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Influence of food system conditions on *N*-acyl-L-homoserine lactones production by *Aeromonas* spp.☆

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Abstract

Eleven of 13 *Aeromonas* strains were shown to produce AHLs. Results of TLC showed that *N*-butanoyl-L-homoserine lactone (C4-HSL) was the main AHL produced in LB medium at 30 °C. The influence of different carbon sources, temperature, pH values and salt concentrations on AHL production was determined in eight *A. hydrophila* and one *A. caviae* strain. Additionally a quantitative study of C4-HSL production by *A. hydrophila* strain 519 under different conditions was performed. Positive results were found in the AHL induction assay for some *Aeromonas* strains in cultures in LB agar incubated at 12 °C after 72–96 h. The induction of the sensor strains by *Aeromonas* spp. occurred in LB medium supplemented with all carbon sources in a concentration of 0.5%. The production of C4-HSL by *A. hydrophila* 519 was found until 3.5% (w/v) of NaCl. For pHs close to the neutrality the C4-HSL production by *A. hydrophila* was evident after 24–48 h of incubation. *A. hydrophila* 519 produced C4-HSL under anaerobic conditions. Also, the AHL production by *Aeromonas* strains was studied in simulate agar of shrimp, fish and some vegetables. The production of AHLs was evident by almost all the test strains in shrimp simulated agar. In fish agar only for one of three fish species tested, positive results were found. Induction assay in vegetables simulated agar showed principally negative results, probably because of the presence of inhibitory compounds in these vegetables.

Keywords: *Aeromonas*; Quorum sensing; *N*-acyl-L-homoserine lactone; Induction assay; Food systems

1. Introduction

Aeromonas spp. are oxidase and catalase positive Gram negative rods belonging to the family *Vibrionaceae*. Aquatic environment is the principal ecosystem of these microorganisms, and they have been found in unprocessed water (Hernández et al., 1997) and raw foods such as seafoods (fish, shrimp), poultry, meat, fresh vegetables (Neyts et al., 2000; Castro-Escarpulli et al., 2003).

Aeromonas spp. have optimum growth temperature ranging from 22 °C to 28 °C and pH limits range from 5.2 to 9.8. The growth of *Aeromonas* spp. is optimal in 1–2% NaCl, however some strains may tolerate concentrations up to 5% NaCl under otherwise ideal conditions (Blair et al., 1999). Important characteristics of these microorganisms are the capacity to grow at low temperatures and under anaerobic conditions which may account for the presence of elevated numbers of *Aeromonas* in fresh raw foods, with a prolonged shelf life under refrigeration temperature (Kirov, 2001).

Members of *Aeromonas* genus have been described as opportunistic pathogens which produce infections and gastroenteritis. Children, elderly and immunocompromised patients are the most affected populations (Kuijper et al., 1989). From this genus strains belong to the species *A. hydrophila*, *A. caviae* and *A. veronii* biovar *sobria* have been associated with gastrointestinal disease (Kirov, 1993; Janda, 1991).

Many bacteria are able to regulate the expression of phenotypic characteristics as a function of cell density, which is called quorum sensing (Miller and Bassler, 2001). This system uses a signal molecule which is produced and released by the cell. When the concentration of the signal molecule reaches a limit (dense population), regulation in the expression of certain genes occurs. In general, the Gram negative bacteria produce *N*-acyl-L-homoserine lactone (AHL) like signal molecules in the cell to cell communication system (Whitehead et al., 2001). The

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components involved in AHL-based quorum sensing systems of the lux-homologous family have been cited by Gram et al. (2002) and the molecular bases of quorum sensing system has been described (Miller and Bassler, 2001; Schauder and Bassler, 2001; Whitehead et al., 2001).

Bacteria use quorum sensing systems to regulate several physiological functions such as symbiosis, virulence, motility, sporulation, biofilm formation, etc. (Winson et al., 1995; Davies et al., 1998; Lindum et al., 1998). Lynch et al. (2002) showed evidence that supports the role of AHL-dependent quorum sensing in *A. hydrophila* biofilm development. The identification of AHL and the cognate LuxRI homologs AhyRI from *A. hydrophila* and *A. salmonicida* were reported by Swift et al. (1997). The regulation of exoprotease production by AHL quorum sensing system in *Aeromonas hydrophila* was studied by Swift et al. (1999).

Compounds present in the food matrix and the storage environment could influence the production of AHLs. Environmental conditions may affect the AHL production, and it is possible that in the presence of quorum (dense population), environmental factors could affect the accumulation of AHL (Fuqua et al., 2001). Fuqua et al. (2001) explained the dynamics of AHLs accumulation. The pH is an important factor that can affect the stability of AHLs. In alkaline conditions, most AHLs are unstable and a degradation of these molecules will occur (Fuqua et al., 2001). The effect of several conditions on AHLs produced by two *Enterobacteriaceae* isolated from fish was studied by Ravn et al. (2002) who found the production of the same type of AHLs under all conditions tested and similar amounts produced in almost all the conditions studied, however the authors reported that the AHLs produced become unstable under alkaline condition (pH > 7.5). Gram et al. (1999) studied the production of AHLs by 154 psychrotrophic *Enterobacteriaceae* strains, isolated from cold-smoked salmon or vacuum-packed chilled meat, finding production of 3-oxo-*N*-hexanoyl homoserine lactone (3-oxo-C6-HSL) and some analogue molecules by 116 of them. These authors reported the production of detectable amounts of AHL when cultured at 5 °C in both LB broth and defined medium supplemented with 4% (w/v) NaCl at densities of 10^6 CFU/g. They also reported AHLs production in cold-smoked salmon stored at 5 °C under N_2 atmosphere.

The goal of this study was to determine the production of AHLs by *A. hydrophila* and *A. caviae* strains and to evaluate the influence of environmental factors on AHL production. Information about the effect of some intrinsic and extrinsic conditions typically encountered in a food system let to understand the dynamic of AHL production by these pathogens in foods.

2. Materials and methods

2.1. AHLs production

2.1.1. Strains and culture conditions

Sensor strains were cultured in LB medium (Difco, Le Pont de Claix, France) solidified with 1.2% agar and supplemented with appropriate antibiotic (in case of *Agrobacterium tumefaciens* NT1 gentamycin 50 μg/ml, (Sigma-Aldrich, Irvine, UK); in case of *Chromobacterium violaceum* CV026 kanamycine 20 μg/ml (Sigma-Aldrich). The *Aeromonas* strains tested for their AHL production were grown in LB medium solidified with 1.2% agar. All the strains were incubated at 30 °C for 24 h. The source of each strain is presented Table 1.

2.2. Screening of AHL production by Aeromonas: induction assay in solid media

Aeromonas strains were tested using the induction method in solid system described by Ravn et al. (2001). Three different sensor systems were used to screen for AHL production, which include *Agrobacterium tumefaciens* NT1 which carries a plasmid pZLR4 and produces a blue color from the hydrolysis of the 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-Gal) in the medium by the β-galactosidase expressed from *traG:lacZ* reporter fusion when induced by particular AHLs (Cha et al., 1998; Ravn et al., 2001); *Chromobacterium violaceum* strain CV026 with the LuxR homologue, CviR, which regulates the production of a purple pigment when induced by particular AHLs (McClean et al., 1997; Ravn et al., 2001); and the inhibition of CviR of *Chromobacterium violaceum* by long chained AHLs resulting in a lack of pigment when compared to the control (McClean et al., 1997; Ravn et al., 2001). Each strain was streaked onto LB medium in parallel to the sensor strain. In the assay with *A. tumefaciens* NT1 the LB medium was supplemented with 50 μg/

Table 1
Bacterial strains

Strain	Isolated from	Source or reference
Chromobacterium violaceum CV026		Ravn et al., 2001
Agrobacterium tumefaciens NT1 (pZLR4)		Ravn et al., 2001
Serratia liquefaciens MG1		Ravn et al., 2001
Pseudomonas aeruginosa PO1		Ravn et al., 2001
Aeromonas hydrophila 064	Faeces	BCCM™ collection (LMG 3771)
Aeromonas hydrophila 566	Faeces	BCCM™ collection (LMG 13662)
Aeromonas hydrophila 569	Faeces	BCCM™ collection (LMG 13442)
Aeromonas hydrophila 519	Meat	LFMFP
Aeromonas hydrophila 489	Fish (Whiting)	LFMFP
Aeromonas hydrophila 497	Meat (Beef steak)	LFMFP
Aeromonas hydrophila 495	Fish (Salmon)	LFMFP
Aeromonas hydrophila 514	Bacon	LFMFP
Aeromonas caviae HG4 438	Bean	LFMFP
Aeromonas caviae 574	Faeces	BCCM™ collection (LMG 13680)
Aeromonas caviae complex 445	Vegetable	LFMFP
Aeromonas caviae HG5B 429	Vegetable	LFMFP
Aeromonas caviae 70	Epizootic of young guinea pigs	BCCM™ collection (LMG 3775)

BCCM™: Belgian Co-ordinated Collection of Micro-organisms. LMG: Laboratory of Microbiology, Ghent University.
LFMFP: Laboratory of Food Microbiology and Food Preservation, Ghent University.

ml X-Gal (Promega, Madison, USA). The production of long chained AHLs was detected by inhibition of the induced *C. violaceum* in LB agar plates supplemented with 500 nM *N*-hexanoyl-L-homoserine lactone (C6-HSL) (Biochemika, Sigma-Aldrich). As positive controls *Serratia liquefaciens* MG1 and *Pseudomonas aeruginosa* PO1 (Ravn et al., 2001) were used in the induction assay with *C. violaceum* and *A. tumefaciens* respectively. For the inhibition of induced *C. violaceum*, *P. aeruginosa* PO1 was used as positive control. As a negative control, *P. aeruginosa* PO1 was used for *C. violaceum* induction. For the inhibition of induced *C. violaceum* and induction assay with *A. tumefaciens* the monitor strains themselves were used as negative controls.

2.3. Determination of AHL type produced by Aeromonas: TLC

The AHL types produced by the *Aeromonas* test strain were determined by extractions with ethyl acetate acidified and thin layer chromatography as is described by Ravn et al. (2001).

TLC was performed in a C_{18} TLC plate (TLC aluminium sheets 20 × 20 cm^2, RP-18 $F_{254}S$, 1.05559) (MERCK, Darmstadt, Germany); the plates were cut of 20 × 10 cm^2 and between 10 and 100 µl of each extract or synthetic standard was applied. The chromatoplate was developed in 200 ml methanol–water phase (60:40) for approximately 2 h. The TLC plate was allowed to dry for at least 10 min while a top layer agar was prepared using either *C. violaceum* CV026 or *A. tumefaciens* NT1. For this, a preculture was done in LB medium and incubated for 24 h at 30 °C, and 1 ml of it was taken to inoculate 50 ml of LB medium. The culture was grown for 24 h at 30 °C and was mixed with either 100 ml of LB agar (1.6% agar) in case of *C. violaceum* or ABT agar (1.6% agar) (Ravn et al., 2001) in case of *A. tumefaciens* which was previously melted and maintained at 46–48 °C. Each medium was supplemented with the respective antibiotic as described before and the ABT-agar for *A. tumefaciens* was mixed with X-Gal (50 µg/ml). The synthetic standards used included *N*-butanoyl-L homoserine lactone (C4-HSL) (Biochemika, Sigma-Aldrich), *N*-hexanoyl-L homoserine lactone (C6-HSL) (Biochemika, Sigma-Aldrich), *N*-octanoyl-L homoserine lactone (C8-HSL) (Biochemika, Sigma-Aldrich), 3-oxo-*N*-hexanoyl-L homoserine lactone (3-oxo-C6-HSL) (Sigma-Aldrich).

2.4. Determination of quantities of C4-HSL produced by Aeromonas: agar well diffusion assay

The amount of C4-HSL produced by the *Aeromonas* spp. test strains was determined using an agar well diffusion assay method as described by Ravn et al. (2001). The ethyl acetate extracts, as described before for TLC analysis, were used and the agar plates were prepared with *C. violaceum* as is described before for TLC-top layer agar. The agar was poured in 20 ml/plate portions, and 60 µl of each extract or C4-HSL synthetic standard added into wells (7 mm) punched in the middle of the agar. The plates were incubated for 24 h at 30°C and the diameter of the C4-HSL induced zone around the wells (Fig. 1) was determined with a vernier caliper. The amount of C4-HSL (nmol/10 ml culture) was calculated using a calibration curve previously set up using a synthetic C4-HSL standard.

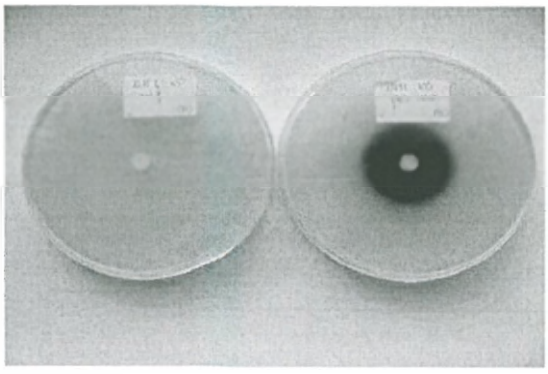

Fig. 1. Agar well diffusion assay with *C. violaceum* as sensor strain. Right: positive control (C4-HSL synthetic standard in ethyl acetate; 120 nmol). Left: negative control (ethyl acetate).

2.5. Effect of several conditions on C4-HSL production by Aeromonas in solid media

The influence of several conditions: carbon source, NaCl, pH and temperature (Table 2) normally found in foods associated with *Aeromonas* spp., on the C4-HSL production by eight *A. hydrophila* and one *A. caviae* strain was evaluated. For this, the induction assay in solid media (LB medium) was used with *C. violaceum* and *A. tumefaciens* as sensor strains. The influence of different environmental conditions on C4-HSL production was studied after 24, 48, 72 and 96 h of incubation. For study at different pHs, the LB medium was acidified with HCl (1 N).

2.6. Effect of conditions on C4-HSL production by A hydrophila strain 519 in broth

A study of C4-HSL production by *Aeromonas hydrophila* under different conditions of pH, NaCl and anaerobic conditions was performed in liquid media. For these, the strain *A. hydrophila* 519 previously checked as positive to C4-HSL was chosen. A preculture was done in LB medium and incubated at 30 °C during 24 h. From this preculture, three serial dilutions were prepared in PPS (1 g/l peptone, 8.5 g/l NaCl) and 100 µl of the last dilution was used to inoculate 100 ml of fresh LB medium in order to obtain an initial level of ca. 10^3 CFU/ml. After incubation, samples were withdrawn for determination of viable cell counts as well as for determination of C4-HSL. The method of extraction with acidified ethyl acetate and the quantification of C4-HSL using the agar well diffusion assay method was used as described before. The conditions used were NaCl concentration, and pHs as shown in Table 3. The anaerobic condition (< 1% oxygen and 9–13% carbon dioxide) was created in a jar with anaerobic system (AnaeroGen ™ Oxoid Limited, Basingstoke, Hampshire, England).

2.7. Induction assay in food simulate agar plates

The induction assay with *C. violaceum* and *A. tumefaciens* was done on a simulate agar for vegetables and seafoods in order to

Table 2
Induction assay to *A. hydrophila* and *A. caviae* strains under different conditions after 96 h of incubation

Growth conditions	*Aeromonas hydrophila* strains																*A. caviae*	
	064		566		569		519		489		497		495		514		438	
	Monitor system		Monitor system		Monitor system		Monitor system		Monitor system		Monitor system		Monitor system		Monitor system		Monitor system	
	C.v.	A. t.	C.v.	A. t.	C.v.	A. t.	C.v.	A. t.	C.v.	A. t.	C.v.	A. t.	C.v.	A. t.	C.v.	A. t.	C.v.	A. t.
Temperature[a]																		
10 °C	−	−	+	−	−	−	−	−	+	−	−	−	+	−	−	−	−	−
12 °C	−	−	+	−	++	−	++	−	++	−	++	−	++	−	+	−	+	++
22 °C	++	−	++	+	++	++	++	+	++	+	++	+	++	+	++	+	++	++
30 °C	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
37 °C	−	++	−	++	−	++	−	++	+	++	+	++	−	++	−	++	−	++
pH																		
5.0	+	−	++	−	++	−	++	−	++	−	++	+	++	+	++	−	+	++
5.5	++	−	++	+	++	−	++	+	++	+	++	+	++	++	++	+	++	++
6.0	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
6.5	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
7.0	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
[NaCl][b]																		
0.5	++	+	++	+	++	++	++	+	++	+	++	+	++	++	++	+	++	++
1%	++	++	++	++	++	++	++	+	++	++	++	++	++	++	++	+	++	++
1.5%	++	++	++	++	++	++	+	++	++	+	−	++	++	++	++	+	+	++
2%	−	++	−	++	−	++	−	++	−	++	−	++	−	++	−	++	−	++
2.5%	−	++	−	++	−	++	−	++	−	++	−	++	−	++	−	++	−	++
3%	−	++	−	++	−	++	−	++	−	++	−	++	−	++	−	++	−	++
3.5%	−	−	−	++	−	++	−	−	−	−	−	−	−	−	−	−	−	++
4%	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
4.5%	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Carbon source																		
Glucose	+	++	++	++	++	++	++	++	++	++	++	+	++	++	++	++	++	++
Lactose	+	−	++	−	++	−	++	−	++	−	++	−	++	−	++	−	++	−
Mannitol	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
Sorbitol	+	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
Mannose	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++

[a] *Serratia liquefaciens* (positive control) was negative in the *C. violaceum* induction at 10 °C and 37 °C. *P. aeruginosa* (positive control) was negative in the *A. tumefaciens* induction at 10 °C and 12 °C. *P. aeruginosa* (negative control) was positive in the induction assay with *C. violaceum* at 22 °C.
[b] *Serratia liquefaciens* (positive control) was positive in the induction assay with *C. violaceum* until 2% NaCl. *P. aeruginose* (positive control) was positive at all NaCl concentrations in the induction assay with *A. tumefaciens*. C.v.: *Chromobacterium violaceum*; A.t.: *Agrobacterium tumefaciens*; (+) weak positive; (++) strong positive; (−) negative.

know if the AHL can be produced at the different food compositions. For vegetables, the method described by Jacxsens et al. (2003) was followed. The vegetables were washed with cold tap water (60 s) and dried for 60 s by means of a manual kitchen centrifuge (ACEA, Manodomestici, Italy). Then, the vegetables were cut with a kitchen knife, and after blending in a food processor (Braun, type 4290, Kronberg, Germany) to obtain juice and pulp, the juice was centrifuged at 16,270 ×*g* during 20 min (Sorvall, RC-5B, Du Pont Instruments, Delaware, USA). The supernatant was heated for 2 h at 80°C to denature enzymes and proteins, and filtered (Retsch, 200 μm, Haan, Germany). Finally the extract was mixed with 1.5% agar and autoclaved for 15 min at 121 °C. To simulate fish and shrimp agar, the method described by Boskou and Debevere (1997) was followed. Briefly shrimps and three species of fish were used. Each one was divided in portions of 200 g, mixed with 400 ml of distilled water, and blended for 1 min in a lab blender (Waring Commercial, Connecticut, USA). The mix was heated in a bath for 1 h at 80 °C cooled and squeezed through a bag of cheesecloth to remove the precipitate. The filtrate was centrifuged at 16,270 ×*g* for 10 min and the supernatant was mixed with 1.5% agar and autoclaved for 15 min at 121 °C. Of each extract the pH was measured in a pH meter (Microprocessor pH meter 763, Knick, Berlin, Germany) with an electrode (Mettler Toledo, Urdorf, Switzerland).

3. Results and discussion

3.1. Detection, identification and estimation of quantities of AHLs produced by Aeromonas test strains

Of 13 *Aeromonas* strains tested, 10 strains produced response in *C. violaceum* and 11 strains caused reaction in *A. tumefaciens*, which indicates the diffusible AHL production (Table 4). The test strains did not cause inhibition of pigment production in the induced *C. violaceum* CV026 system, thus indicating no production of long chain AHLs by these strains. By means of a TLC

analysis the main AHL produced was tentatively identified as C4-HSL, according to the Rf value (~0.64) and the shape of the spot. A similar R_f value was reported by Ravn et al. (2001) for C4-HSL produced by *Serratia liquefaciens* MG1. The TLC results for *A. hydrophila* 519 are shown in Fig. 2. The induction of *A. tumefaciens* by *A. hydrophila* strains was detected in spite of some authors reporting that *A. tumefaciens* does not sense C4-HSL (Shaw et al., 1997). The *A. tumefaciens* sensor strain may have sensed other AHLs such as C6-HSL, although C6-HSL was not detected by TLC in the *Aeromonas* extract. The C6-HSL standard was visualized by TLC (Fig. 2). The production of C6-HSL in *Aeromonas* spp. was described by Swift et al. (1997) who identified the major AHL signal purified from *A. hydrophila* and *A. salmonicida* culture supernatants as C4-HSL and a second minor signal as C6-HSL with a ratio of approximately 70:1.

Table 3
C4-HSL production by *A. hydrophila* 519 under the different conditions

Growth conditions	Time (h)	CFU/ml	C4-HSL nmol/10 ml
[NaCl]			
0.5%	24	1.19×10^{9}	130
	48	9.36×10^{9}	370
	72	6.65×10^{9}	290
1%	24	1.19×10^{9}	<40[a]
	48	1.13×10^{10}	260
	72	2.79×10^{9}	280
1.5%	24	7.60×10^{8}	ND
	48	2.53×10^{9}	140
	72	1.73×10^{9}	240
2%	24	7.90×10^{8}	<40
	48	1.38×10^{9}	<40
	72	1.58×10^{9}	290
2.5%	24	4.60×10^{8}	<40
	48	4.80×10^{9}	<40
	72	7.80×10^{8}	105
3%	24	7.50×10^{7}	<40
	48	1.54×10^{8}	120
	72	1.59×10^{8}	70
3.5%	24	1.07×10^{7}	<40
	48	4.80×10^{8}	80
	72	1.44×10^{8}	80
4%	24	2.90×10^{7}	<40
	48	7.00×10^{6}	<40
	72	1.90×10^{7}	<40
4.5%	24	1.00×10^{4}	<40
	48	4.90×10^{7}	<40
	72	4.00×10^{6}	<40
pH			
6.5	24	2.50×10^{9}	280
	48	3.11×10^{9}	310
	72	1.72×10^{9}	255
6.0	24	1.60×10^{9}	220
	48	4.60×10^{9}	310
	72	2.11×10^{9}	60
5.5	24	5.20×10^{6}	<40
	48	7.80×10^{8}	<40
	72	2.45×10^{9}	50
Anaerobiosis			
	24	1.16×10^{9}	90
	48	2.24×10^{9}	185
	72	1.51×10^{9}	420

(Incubation temperature 30 °C).
[a]Lower detection limit (40 nmol/10 ml) of the agar well diffusion assay using synthetic standard C4-HSL and *C. violaceum* as the sensor strain. ND: not determined.

Table 4
Screening of AHL production by *Aeromonas* test strains

Strain	Sensor strain		
	C. violaceum CV026 induction assay	*A. tumefaciens* NT1 assay	*C. violaceum* CV026 inhibition assay
064	+	++	–
566	++	++	–
569	++	++	–
519	++	++	–
489	++	++	–
497	++	++	–
495	++	++	–
514	++	++	–
438	+	+	–
574	–	–	–
445	++	++	–
429	–	+	–
70	–	–	–

(+) weak positive; (++) strong positive; (–) negative.

One *A. caviae* strain produced a compound with chromatographic properties (typical tale sharp and R_f value 0.57) corresponding to the 3-oxo-C6-HSL synthetic standard (results not shown). This is, however only a preliminary identification and must be confirmed by other methods such as high pressure liquid chromatography, mass spectrometry, nuclear magnetic resonance and infrared spectroscopy (Shaw et al., 1997; Brelles-Mariño and Bedmar, 2001). No former reports were found about AHL production by *A. caviae*.

According to the TLC results, C4-HSL was considered as the principal AHL produced by the tested *Aeromonas* strains and the major contributor to the induced areas surrounding the wells in the diffusion assay. Therefore a calibration curve with a C4-HSL synthetic standard was set up (Fig. 3) and an estimate of the quantities of C4-HSL was calculated. The detection limit for quantification was ca. 5 nmol (40 nmol/10 ml of sterile supernatant). The amount of C4-HSL produced by 10 ml of culture of these C4-HSL producing *A. hydrophila* (8 strains) and *A. caviae* (1 strain) after 24 h incubation at 30 °C ranged from ca. 25 to ca. 200 nmol.

3.2. Effect of different conditions on AHL production by *A. hydrophila* and *A. caviae* strains in solid media

The results of the induction assay for the *Aeromonas* test strains under different conditions are presented in Table 2. The induction of sensor strains occurred mainly on the optimal growth conditions. Also 8 of 9 test strains showed AHL production at 12 °C, after 96 h of incubation. Ravn et al. (2003) tested the influence of temperature (5 °C, 25 °C) and other conditions on the AHL profile and production rate of a *Serratia proteamaculans* and an *Enterobacter agglomerans* strain. Both produced the same types of AHLs under all the tested conditions. *A. hydrophila* is able to grow in food products kept at refrigeration temperatures and evidence exists that *Aeromonas* species can produce virulence factors (compounds of an organism that determine its capacity to

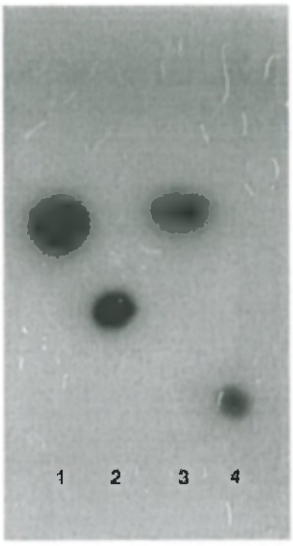

Fig. 2. Thin layer chromatography of *A. hydrophila* 519 visualized using *C. violaceum* CV026. 1. *N*-butanoyl-L-homoserine lactone (1.8 mM). 2. *N*-hexanoyl-L-homoserine lactone (1.75×10^{-2} mM). 3. *Aeromonas hydrophila* 519 extract. 4. *N*-octanoyl-L-homoserine lactone (0.48 mM).

cause disease) under this condition (Kirov et al., 1993; Tsai et al., 1997; González-Rodríguez et al., 2004). The effect of different carbohydrates on AHL production showed induction of sensor strains in LB agar supplemented with all carbon sources at a concentration of 0.5%. Only in the assay with LB agar plus lactose and X-Gal the results were negative. This is probably due to the induced galactosidase activity which is used to split the lactose, present in excess compared to the X-Gal, in glucose and galactose; Ravn et al. (2003) reported no effect on the specific 3-oxo-C6-HSL production by a *S. proteamaculans* strain by addition or change of carbon sources.

In the induction assays under different NaCl concentrations, positive results with *A. tumefaciens* were observed with 3% and 3.5% NaCl. *C. violaceum* can no growth at levels higher than 2%, which indicate its limited use in this assay.

This rapid screening in solid media allows having an idea about the conditions normally found in food systems in which *Aeromonas* sp. could produce the signal molecule. However only qualitative results (induction or not) can be obtained, and no relative differences among degrees of induction can be concluded. In addition, positive results depend upon the ability of the sensor strains to grow and express the AHL dependent phenotype under adverse conditions. This indicates that the AHL production per se is no affected, but the detection will depend on the sensor strain used. Therefore, initiative was taken, to determine C4-HSL quantitatively in extract of culture supernatants of *Aeromonas* test strains grown under different conditions commonly encountered in foods.

3.3. Production of C4-HSL by *A. hydrophila* 519 in liquid media under different conditions

Table 3 presents the results obtained for C4-HSL production by *A. hydrophila* 519 under different conditions. In general the results show a strong relation between high population levels (10^8–10^9 CFU/ml) and AHL production. Our data agree with previous reports about AHL quorum sensing systems in bacteria such as *Vibrio fischeri* LuxI/LuxR bioluminescence system which were reviewed by Fuqua et al. (2001) and Miller and Bassler (2001). Under sub-optimal conditions more time of incubation was necessary in order to obtain the quorum (dense population) necessary for C4-HSL production.

3.3.1. Sodium chloride

At 4% and 4.5% (w/v) NaCl, no C4-HSL production was notified. This result is correlated with a low colony count (maximum of 1.9×10^7 and 4.0×10^6 CFU/ml respectively). In general, the higher the NaCl concentration (from 0.5% to 3.5%), the lower the C4-HSL production (from ca. 370 nmol/10 ml to 80 nmol/10 ml). C4-HSL production was detected at 1 to 3.5% only after 48–72 h although under these conditions cultures in LB medium reached high numbers (4.6×10^8–4.6×10^9 CFU/ml) earlier without C4-HSL being detected using the agar well diffusion assay. This may be due to the high detection limit of the method. The degree of variability between results obtained in extractions that were performed in duplicate and the fact that the measurement of the halo around the well in the diffusion assay may show some variation dependant upon the analyst, indicate the limitations of the agar well diffusion method as a reliable quantitative method for C4-HSL. Therefore, the C4-HSL concentrations mentioned should be interpreted as an estimate of the real value. The agar well diffusion should be regarded as a semi-quantitative method rather than an exact quantitative method.

3.3.2. pH

With regard to C4-HSL production at different pH values, C4-HSL concentrations of ca. 310 nmol/10 ml were found after 48 h of incubation at pH 6 and 6.5. These results correlated to viable cell counts of $> 10^9$ CFU/ml. At pH 5.5 only after 72 h of incubation detectable C4-HSL levels were found, which was the time when the culture reached 9 log CFU/ml. Only acid conditions were taken up in the experimental setup as it is known that at alkaline pHs (7.5) AHLs are unstable (Fuqua et al., 2001; Ravn et al., 2003).

3.3.3. Anaerobic conditions

Aeromonas spp. is a facultative anaerobic microorganism, able to grow under limitation of oxygen in the environment. Detectable

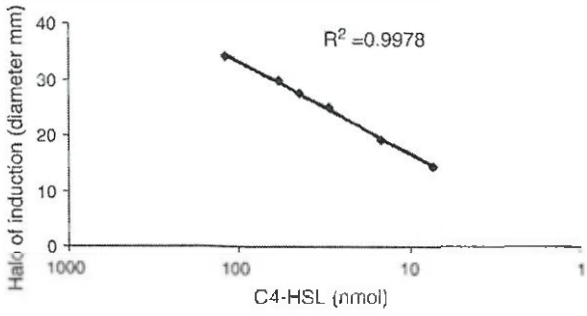

Fig. 3. Standard curve, relating diameter of induced zone in *C. violaceum* agar well diffusion assay with concentrations of synthetic C4-HSL standard.

Table 5
Induction assay to *A.hydrophila and A. caviae* in simulated vegetable agars

Simulated food agar	*Aeromonas hydrophila* strains																*A. caviae*	
	064		566		569		519		489		497		495		514		438	
	Monitor system C.v.	A. t.	Monitor system C.v.	A. t.	Monitor system C.v.	A. t.	Monitor system C.v.	A. t.	Monitor system C.v.	A. t.	Monitor system C.v.	A. t.	Monitor system C.v.	A. t.	Monitor system C.v.	A. t.	Monitor system C.v.	A. t.
Broccoli (*Brassica oleracea*)	–	–	–	–	–	–	–	–	+	–	+	–	–	–	–	–	–	–
Parsley[a] (*Petroselium crispus*)	–	–	–	–	–	–	+	–	+	+	–	–	++	++	+	–	–	–
Parsley[b]	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Spinach (*Spinacia oleracea*)	–	–	–	–	–	–	–	++	+	++	–	++	+	–	+	–	–	+
Mixed[c] lettuce (*Lactuca sativa*)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Sprouts (*Brassica oleraceae* var. *gemmifera*)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

[a,b]Two types of parsley were used. [c]Lettuce types: endive, krul, radicchio, lloló roso (*L. sativa* var. *crispa*), blonda and oak leaf lettuce (*Cichorium intybus*).
C.v.: *Chromobacterium violaceum*; A.t.: *Agrobacterium tumefaciens*; (+) weak positive; (++) strong positive; (−) negative.

C4-HSL levels were found under anaerobic conditions with increments throughout the incubation time. Gram et al. (1999) reported the production of detectable amounts of AHL by psychrotrophic *Enterobacteraceae* when cultured in pieces of salmon, which were incubated at 5 °C under N_2 atmosphere, simulating the low oxygen conditions of vacuum packing. The capacity to produce AHL in anaerobic conditions and at low temperatures by this *A. hydrophila* strain shows that the quorum sensing system could be relevant in an *Aeromonas* population in foods kept under these conditions.

Quorum sensing has been described in many important Gram negative bacteria associated with foods e.g. *Enterobacter*, *Pseudomonas* and *Serratia* (Gram et al., 1999; Steidle et al., 2002; Christensen et al., 2003). However few studies involved relevant conditions present in the food matrix and food environment. Our study is a contribution to understand the effect of some extrinsic and intrinsic parameters, present in foods related with *Aeromonas hydrophila*, on the production of AHL.

3.3.4. AHL production in food simulate agar

Aeromonas spp. have been isolated from both vegetables and seafoods (Neyts et al., 2000; Castro-Escarpulli et al., 2003). Gram et al. (2002) in a review showed positive results for the presence of AHL in bean sprouts stored at 5 °C and in fish products. The AHL induction in simulated food agars allow to know if these molecules can be produced in real food conditions. Tables 5 and 6 show the results of AHL induction in simulated food agar plates. Eight of nine test strains were positive in the induction assay on shrimp agar plates. Only one (Dory, *Zeus fabe*) of the three types of tested fishes showed positive results. These differences could be due to the instability of the AHLs at alkaline pH (pH>8) of the agars. Shrimp and Dory fish agars had a pH around 7. In vegetable agar plates, only few *Aeromonas* strains gave positive results for parsley, spinach and broccoli. No production of AHL was detected in the rest of vegetables tested. The pH values of all simulate vegetable agars were between 5.6 and 6.6 which is, as shown in Table 2, not inhibitory to C4-HSL production.

Table 6
Induction assay to *A. hydrophila* and *A. caviae* in simulated shrimp and fish agars

Simulated food agar	*Aeromonas hydrophila* strains																*A. caviae*	
	064		566		569		519		489		497		495		514		438	
	Monitor system C.v.	A. t.	Monitor system C.v.	A. t.	Monitor system C.v.	A. t.	Monitor system C.v.	A. t.	Monitor system C.v.	A. t.	Monitor system C.v.	A. t.	Monitor system C.v.	A. t.	Monitor system C.v.	A. t.	Monitor system C.v.	A. t.
Shrimp	–	–	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	+
Dory fish (*Zeus farber*)	–	–	+	–	+	++	+	++	+	+	+	++	+	++	+	++	–	–
Nileperch (*Lates niluticus*)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Whiting (*Merlangius melangus*)	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–

C.v.: *Chromobacterium violaceum*; A.t.: *Agrobacterium tumefaciens*; (+) weak positive; (++) strong positive; (−) negative.

The causes of lack of AHL production can be multiple. As noticed by visual inspection some of the test strain could not grow on some of these agars or may not have reached the population density necessary for the AHL production in detectable levels. Some compounds, naturally present in plant material, could have had some inhibitory effect on the test strains' growth. Hao et al. (1998) found that eugenol and pimento leaf extract significantly inhibited the growth of *A. hydrophila* in refrigerated cooked poultry. Inhibitory effect of essential oils and other compounds present in plant products on food-borne pathogens have been described (Cowan, 1999; Bagamboula et al., 2004). Also some substances present in plant tissues can affect the production of AHLs. Polyphenolic compounds (widely distributed in plants) can interfere with bacterial quorum sensing (Huber et al., 2003). Therefore, our negative results show the necessity to acknowledge possible substances present in these vegetables that can inhibit responses in the sensor strain used. The present study shows that indication of AHL production in defined and controlled cultured media cannot be extrapolated to the more complex food conditions. Food simulate agars represent a closer approximation of the real food conditions. Care must be taken however in the analysis of the results of tests in complex media. False positive results can also be obtained due to compounds that mimic bacterial AHL-like activities. Plant exudates from pea (*Pisum sativum*) secrete substances that mimic bacterial AHL-like activities in some reporter strains and affect population density-dependent behaviour in associated bacteria. These compounds can stimulate AHL-regulated behaviour in some strains while inhibiting such behaviours in others (Teplitski et al., 2000; Bauer and Teplitski, 2001). Gao et al. (2003) reported the production of at least 15 to 20 substances by the legume *Medicago truncatula* capable of specifically stimulating or inhibiting responses in quorum sensing reporter bacteria. In order to make decisive conclusions in assays with simulate food agars the simultaneous use of different sensor strains and appropriate medium controls are recommended.

4. Conclusions

It was shown that the *Aeromonas* test strains were able to produce AHLs (in particular C4-HSL) under a variety of conditions of temperature, pH, and NaCl concentrations commonly encountered in foods associated with this microorganism when multiplication to high enough numbers (dense population) was reached. Further study on the kinetics of AHL production is being performed in our laboratory at present. The use of simulate food agar is a practical, easy and fast way to screen the production of AHL by bacterial species in conditions normally found in foods and relevant information for AHL production in food systems can quickly be obtained. Studies on the actual compounds in foods responsible for inhibition or stimulation of AHL production in Gram negative bacteria need further attention. Insight in the ability of *Aeromonas* strains to produce AHLs under conditions normally encountered in food products or used for food preservation, should enable a better understanding of their behaviour in food products.

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