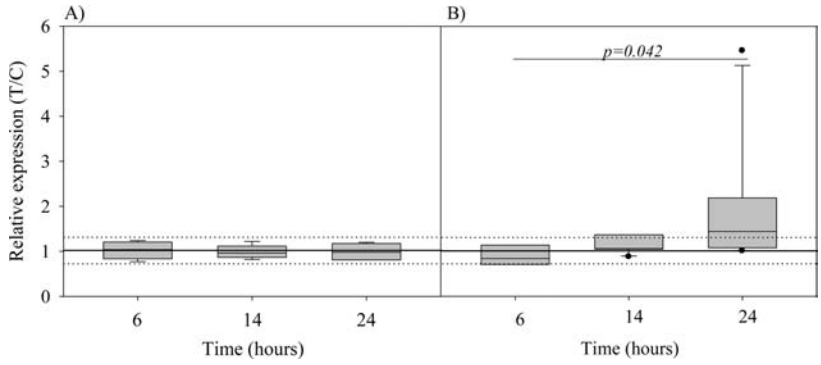

Figure 4. Differential expression of (A) *yenI* and (B) *yenR* in *Y. enterocolitica* following exposure to the orange extract (200 μg/mL) for 6, 14, and 24 h during bacterial growth at 30 °C. The relative change in expression in treated cells (ratio treated/control, T/C) was calculated against control cells at the same time points. Dashed lines mark the minimum (0.7) and maximum (1.3) ratio values estimated to be intrinsic variability. Values are the mean ± SD (displayed as error bars) of at least four independent experiments (n = 3 replicates per experiment). Significant differences (P values < 0.05) between time points are indicated.

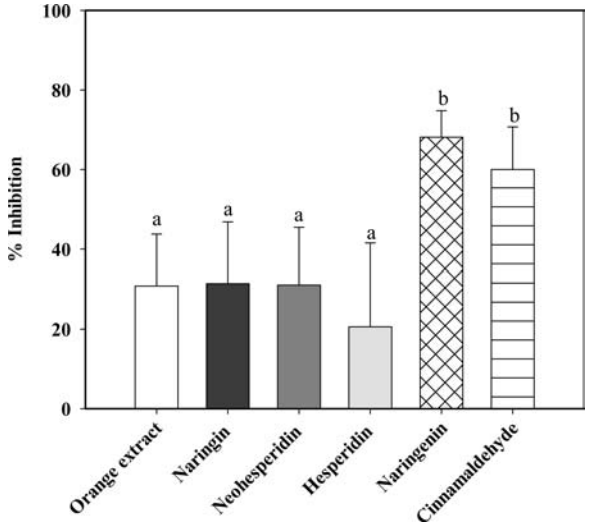

Figure 5. Percentage of inhibition of biofilm formation by *Y. enterocolitica* treated with the orange extract, naringin, neohesperidin, or hesperidin for 24 h. Naringenin (95 μg/mL) and cinnamaldehyde (50 μg/mL) were included as positive controls. Values are indicated as the percent of inhibition with respect to the control untreated cultures and are the mean ± SD (displayed as error bars) of three independent experiments (n = 2 replicates per experiment). Bars labeled with different letters indicate significant differences at P < 0.05. Values are all significantly different from untreated controls (P < 0.05).

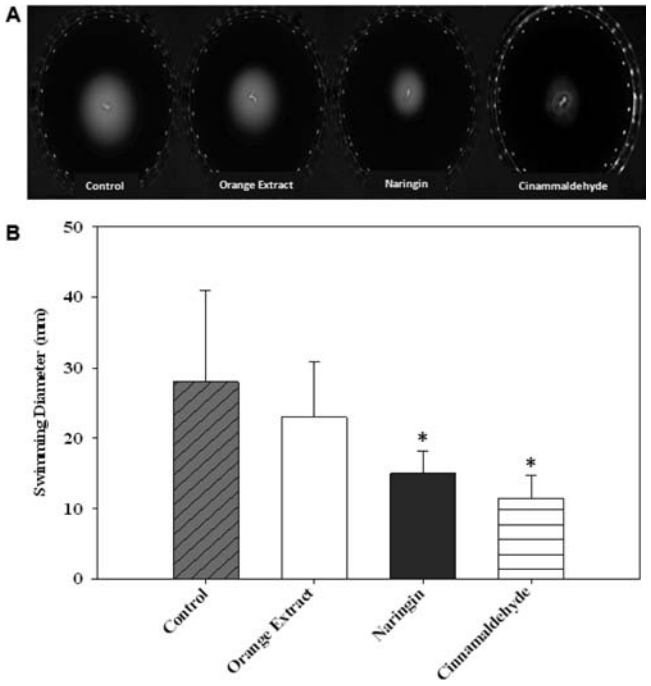

Figure 6. Swimming motility in *Y. enterocolitica* (grown at 25 °C on 0.3% swim agar plates, which were point inoculated). Motility was determined by measuring the swimming diameter (mm) in untreated (control) plates and plates treated with the orange extract (A) or naringin (B) at 400 μg/mL for 24 h. Cinnamaldehyde (50 μg/mL) was included as a positive control. Values are the mean ± SD (displayed as error bars) of at least four independent experiments (n = 3 replicates per experiment). Significant differences (P < 0.05) with control samples are indicated.

flavanones, with inhibition mean values between 20 and 30%. No significant differences were found, however, among the different compounds. Direct addition of 3-oxo-C6HSL and C6HSL (20 μg/mL each) to the assay did not reverse the inhibition induced by naringin, naringenin, or cinnamaldehyde.

Inhibition of Motility in *Y. enterocolitica* by the Orange Extract and Naringin. We next evaluated the effect of the orange extract and of naringin, neohesperidin, and hesperidin (at 200 and 400 μg/mL) on the motility of *Y. enterocolitica* by analyzing the effects of these compounds on swimming and swarming at 25 °C. None of the compounds tested had any effect on swarming, and only naringin exhibited a significant inhibitory effect on swimming at 400 μg/mL (46.0 ± 10%; Figure 6). Cinnamaldehyde, which was included as a positive control, also strongly inhibited swimming motility in *Y. enterocolitica* (59.0 ± 11.5%). Direct addition of 3-oxo-C6HSL and C6HSL (20 μg/mL each) to the assay did not reverse the inhibition induced by naringin or cinnamaldehyde.

Effect of the Orange Extract and Naringin on *flhDC*, *fliA*, and *fleB* Expression. We first measured the expression changes for these three flagellar-related genes in control cultures of *Y. enterocolitica* at 25 °C and at three different time points of the bacterial growth curve, 6, 14, and 24 h, which corresponded to the mid lag phase and the beginning of the exponential and stationary phases (Figure 7). Results indicate that the mRNA levels of these genes are slightly induced after 14 and 24 h of growth.

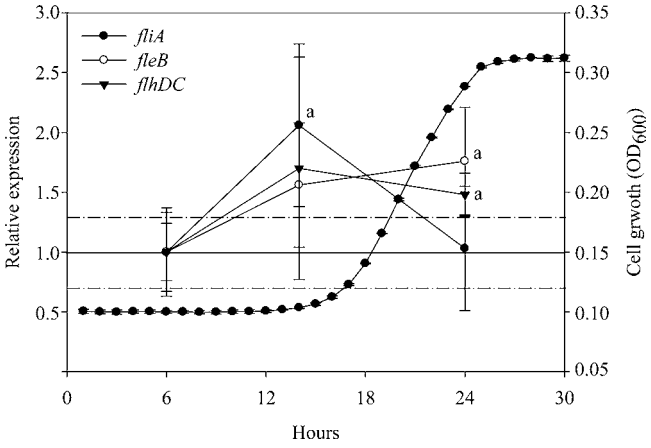

Figure 7. Relative gene expression levels in *Y. enterocolitica* of *flhDC*, *fliA*, and *fleB* at 25 °C as a function of cell growth. Dashed lines mark the minimum (0.7) and maximum (1.3) ratio values estimated to be intrinsic variability. Values are the mean ± SD (displayed as error bars) of at least four independent experiments (n = 3 replicates per experiment). Bars labeled with different letters indicate significant differences against values at 6 h ($P < 0.01$). Bacterial growth is shown as absorbance at 600 nm, and values are the mean ± SD (displayed as error bars) of n = 4 replicate wells.

Changes in the expression of these three genes after exposure to the orange extract or to naringin are shown in Figure 8 (values above 1.3 or below 0.7, expression noise, are indicated by dashed lines). The most significant changes were found for *flhDC*, which was up-regulated in *Y. enterocolitica* after exposure to 200 and 400 μg/mL of orange extract for 6 h (relative expression treated/control = 3.8 ± 2.9 and 2.4 ± 0.7, respectively) and 24 h (2.7 ± 1.5 and 2.3 ± 0.7, respectively), whereas at 14 h the levels of this transcript were within the range of the expression noise values (Figure 8A). Similarly, exposure to naringin also caused a significant induction in the expression of this gene at 6 h (3.4 ± 0.6 and 1.5 ± 1.2) and 24 h (2.1 ± 0.8 and 1.5 ± 1.0) both at 200 μg/mL and at 400 μg/mL of the flavanone, respectively (Figure 8B). Although gene expression changes for *fliA* did not reach significance, ratios were all above the limits of intrinsic variability, indicating an apparent general up-regulation of the expression of this gene after the treatments (Figure 8C,D). The expression of *fleB* was slightly (although not significantly) up-regulated following 14 h of exposure to the extract (1.7 ± 0.5 at 400 μg/mL) (Figure 8E) or to the flavanone (1.9 ± 1.9 and 1.6 ± 1.4 at 200 and 400 μg/mL, respectively) (Figure 8F). At 24 h, the expression of *fleB* was found to be significantly down-regulated by the orange extract (0.6 ± 0.1, $P < 0.05$) at the highest concentration tested (400 μg/mL).

DISCUSSION

The Centers for Disease Control and Prevention reported that ~60% of all infections in developed countries are caused by

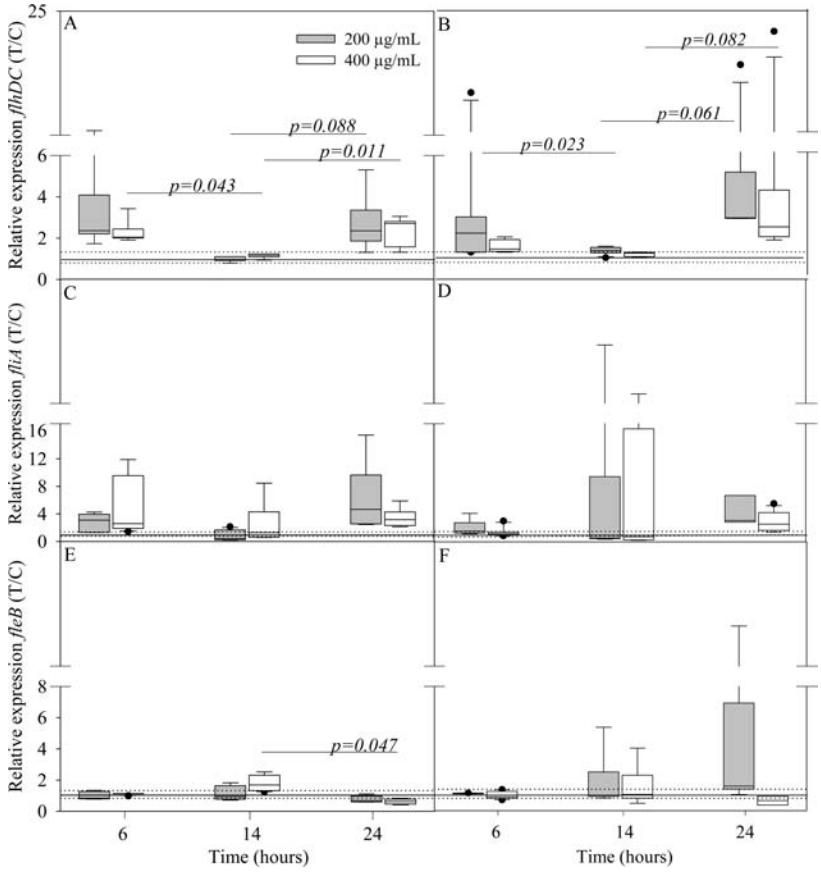

Figure 8. Differential expression of *flhDC*, *fliA*, and *fleB* in *Y. enterocolitica* at 6, 14, and 24 h of growth and 25 °C in the presence of soluble orange extract (200 and 400 μg/mL) (A, C, and E) and naringin (200 and 400 μg/mL) (B, D, and F). Dashed lines mark the minimum (0.7) and maximum (1.3) ratio values estimated to be intrinsic variability. Values are the mean ± SD (displayed as error bars) of at least four independent experiments (n = 3 replicates per experiment). Significant differences (P values < 0.1 or < 0.05) between time points are indicated.

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biofilms, which are bacterial communities that settle and proliferate on surfaces and are covered by an exopolymer matrix. A high proportion of chronic infections, often untreatable, are accompanied by the formation of biofilms with a high resistance to antibiotics.[26] In this context, the search for natural compounds able to attenuate bacterial pathogenicity rather than bacterial growth has become a priority. Former studies have shown that different fruit and vegetable extracts as well as some of their phenolic components or derivatives are able to inhibit the synthesis of QS autoinducers and (or) to attenuate QS-associated processes such as motility, biofilm formation, or secretion of virulence factors.[6,7,15,19−22,27−30] Recently, the anti-QS properties of several citrus flavonoids against *E. coli*, *V. harveyi*, and *P. aeruginosa* have been reported.[19,20] In these studies, the nonglycosylated flavanone naringenin (aglycone) has emerged as an efficient QS inhibitor. However, in some citrus fruits such as oranges as well as in some of their derived products, the main flavanone components are the *O*-glycosylated derivatives such as hesperidin and naringin.[2] In this study we show that a soluble orange extract as well as its main flavanone components, the glycosides naringin, neohesperidin, and hesperidin, exert a QS-inhibitory effect against *Y. enterocolitica* as shown by a significant reduction in the levels of autoinducer molecules (AHLs) produced by the bacteria and a significant inhibition of the formation of biofilm. In addition, naringin was also able to inhibit the ability of *Y. enterocolitica* to swim on agar plates.

Following a dietary approach, the intestinal pathogen *Y. enterocolitica* was exposed to low doses of the extract or the flavanones (200 μg/mL, equivalent to ∼320−350 μM). These quantities are within the range of the levels that can be detected in the intestine even several hours after oral intake of these compounds.[5] At these concentrations, the orange extract and the individual flavanones moderately inhibited the formation of biofilm by *Y. enterocolitica* (20−30%). Similar values can be found in the literature for these and other natural flavonoids and citrus components. In the same way, naringin, neohesperidin, and hesperidin at 100 μg/mL inhibited the formation of biofilm in *E. coli* by approximately 50, 40, and 5%, respectively.[19] Also, several citrus limonoids at 100 μg/mL caused approximately between 20 and 35% inhibition of *E. coli* biofilm formation,[29] or the flavonoid catechin, at concentrations 10 times higher (1000 μg/mL), inhibited biofilm formation in *P. aeruginosa* by only 45%.[30] The anti-QS effectiveness of the compounds can vary considerably depending not only on the concentrations used but also on the bacterial species investigated. We have shown that naringin can inhibit biofilm formation, motility, and AHLs production in *Y. enterocolitica*; however, this compound was not able to inhibit QS-controlled virulence factors in *P. aeruginosa* (PAO1).[20] In a similar fashion, naringenin, which efficiently inhibited the formation of biofilm in *E. coli* and *V. harveyi* (approximately 70−90% inhibition at 100 μg/mL),[19] was not able to inhibit the formation of biofilm by *Salmonella typhimurium* at concentrations between 1.56 and 100 μg/mL. However, naringenin, at 200 μg/mL, induced the formation of biofilm in these bacteria.[28] Our data and others indicate that flavanones can influence bacteria QS-related mechanisms in different ways depending on the type of bacterial cells and on the concentration used.

Inhibition values between 20 and 40% may appear to be moderate or less significant when compared to other more effective compounds that may cause inhibition values as high as 90%.[31] However, it is not a trivial issue to establish whether an effect is "biologically significant", because this will depend on the specific biological effects investigated. From the point of view of intestinal infections by pathogens such as *Y. enterocolitica*, modest effects by dietary compounds may become relevant in the long term. As it has been repeatedly shown in the field of polyphenols bioactivity, exposure of cultured cells or animal models to dietary low levels of plant-derived polyphenols causes, in general, very moderate effects in contrast to treatment with high doses of xenobiotic compounds or drugs (pharmacological approach).[32] Our results suggest that regular consumption of micromolar concentrations of the orange extract or the flavanones may exert moderate anti-QS effects in the gut.

Because the orange extract and its main flavanone components interfered with the QS system in *Y. enterocolitica*, we hypothesized that this may be reflected at the transcription level of QS-related genes. We first investigated the effect of the orange extract on the expression levels of *yenI* and *yenR*, which are involved in the synthesis of AHLs and their regulation in *Y. enterocolitica*.[13,25] Our results exhibited a high variability probably caused by expression noise, which includes "intrinsic noise" driven by random fluctuations in mRNA synthesis and degradation and "extrinsic noise", which originates from external factors.[33] Previous studies reporting QS-gene expression changes associated with bacterial cell exposure to flavonoids also showed a high variability in the results with coefficient variation values as high as 45% in some cases.[20] With regard to our experimental conditions, replicated bacterial cultures in shake flasks have been reported to produce protein expression profiles more variable than those bacteria cultivated under more controlled environments, that is, bioreactors or chemostats.[34] To partially filter out some of this noise, we had determined the variability in the expression of the selected genes in control batches under our culture conditions.[21] Thus, gene expression ratios between 0.7 and 1.3 were established as intrinsic noise and excluded from the results. In general, results reported in the literature on QS-associated gene expression regulation by exogenous phenolic compounds are still scarce and incomplete. Certain flavonoids such as catechin or naringenin have been reported to inhibit QS, and this inhibition was associated with a down-regulation in the expression levels of AHLs synthase and regulator genes.[7,20] A closer view of the reported changes shows, however, that gene expression regulation is clearly a time-dependent process and that results are markedly affected by the time point at which gene expression is measured. Large differences in gene expression changes have been shown even within 2 h.[19] The down-regulation of *lasI* and *lasR* by catechin and naringenin in *P. aeruginosa* was reported at 8 and 18 h of growth.[20] Our data clearly display a significant tendency to an induction in the expression of *yenR* following 24 h of exposure to the orange extract (Figure 4). In agreement with these results, other phenolic compounds such as urolithin-B, an ellagitanin-derived metabolite, also inhibited the production of AHLs in *Y. enterocolitica* but increased the mRNA levels of *yenR* after 24 h of exposure.[21] Although we do not have a clear explanation for this, we hypothesize that if a down-regulation of *yenR* had occurred following exposure to the orange extract, this might have taken place at earlier stages of the bacterial cell growth and that the posterior induction seen at 24 h may be a counteracting response of the bacteria.

We next sought to determine whether treatment with the orange extract or naringin influenced the expression of *flhDC*,

fliA, and *fleB*. These three genes are all members of the flagellar transcriptional hierarchy in *Y. enterocolitica* and have been reported to be involved in bacterial motility and biofilm formation.[13] FlhDC is the master regulator of this flagellar regulon encoded by the *flhDC* operon, which is at the top of the hierarchical cascade and affects the expression of other flagellar genes such as *fliA* and *fleB*.[25] *FlhDC* expression in *Y. enterocolitica* reaches maximum levels at 25 °C, but is largely reduced at temperatures above 25 °C.[35] Also, in *Y. enterocolitica*, temperatures below 30 °C induce the coordinate expression of the flagellin genes (*fliA* and *fleB*).[2] Concomitantly, we examined the expression of *flhDC*, *fleB*, and *fliA* at 25 °C. Other authors have reported a similar experimental design.[13] Our data show that exposure of *Y. enterocolitica* to the orange extract or to naringin at 25 °C was associated with a time-dependent up-regulation of the transcription levels of *flhDC* as well as a general induction in the expression of the alternative sigma factor, *fliA*, at the three time points tested. We also detected some small regulatory changes in the levels of the class III flagelin gene, *fleB*. The transcriptional modulators of *flhDC* in *Y. enterocolitica* and their impact on the regulation of flagellar genes are not yet well understood. Various regulatory factors or mechanisms might contribute to the modulation of *flhDC* transcription in response to environmental factors. Very recently, it has been reported that OmpR, a pleiotropic transcription factor which responds to environmental signals, directly activates the expression of *flhDC* in *Y. enterocolitica*.[35] Importantly, the expression of *flhDC* also affects the expression levels of nonflagellar genes, including genes involved in metabolism. Nutritional conditions can also regulate the expression of the flagellar regulon, and the presence of short-chain fatty acids (dietary derived compounds) in the growth medium was found to increase *flhDC* expression in *Y. enterocolitica*.[36] We may speculate that in the presence of the flavanone-enriched orange extract or naringin in the media, the OmpR regulator might activate *flhDC* transcription. Further research into the regulatory role of OmpR on *flhDC* transcription is needed to understand the response of *Y. enterocolitica* to environmental signals such as exposure to natural anti-QS molecules.

The results presented here show that a natural orange extract enriched in flavanones (mainly naringin, neohesperidin, and hesperidin) at doses that can be achieved in the gut through the diet exhibits a moderate antipathogenic activity against *Y. enterocolitica* by reducing the production of *N*-acylhomoserine lactones and the formation of biofilm. These responses were found to be associated with the induction of two important QS-regulatory genes, *yenR* and *flhDC*. Other genes or alternative mechanisms, such as inhibition of the synthesis of AHLs at the protein level, inhibition of the transport and secretion of AHLs to the media, or enzymatic degradation of AHLs might be involved in the observed responses. Many natural extracts (fruits, herbs, spices) have been suggested to inhibit QS by a combination of mechanisms.[27] As previously reported for naringenin,[20] an exogenous supply of the AHLs to the culture media was not sufficient to compensate for the inhibitory effects induced by naringin on motility and biofilm formation, suggesting that other possible mechanisms such as antagonism of AHLs receptors or inhibition of targets downstream of AHL-receptor binding[27] may also be involved in the observed inhibitory effects. All of these other possible mechanisms need to be further investigated to clarify the anti-QS mechanisms of flavanones and other similar natural compounds.

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Notes

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