Chapter 4. Influence of *luxS* gene on biofilm formation and biocide resistance of *L. innocua* UC 8410

[4.1]. Introduction

Bacteria possess several mechanisms not only to perceive modifications and changes of environmental conditions, but same systems could be used also to communicate with other individuals within same bacterial species o with different genera and/or species. Similar processes consist in signal transduction of information like pH or temperature and can modulate expression of metabolic pathways (e.g. from synthesis of toxins until expression of catabolic enzymes). Most exploited and wide-spread mechanism is *quorum sensing* (QS): bacteria secrete diffusible small metabolites, whose accumulation until a limit value (threshold) lead to activation or modulation of genetic mechanisms. Because the threshold value refers to a certain level of bacterial density, this mechanism is known as "quorum sensing".

Both Gram negatives and positive possess similar functions, even if there is a certain variability in terms of signal molecule employed (or autoinducers, AI): while Gram negative use acyl homoserine lactones (AHSL), Gram positive employ modified peptides (Lazdunski, et al. 2004; Turovskiy, et al. 2007; Karatan and Watnick, 2009). Despite this difference, both the two groups share a common mechanism of quorum sensing involving *LuxS* enzyme, which has been found in more than 55 species, suggesting a possible role as universal code for interspecies "talking" (Vendeville, et al. 2005). This mechanism will be deeply discussed in further pages.

One of reason of interest towards this topic can be found in its intriguing complexity, especially if associated to bacterial communities: when these consortia are established (especially between diverse species), within same species multiple signals could be produced and affect same response circuit (despite it occurs through three different pathways) (Jayaraman and Wood, 2008). This implies that same species can respond to diverse stimuli, but information carried by the signal could be different and provoke different changes in behavior. Another important trait of these systems of signaling is the high specificity between signal and corresponding response sensor, which is demanded to preserve the value of information within the signal itself and the fidelity of induced response (Jayaraman and Wood, 2008). Further explanation could be found in paragraph 4.4.

[4.2]. Quorum sensing and bioluminescence in *V. fischeri*

First Lux-based QS system was referred to bioluminescence of Gram-negatives symbiontes (*V. fischeri*, Fig. 4.2) present within light organs of some marine organisms (Fig. 4.1). Interestingly bioluminescence was inducteds during symbiotic relationships (Lupp, et al. 2003), but it is instead absent during planktonic growth. This metabolic pathway involves two proteins, LuxI and LuxR: LuxI catalyzes synthesis of N-3-oxo-hexanoyl homoserine lactone (3-oxo-C6-HSL), which, after outer outside diffusion, is gathered in the environment, if bacterial density could be high enough.

These molecules are sensed by LuxR, which contains a response regulator domain and binds *lux* operon promoter inducing expression of luciferase and thus bioluminescence phenomenon (Turovskiy, et al. 2007). Schematic representation of this system is reported in Fig. 4.3. This phenomenon does not increase gradually: as reported in Jayaraman and Wood (2008), the positive feedback of light production is set off by negative feedback regulatory mechanism on *luxR*, which leads to depression of LuxR amount and consequent expression of uciferase genetic determinants.

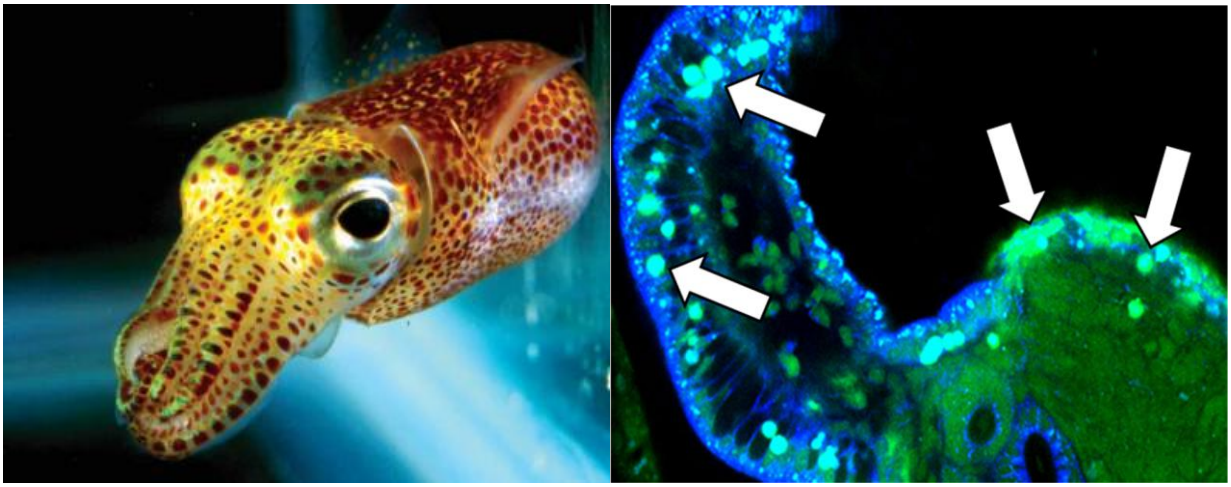

Fig. 4.1: Adult Hawaiian bobtail squid (left image from) and particular with lunminescent bacteria (pointed by white arrows) (from https://discovermagazine.com/photos/1-8-marine-creatures-that-light-up-the-sea, last access on 31/10/2010).

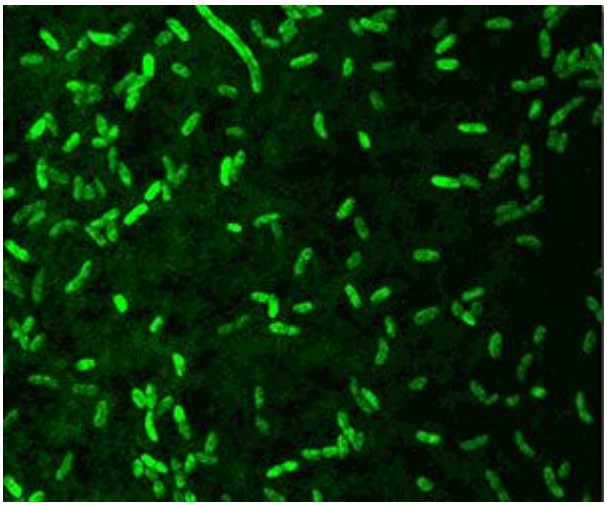

Fig. 4.2: Fluorescently stained *Vibrio fischeri* cells. (Microbe-wiki, Last acces 31/10/2010).

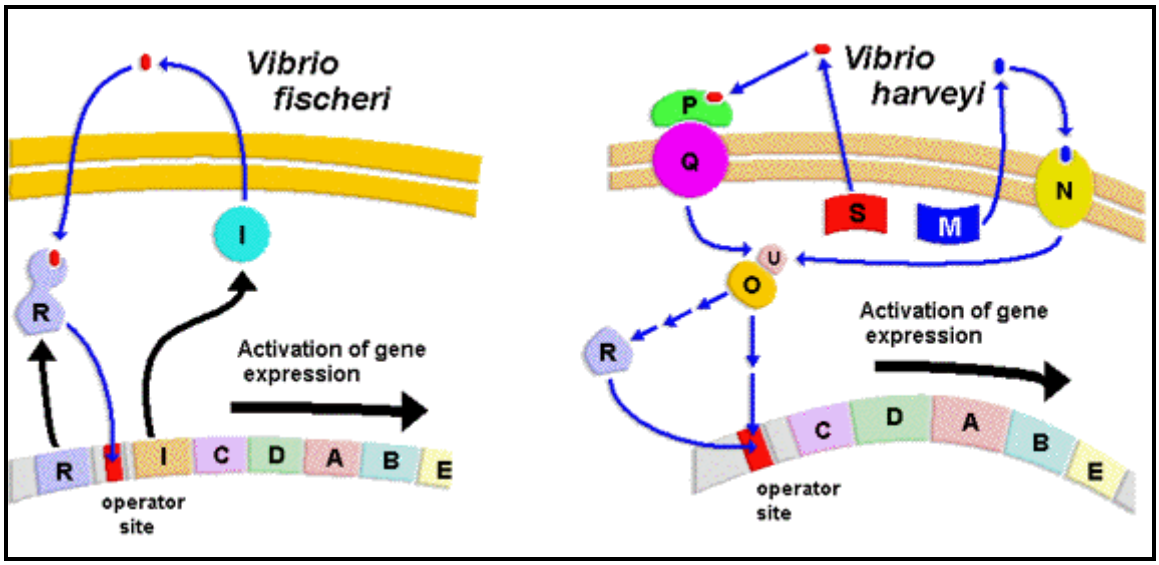

Fig. 4.3: Quorum signal processing in *V. harvey* in comparison with *V. fischeri* (from http://www.photobiology.info/Lin.html, last access on 20/11/2010).

[4.3]. Quorum sensing in *V. harvey*

Most exploited species in studies concerning QS signalling was *V. harvey*, belonging to the same family of previously mentioned *V. fischeri*. Interest towards this bacterium is related to contemporary presence of multiple QS, which can modulate same QS regulon. Surprisingly each of them has its own autoinducer (AI)-synthase as well as a specific sensor hystidine kinase. Here is exposed a concise description of mentioned processes, which are distinguished on the basis of signal molecule involved.

With regard to AI-1, the signal molecule is 4-hydroxyl C4 homoserine lactone and is produced by autoinducer synthase LuxLM. This molecule, after its intake inside the cell, it binds its specific sensor system LuxN. AI-2 are instead synthesized by LuxS and are sensed in the cellular periplasm by LuxP, a solube periplasmatic protein: the binding between these two molecules led to a complex LuxP/AI-2, which interacts with LuxQ. Both LuxN and LuxQ are two hybrid sensor kinases, which contain periplasmatic sensory domains and cytoplasmatic histidine kinase and response regulator domains. In presence of low cellular densities, both LuxN and LuxQ exhibit their kinase activity transferring phosphate groups to the phosphotransferase LuxU. LuxU transfers the acquired phosphate to the response regulator LuxO. In presence of high cellular densities, the phosphate flow direction is reversed from LuxOn to LuxN and LuxQ, where the phosphate hydrolysis takes place.

Dephosphorylated LuxO led to inactivation of expression ofd repressor X, while the transcriptional activator LuxR binds the operon *luxABCDE* promoter activating *lux* operon transcription and thus light production (Mok, et al. 2003). Beside o thse system, *V. harvey* can also responds to autoinducer CAI-1: in this system recently exploited was found in *V. cholerae*, from which the name was derive, signal producer is CqsA, while its related sensor is CqsS. CAI-1 belong to a new class of uncharacterized signalling molecules and is mainly found in *Vibrio* spp. And other closely related marine bacteria. Binding between CAI-1 and CsqS releases phosphate groups to LuxU and above mentioned signal cascade take place (Vendeville, et al. 2005). In *V. cholerae*, AI-2 and CAI-1 were demonstrated to downregulate the expression of virulence factors as much as other virulence-related genes, among which some of them affect biofilm formation. Possible explanation of these effects could be that, when high cellular densities are reached, this parasite leaves its host to disperse itself in the environment, in order to find new nutrients sources (Turovskiy, et al. 2007)

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[4.4]. Quorum sensing signalling in diverse bacterial species

Despite the wide-spread presence in other Gram-negative bacterial species, AHL differs in acyl chain length, extent of saturation and local substituents on C_3; AHL can possess also other additional properties [e.g. Kaufmann et al. (2005) described N-(3-oxododecanoyl) homoserine lactone in *P. aeruginosa* as able to express bactericidal characteristics, even if it cannot affect the producing microbe].

Quorum sensing in Gram-negative bacteria employs a regulatory circuit with many similarities with the one present in *V. fischeri*. A clear example comes from *P. aeruginosa*: LasI synthesize HSL for the corresponding response sensor LasR, which lead to activation of target genes. Beside LasI/LasR mechanism, in same cell is present a second similar and contemporary mechanism (RhlI/RhiR). Gene regulated by quorum sensing sensor encode for virulence determinants like elastase and protease, which are involved in infection process. Other species, (e.g. *Burkolderia cepacia*, *Yersinia enterocolitica* and *Agrobacterioum tumefaciens*) possess analogue system, even full the wide range of genetic function affected by quorum sensing is still not completely clear) (de Kievit and Iglevski, 2000; Lazduski, et al. 2004; Waters and Bassler, 2005).

Gram-positive species possess analogue density-based systems, where autoinducer is a modified peptide (autoinducing peptide, AIP). This molecule cannot freely move in and outside the cell, but, after structural modifications, it is exported through ATP-binding cassette (ABC)-like transporter or similar machinery. AIP do not bind with a cognate receptor like AI mechanism, but their signal transduction involves a hystidine kinase sensor, from which information is transmitted, through a phosphorylation cascade reactions, to response regulator. This latter one binds to the promoter of target genes to modulate genetic expression. All genes involved in this process (including gene encoding signal precursor, membrane-bould sensor and transporter complex) are located in same genetic cluster. Most reported example is *comABCDE* machinery of *S. pneumoniae*, even if AIP-base signal processing has been identified in other Gram-positive bacteria such as *Bacillus* spp. or *Staphylococccus* spp. (de Kievit and Iglevski, 2000; Lazduski, et al. 2004; Waters and Bassler, 2005).

[4.5]. Specificity of AI-based signalling

Despite the wide-spread diffusion of QS-based signalling process in Gram-negative bacteria, AI express diverse effect on bacterial phenotype, suggesting that bacteria can discriminate AI signal produced by different bacterial genera present in same environmental niche. Specificity of information could act at two different levels or in the interaction between the signal molecule and its corresponding receptor either through some regulatory process during the bond between the AI-linked LuxR-like protein and target gene.

Receptor proteins for AI carry their two functions (binding with AI and activation of correlated genetic cluster) through their two domains: amino-terminal end of LuxR-like proteins are dedicated to AI linkage, while carboxy-terminal end express the property to bind promoter sequence of target genes. Because DNA-binding domain is highly conserved and commonly shared among all LuxR-like proteins known until now, binding between AI and its cognate receptor seem to be the effective discriminating step. Moreover, DNA-binding activity cannot occur, if AI does not remove structural interference (Choi and Greenberg, 1992), and same binding with AI can alter LuxR stability, as previous studies on TraR has confirmed that this protein is not anymore susceptible to proteolysis after linkage with signal AI. All these experimental evidences allow recognizing the importance of binding between AI and corresponding sensor protein in QS mechanism.

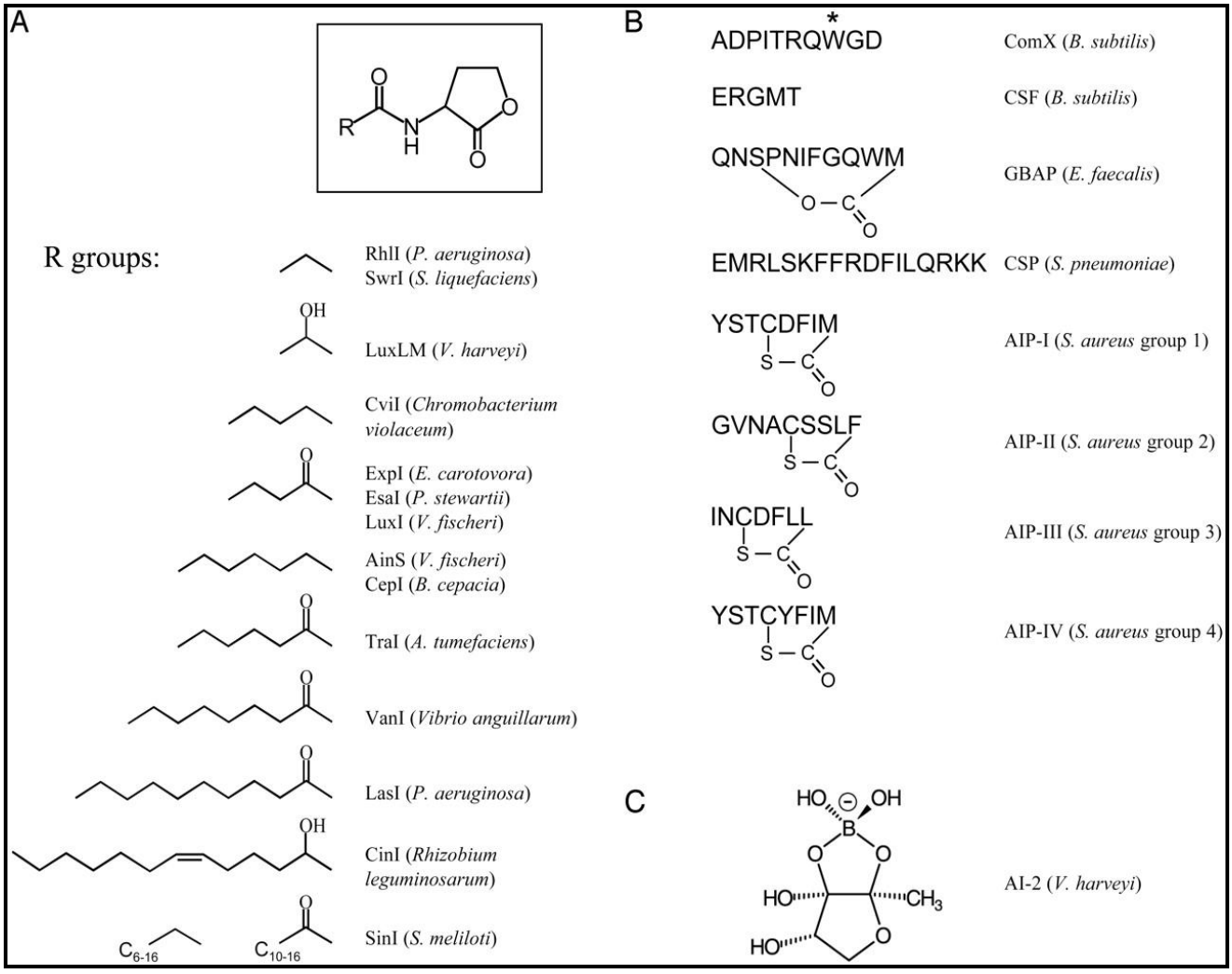

Fig. 4.4: Different AI (A) and AIP (B) from diverse bacterial species in comparison with AI-2 from *V. harvey* (C) (Michiko and Bassler, 2003)

This conclusion implies that involved sensor proteins should be able to distinguish AI structure. Comparing the structure of different AI molecules (Fig. 4.4), it is possible to observe that the only variable element consists of the acyl side chain. This element could vary in terms of length, structure and entity of substitutions: all these properties ensure that sensor proteins can recognize a limited number of signal molecules present in the environment and distinguish the information contained in AI molecular structure. Analogue scheme could be observe with regard to AIP, where specificity of signal is achieved through linkage between the AIP and its corresponding sensor kinase.

Different QS process could be at the same time present within same bacterial cell, as clearly already shown in *V. harvey*. This means that, despite the high specificity of single QS mechanism, non specific signalling due to multiple species cross-talking could take place with consequent interference on genetic expression and thus phenotype. For example, *P aeruginosa* increase biofilm formation in presence of indole produced by *E. coli*, while same *E. coli* are opposed in same phenotypic trait by acyl_HSL secreted by *P. aeruginosa* (Lee, et al. 2007). Interestingly it is possible to observe also unidirectional interactions due to multiple species cross-talking, as shown when *P. aeruginosa* and *Burkholderia cepacia* were grown together (Riedel, et al., 2001).

A suitable explanation for this apparently unclear complexity in cell communication systems could be found from a multicellular point of view: because in natural environments most of biofilms (such as oral plaque or gastrointestinal tract) consist of diverse bacterial species which act cooperatively between each other, a suitable system for communication is required for establishment of the sessile community and for its maintenance. This does not exclude the presence of a sort of universal way of "talking": to date only one signal molecule has been found in diverse bacterial genera, LuxS, which has led to generally accepted conclusion that AI-2 have important role in interspecies communication

[4.6]. Role of *luxS* and AI-2 in cell methabolism

LuxS acts, within bacterial cell, as component of activated methyl cycle (AMC), which, starting from S-adenosyl-L-methionine (SAM), produces activated methyl substituents, necessary for protein methylation, RNA, DNA and other metabolites. This reaction is catalyzed by dedicated transmethylases with consequent formation of S-adenosyl-L-methionine (SAH). Because of its highly toxic characteristics for the cell, species of both kingdoms Archaea and Eukarya employ a one-step pathway of detoxification through conversion of SAH into adenosine and homocysteine: the involved enzyme is SahH (Fig. 4.5), where the discussed step is blue-colored).

In order to achieve detoxification of SAH, there is an alternative possible process, demonstrated in all *Firmicutes* and some species of proteobacteria: in this case SAH is converted in adenosine and S-ribosyl homocysteine (SRH) by Pfs and LuxS transforms SRH into homocysteine (HCY) and 4,5-dihydroxy-2,3-pentanedione (DPD). This latter one is the effective precursor of AI-2, which can be formed through different internal ciclizations due to unstable nature of DPD. These reactions are underlined by red arrows in Fig. 4.5.

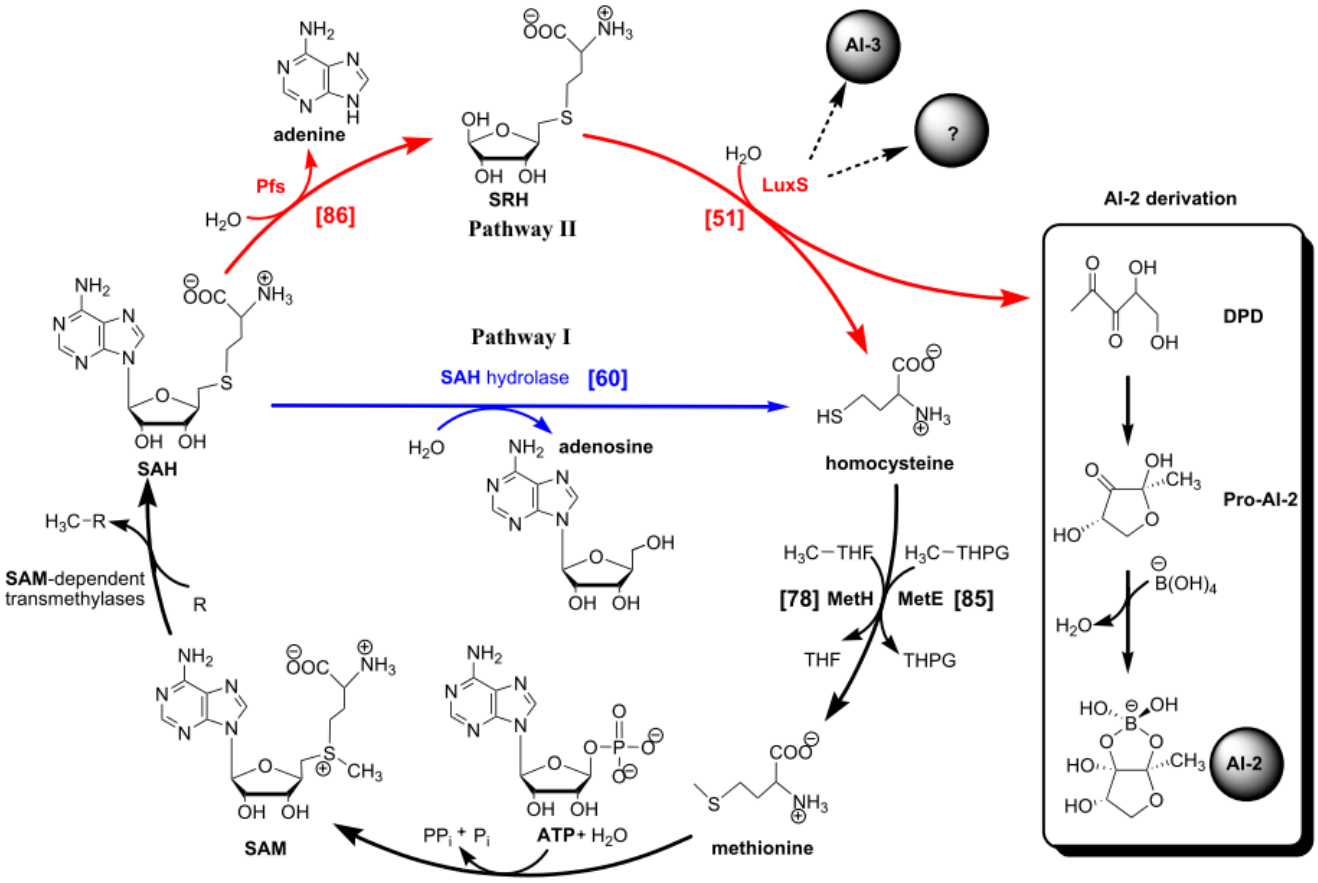

Fig. 4.5: Schematic representation of AMC Cycle. Abbreviations: SAM = S-adenosyl-methionine, SAH = S-adenosyl-homocysteine, SRH = S-ribosyl-homocysteine, DPD = 4,5-dihydroxyl-2,3-pentanedione, Pro-AI-2 = Ai-2 precursor, AI-2 = autoinducer 2, AI-3 = autoinducer 3. Numbers in the brackets represent number of organism which has demonstrated to possess that enzyme.

Begin of research on *luxS* could be found in Greenberg et al. (1979), where culture surnatant of diverse non bioluminescent microbes caused bioluminescence of *V. harvey*. After identification of AI-1 and its related system, researchers hypothesized the contemporary presence of an additional autoinducer-based process which allows interspecies communication with *V. harvey*. Demonstration of this was given by Bassler et al. (1997), where AI-2 was presented as "interspecies communication signal". After few years, structure of AI-2 and its corresponding receptor LuxP was determined. Once AI-2, diverse studies were conducted on presence and effects off *luxS* in several bacterial species, whose modified phenotypes were attributed to *luxS*-based QS signalling. Until now more than 70 diverse bacterial species are known to produce AI-2 and *luxS* has been identified in many bacterial genomes sequenced, leading to the general conclusion that AI-2 are accepted by scientific community as "universal cell-to-cell signal in prokaryotic microorganism" (Turovskiy, et al. 2007).

[4.7]. Biofilm formation and *LuxS*

Researches concerning AI-2 and QS have been in the most recent period associate to another well exploited topic, biofilm formation on surfaces. Different authors have exploited these two themes in their published reports, some of which are briefly summarized in the following lines. Belval et al. (2006) examined the influence of AI-2 on attachment to surfaces by *L. monocytogenes* EGD-e. Compared to wild-type, the *ΔluxS* strain produced denser biofilms on stainless steel. External additions of AI-2 did not alter number of adhered cells, although SRH could affect significantly biofilm cellular density of both wild-type and mutant strains. The authors conclude that altered phenotype is more probably to attribute to an accumulation of SRH instead of QS gene disruption. Similar conclusion were demonstrated with regard to *Lactobacillus reuterii*, by Tannock et al. (2005), where a *luxS* mutant of *L. reuteri* 100-23 was constructed and evaluated for adhesion capacity on plastic surface. Additionally *luxS* disrurption seem to have wider range of effect as ATP content of mutant was 35% lower than parental strain.

Doherty et al. (2006) showed that *luxS*-null mutant of *S. aureus* did not differs significantly from parental strain, when it was grown in rich growth medium. Also virulence and hemolysis were not affected from gene knock-out as well as biofilm formation, although growth in sulfur-limited condition was heavily compromised. Authors hypothesized that AMC cycle was substituted by intake of methionine from environment with consequent modification of metabolism. From test of competition involving wild-type and *luxS* null strain, authors concluded that cell grown in coculture have same pool of autoindicers and modified phenoptype could not be attributed to QS. LuxS has been demonstrated by Schauder et al. (2005) to have similar observed effect also in *N. meningitidis*. Beside effect of biofilm, *luxS* seem to have more wide range influence on general metabolism. Sperandio et al. (2006) from their study of trascriptomics on *luxS*-null mutant have demonstrated an altered phenotype in biosynthesis, metabolism and transport of aminoacids as well as in carbon compounds metabolism, but comprehension of actual range of *luxS* influence is still far from complete exploitation.

[4.8]. Technical approaches to study of biofilms

Biofilms represent a quite extensively exploited topic: Djordevic et al. (2002) and Harvey et al. (2007) employed a microtiter plate assay to assess quantitatively the adhesive properties of *L. monocytogenes* strains in their investigations, as well as Kushwaha and Muriana (2009) did. Another quite often used device was stainless

steel coupon (Kalmokoff, et al. 2001; Mai, et al. 2007; Fuster-Valls, et al. 2008), where testing strains were grown at different temperatures and with diverse growth media to evaluate the relevance of such parameters. Other scientists (Sternberg, et al. 1999; Heydorn, et al. 2000; Stoodley, et al. 2001; Perni, et al. 2006,) use a dynamic approach: tested surfaces were put into flow chambers or cells, where nutrients solution was flown at defined fastness and, through suitable microscopy strumentation, kinetics and developing architecture of biofilm were observed.

Unfortunately both these approaches have intrinsic disadvantages, which make them not perfectly suitable to simulate the process of bacterial setting on surfaces. Through static devices bacterial adhesivity capacity on different material can be investigated, but, instead of biofilm, cells deposition is supposed more probably to take place. Furthermore, shear forces and other factors related to flow rate and mobility in fluids environments are absent in similar conditions, leading to modifications of architecture and complexity of biofilm architecture: cells are enveloped in a multilayered structure of organic matter, which can increase just its thickness within certain limits. When subjected in turbulent regime, voids within the matrix and tower-like complexes are observed and detachment of cells-organic matter occurs.

This aspect is strongly related to antimicrobial resistance: in a multilayered complex biocide cannot penetrate the whole thickness acting only in top levels, while in dynamic regimen cells can significantly modify their metabolic pathways expressing the so-called "`biofilm phenotype", physiological state in which cells are less susceptible to antimicrobials and more suitable to grow in absence or low levels of nutrients. Equipment like flow chambers can overcome this limit by application of flow rate of solution through a peristaltic pump, but the final result is a qualitative or semi-quantitative measurement because of monitoring all the stages of the process by epifluorescence microscopy or other optical techniques.

Aim of the present work was investigating on influence of *luxS* on biofilm formation. To carry out this purpose, *luxS* in-frame deletion was performed on *L. innocua* UC 8410 through Campbell-like single crossover event. Both wild type and *luxS*-null mutant were evaluated for surface adhesivity both in static and dynamic conditions as well as for biocide resistance. Also modifications of other traits of interest (e.g. hydrophobicity) due to applied genetic deletion were evaluated.

[4.9]. Materials and methods

I. Test materials

Tested materials used are described in par. I, section 2.5, Chapter 2.

II. Disinfectants

Benzalkonium chloride (BC) (50% in water, Fluka) and peracetic acid (PAA, 40% in acetic acid: water, Fluka) were prepared at concentrations of 200 µg/ml and 5% (equal to 5,8 mg/ml), respectively, as routinely used for sanitation in food processing environments.

III. Bacterial strains and plasmids

Three isolates of *Listeria innocua* (CLIP 11262, UC 8409 and UC 8410) were cultured in BHI broth at 37°C. *E. coli* TB1 was grown in Luria-Bertani (LB) at 37°C in shaking conditions. *L. innocua* 7117 (*Listeria innocua ΔluxS* UC 8410) was grown at 37°C in BHI supplemented with 32 µg/ml erythromycin. *V. harvey* BB117 (ATCC®BAA-116) was grown in aerobic conditions in Marine Broth (MB, Difco) at 30°C. Cloning procedure has involved p-GEM T-easy Vector (Promega) and pRV300.

IV. DNA Manipulation

Single colonies from ALOA (Agar Listeria & Ottaviani Agosti, Biolife Italiana Spa) were used as inoculums for 10 ml BHI broth. 1 µl of an overnight subculture of *Listeria innocua* was treated with 19 µl of *micro*Lysis (Labogen), as indicated by the manufacturer. 1 µl of the resulting solution was used as DNA template in 50 µl PCR reaction using GoTaq Green Master Mix (Promega), 0.4 µM *luxS* primers (LuxS1-LuxS2). *luxS* primers sequence and PCR thermal conditions are reported in Tab. 4.1. In parallel,

PCR of *iap* was performed on same samples following protocol reported in Bubert et al. (1997) as control.

PCR reaction	Iap	LuxS
Primer	Bact1-Lis1B	LuxS1-LuxS2
Sequence	(Jordan, et al. 2008)	LuxS1: CCAAATAAAGAACATATGGAAATGC LuxS2: ACATTTTTCCATTCGCTTCG
MgCl2	1.5 mM	3 mM
Nucleotides	0.2 mM	0.2 mM
Initial Denaturation	5 min, 95°C	5 min, 95°C
Denaturation	1 min, 95°C	1 min, 95°C
Annealing	1 min, 60°C	45 s, 50°C
Extension	45 s, 72°C	1 min. 72°C
Number of cycles	35	35
Final extension	5 min, 72°C	5 min, 72°C

Tab. 4.1: Primers and temperature ramps used in PCR reactions

V. Cloning procedure

The amplified fragment was purified by microClean (Labogen) and quantified by Marker II (Roche). The obtained fragment was inserted in p-GEM T-easy vector (Promega) according to manufacturer protocols, obtaining the plasmid pPC 7048. The so constructed cloning vector was electroporated in electrocompetent *E. coli* TB1 at 200 Ω, 25 µF and 12.5 kV/cm (time constant = 4.0÷4.4) in 0.2 cm electroporation cuvette (Biorad). The final mixture was plated by spreading onto LB agar plates supplemented with 100 µg/ml ampicillin. The selected colonies were cultured in 10 ml LB broth incubated at 37°C in shaking overnight and the plasmid was extracted by Wizards SV MInipreps (Promega). Gene insertion in pGEM T-easy Vector was verified by PCR. 10 µl plasmidic DNA were digested with *SalI* at 37°C for 3 hours and loaded into 1x agarose gel in 1x TAE.

The 240 bp band was cut and purified by GenElute™ Gel Extraction Kit (Sigma-Aldrich). The so treated fragment was ligated in pRV300, previously digested with *SalI* and dephosphorylated by calf alkaline phosphatase (Promega). 1 µl of treated plasmid was mixed with 3 µl of 240 bp fragment and ligated by using T4 ligase (Promega). The final mixtures were incubated at 4°C over-night and electroporated in *E. coli* TB1, as previously described. The so treated *E. coli* was plated by spreading onto LB agar plates containing 100 µg/ml erythromycin and incubated over-night at 37°C. One colony was picked up to inoculate 10 ml LB containing 100 µg/ml erythromycin. The so constructed shuttle

vector was extracted as above mentioned and verified by a PCR screening with the primers T7 promoter-LuxS2 and LuxS1-LuxS2.

VI. Preparation of electrocompetent cells

Electrocompetent *L. innocua* cells were obtained using procedure of Monk et al. (2008) with slight modifications. A shaken overnight BHI culture was diluted 1:100 in 500 ml of BHI containing 500 µM sucrose (BHIS), resulting in an initial optical density at 600 (OD_{600}) of 0.01 ÷ 0.02, and then grown to an OD_{600} of 0.4 ÷ 0.5. Cells were cooled on ice for 10 min and centrifuged (5,000 *x g* for 10 min at 4°C). Cell pellets were resuspended in 500 ml of ice-cold sucrose-glycerol wash buffer (SGWB) (10% glycerol, 500 µM sucrose; pH adjusted to 7 with NaOH; filter- sterilized) by swirling on ice. Cells were centrifuged two more times; they were resuspended in 175 ml of SGWB after the first centrifugation and in 50 ml of SGWB after the second centrifugation. Cells were centrifuged (3,000 *x g* for 10 min at 4°C) and resuspended in 20 ml of SGWB. Cells were finally centrifuged, the final volume was adjusted to 2.5 ml by pipetting, and 50 µl aliquots were frozen at -80°C.

VII. Electroporation into *L. innocua*

A 50 µl aliquot of electrocompetent cells was mixed with 2 of plasmid DNA and incubated on ice for 5. The mixture was transferred to a chilled 1 electroporation cuvette (Bio-Rad) and pulsed at 10 kV/cm, 400 Ω and 25 µF. Time constants between 7 and 8 were observed with the protocol described above. To regenerate the cells, 1 ml of room temperature autoclaved BHIS was pipetted immediately into the cuvette and incubated statically at 37°C for 1,5h. Regenerated cells were serially diluted and plated on BHI agar containing erythromycin.

VIII. Biofilm formation in two different conditions of growth

Overnight liquid subculture of both *L. innocua* UC 8410 and *L. innocua* UC 7117 were tested in parallel for surface adhesion both in static and in dynamic conditions. Experimental procedure, as well as used apparatus, to evaluate strain adhesion during static incubation is reported in par. III of section 2.6, while par. IV of above mentioned section describes protocol for biofilm development on surfaces immersed in a milk flow.

IX. Bactericidal efficiency of disinfectants

Both *L. innocua* UC 8410 and *L. innocua* UC 7117 were grown in biofilm state using apparatus described in par. III of section 2.5. Bactericidal effectiveness of above mentioned biocides on planktonic and sessile cells was evaluated on aliquots of nutrient solution (milk) and tested surfaces (1 cm wire),, respectively during 24 h monitoring period by using UNI EN 1040:2006 protocol.

X. Monitoring inhibitory effect of selected antimicrobials during growth kinetic

Bacterial cultures were washed and adjusted with 0.9% NaCl in order to get OD_{625} of 0.080÷0.100. 100 µl of cell suspension were added into a 100 wells honeycomb plates (Honeycomb, ThermoLabsystems, Finland), where 300 µl of test medium and 4 µl of testing antimicrobial were added. Plates were incubated for 24-48 h at hours with low shaking and reading of optical density were performed every 15 min by using a wide band filter (420-580 nm). At least two independent experiments were performed with approximately 3-5 replicates for each of them, while data elaboration was conducted on average of each replicate. Test medium was BHI broth.

XI. Microbial adhesion to solvents (MATS)

This hydrofobicity assay was performed as described by Briandet et al. (1999): 18h liquid subculture was harvested at 2500 *xg* for 5 min and washed twice with 0.85 % NaCl in water. Cells suspension was divided in 2.4 aliquots, to which 0.4 ml of hexadecane (Sigma-Aldrich) was added. The mixture was vortexed for 1 min and then incubated at room temperature for 15 min, to allow complete separation of the two phases composing the mixture. 1 ml was taken from the mixture and optical density at 400 nm was measured. Results were collected from three replicates obtaining by using two independent subcultures and were expressed as percentage of affinity with solvent by using the equation:

$$\% \text{ affinity with the solvent} = 100 \times [1-(A/A_0)]$$

XII. AI2 bioluminescence assay

Bioluminescence assay for quantitative detection of AI-2 was performed as described from Vilchez et al. (2007). Briefly, an aliquot form -70°C stock was grown onto an AB medium plate over-night. Plate was washed with fresh AB medium and then cultured in AB-Fe medium (AB medium supplemented with iron solution) for 1.5 in shaking conditions. Once checked optical density, culture was diluted in order to get reference value (3000-5000 CPS). All measurements were performed in triplicate using Victor 1420 Multilabel Plate Reader (Perkin Elmer).

In parallel method described by Bassler et al. (1993) was carried out with slight modification. A over-night subculture of *V. harvey* BB117 in 3 ml of Marine Broth (MB, Difco) was diluted 1:5000 in fresh MB and used as working solution. In parallel testing solutions were prepared as following. 1%-inoculated liquid subculture was harvested at 2500 *xg* for 10 min at room temperature. Cell surnatant of *L. innocua* was recovered, adjusted to pH 7 and sterilized through filtration with 0.45 µm membrane filter. The so-treated surnatant was divided into aliquots and kept at -20°C. 900 µl of working solution were mixed with 100 µl of sterile pH-adjusted cell surnatant and incubated for 6 h. Measurement was performed through a systemSURE ™portable luminometer (Celsis•Lumac, Cambridge). As negative

control, pH-adjusted and filter-sterilized MB was used. All obtained data were obtained from three replicates.

XIII. Statistical analysis and treatment of data

Data were subjected, through SPSS 14.0, to One-way ANOVA, *t* test and post-hoc analysis, in order to detect significant parameters and to give a quantitative definition of influence of selected parameters (i.e. strain, temperature, nutrients, surface material).

[4.10]. Results

I. *luxS* gene inactivation in *L. innocua*

As first step, the presence of *luxS* was verified into the studied strains (Fig. 4.6). Primers used (LuxS1-LuxS2) were constructed basing on the sequences of *luxS* of *L. innocua* CLIP 11262present in Genebank. Both UC 8409 and UC 8410 harbor this gene, as confirmed by the sequence analysis of the amplified fragment, as reported in Fig. 4.7.

In order to evaluate its influence on biofilm formation, *luxS* gene disruption was performed by a Campbell-like gene inactivation. To achieve this goal, the plasmids pRV300 was used as shuttle vector in *L. innocua*.

A 240 bp fragment of *luxS* gene (from bp base 135 to bp base 455) was amplified in *L. innocua* UC 8410, cloned in the multi-cloning site site of pGEM®-T Easy vector and then introduced in the vector pRV300, a plasmid harbouring an origin of replication suitable for *E. coli* but not for *Firmuctes* as *L. innocua* (Leloup et al. 1997) as well as the gene for erythromycin resistance. The knock-out vector harboring the 240 bp *luxS* fragment was replicated in *E. coli* TB1.PCR screening was conducted on plasmids isolated from *E. coli* TB1 to verify that they were properly constructed: primers used were able to amplify the sequence between the plasmid promoter (T7 for) and investigated gene. The verified vector, named pPC 7051, was then electroporated into electrocompetent *L. innocua* cells.

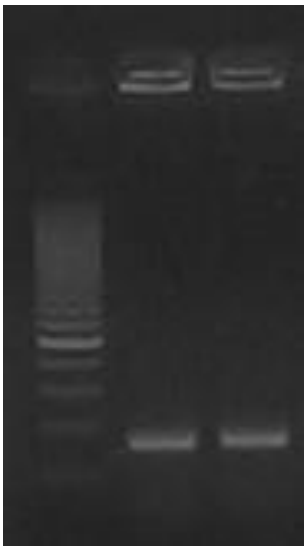

Fig. 4.6: PCR to verify presence of *luxS* in examined bacteria. Lane 1: marker 200 bp, lane 2: *L. innocua* UC 8410, lane 3: *L. innocua* UC 8409. Size of amplicone was 320 bp.

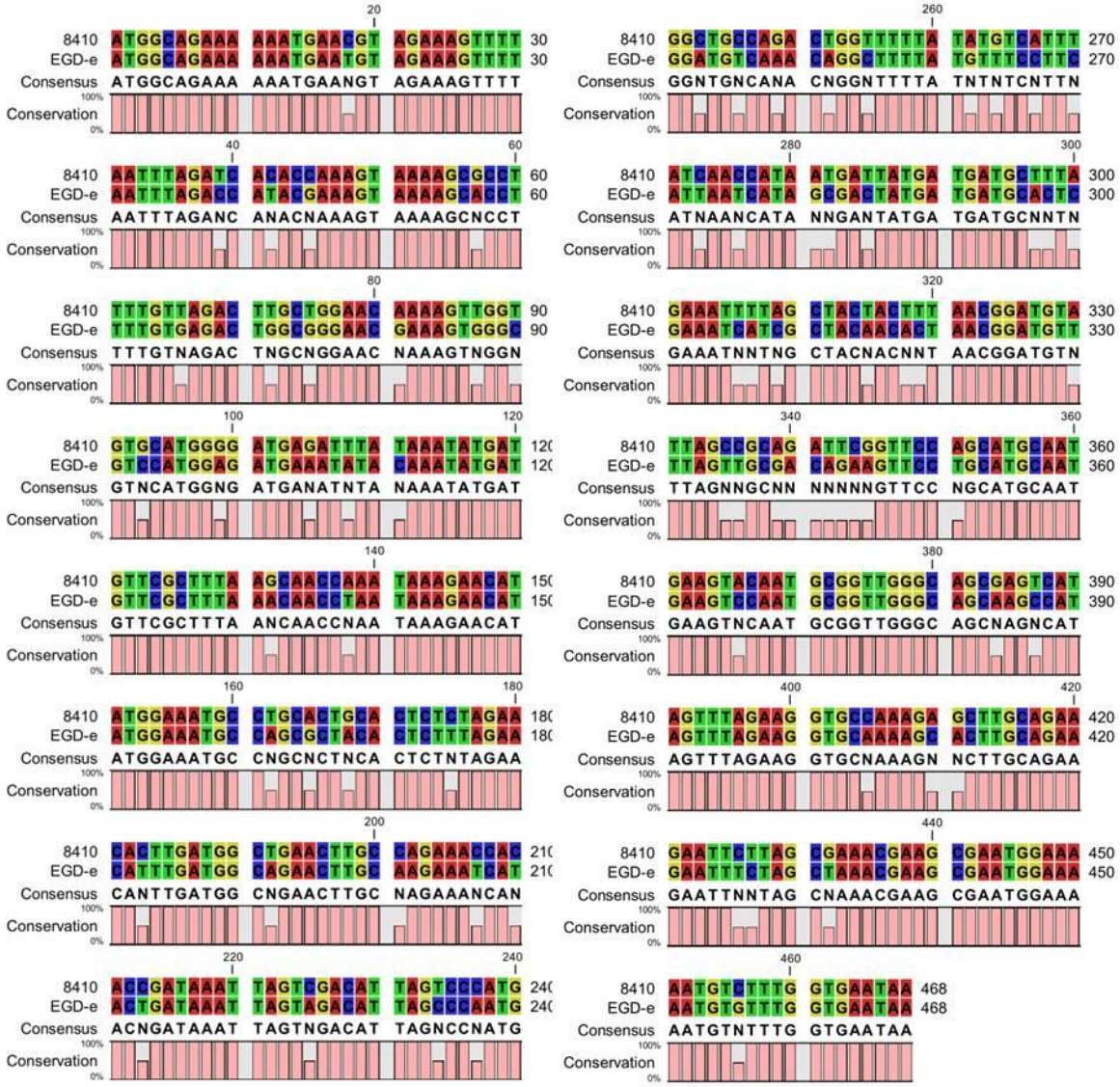

Fig. 4.7: *luxS* alignment obtained by comparison of *luxS* sequences present in *L. innocua* UC 8410 and in *L. monocytogenes* EGD-e through CLC Sequence viewer 6.0. Bars represent percentage of homology, while nucleotides sequence is reported in letters of different colors.

Once regenerated with sucrose supplemented medium, cells were plated on agar plates supplemented with 5 µg/ml of erythromycin agar plates and were then screened by PCR assay to verify plasmid integration in bacterial chromosome and thus genetic disruption of target gene: the couple of primers T7-LuxS2 was able to amplify the sequence between the T7 promoter and the inserted fragment harboured by the knock out vector (380 bp), while a 320 bp was the amplicone of chromosomal luxS gene obtained by using the primers LuxS1-LuxS2 (Fig. 4.8). *luxS*-deleted *L. innocua* strains were coded as UC 7117.

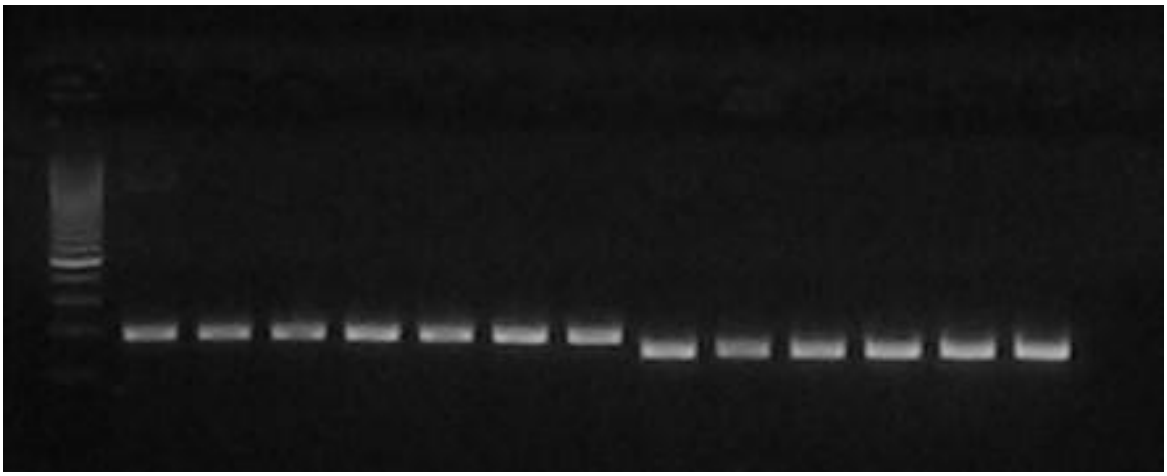

Fig. 4.8: Verify of genetic knock-out of *luxS* in all obtained mutant strains of *L. innocua* UC 8410. Lane 2-8 are PCR products obtained by using T7-LuxS2, while lanes 9-14 correspond to luxS amplicone produced with primers LuxS1-LuxS2.

II. Effect of *luxS* gene disruption through MATS and bioluminescence

To verify the physiological effect of *luxs* inactivation, the bioassay for AI-2 detection through the bioluminescence of *V. harvey* was performed on the cell supernatant of both L. innocua UC 8410 and UC 7117. For this purpose a culture of *V. harvey* in stationary phase was exposed to filter-sterilized cell surpernatant, whose pH was adjusted to pH 7. Results are reported in figure. Overnight subculture of *L. innocua* UC8419 was demonstrated to produce a signal of 0.10 RLU, while this feature was completely abolished in the *luxS*-null mutant UC 7117 . Results collected are reported in Fig. 4.9.

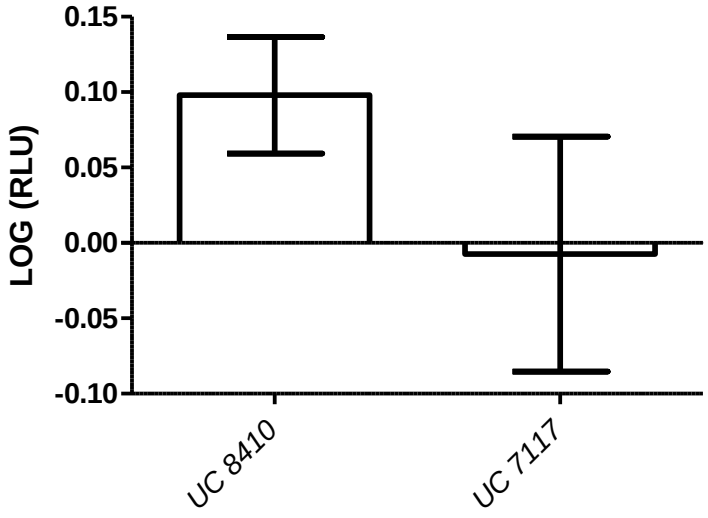

Fig. 4.9: Bioluminescence assay conducted on *L. innocua* strains UC 8410 and UC 7117 using the protocol of Bassler et al. (1993).

Moreover, the autoinducer production was monitored during 24 h of growth through bioluminescence of *V. harvey* BB170 with AB medium as previously reported (Vilchez, et al. 2007). Results (

Fig. 4.10) showed clearly that UC 7117 had a sharply decrease ability in producing AI-2 compared to wild type version.

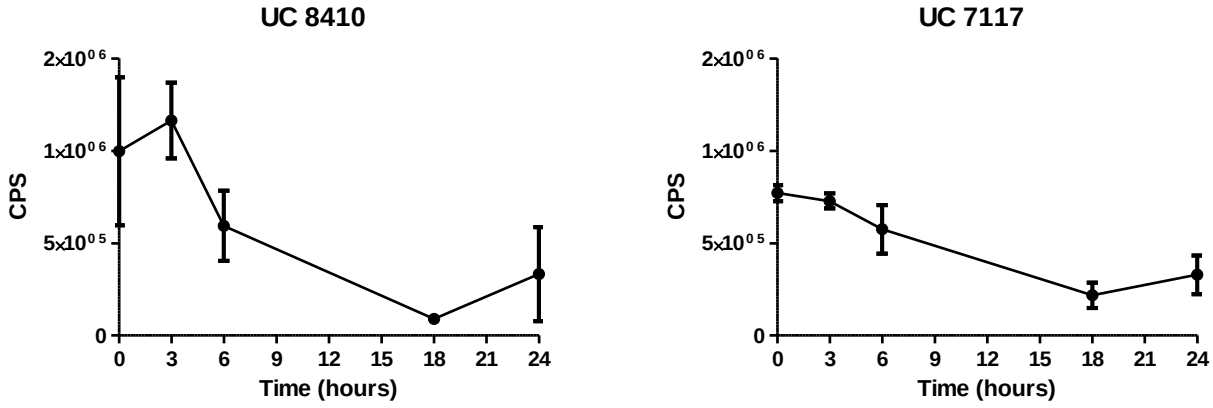

Fig. 4.10: Bioluminescence assay for AI-2 detection on sterile-filtered cell surnatant from UC 8410 and UC 7117. Data are expressed as average ± standard error mean of three replicates.

Moreover the effect of genetic mutation on cell hydrophocbicity was investigated through microbial adhesion to solvents (MATS) assay. This method was performed with hexadecane, as reported by Briandet et al. (1999), and results are shown in Fig, 4.11.

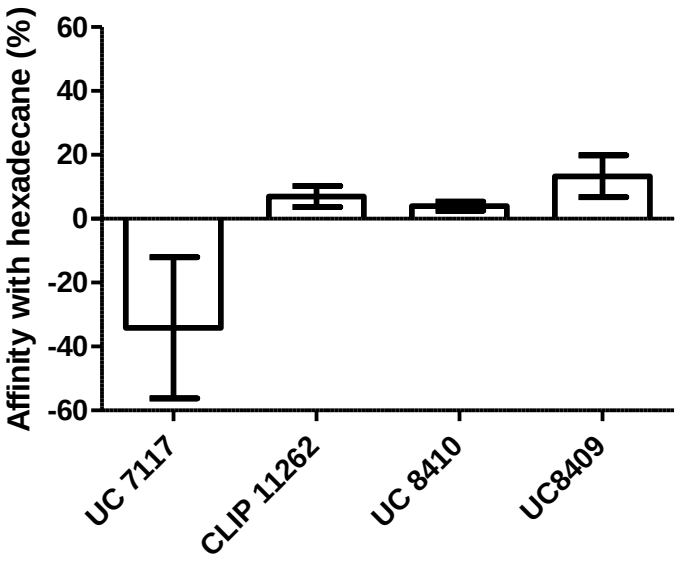

Fig. 4.11: MATS assay conducted on tested *L. innocua* strains. CLIP 11262 is *L. innocua* strain whose *luxS* sequence was used in cloning procedure, while UC 8410 was parental strain used in cloning procedure. Bars represent average of at least three replicate for each sample ± standard error mean.

UC 8410 were compared to other available strains of same species and with its *luxS* null derivative l, to assess is Relevant discrepancies were observed among tested isolates (even if belonging to same environmental niche). Most of tested strains showed low affinity to hexadecane, demonstrating that cells can be correctly assumed as strongly hydrophilic. UC 8409 revealed higher affinity to employed solvent (17%, respectively), which was considered as not significant in statistical elaboration. Although *luxS*-null strains expressed quantitatively different hydrophobicity from their wild-type analogue, *L. innocua* UC 7117 has revealed marked hydrophilic properties, while its parental strain showed slight hydrophobicity.

III. Role of *luxS* in biofilm formation on abiotic surfaces

The adhesion of *L. innocua* UC 8410 and its *luxS*-null mutant *L. innocua* UC 7117 was evaluated within a 24 h both in static conditions. In this case the experimental procedure reported in par. III, sect. 2.5 of Chapter 2 was performed using BHI broth, instead of dBHI. During the 24 h monitoring period, *L. innocua* UC 7117 demonstrated to adhere on SS coupon in lesser amounts when compared to UC 8410 (Fig. 4.12), although this difference ranged between 1-2 logarithms during the whole period of analysis.

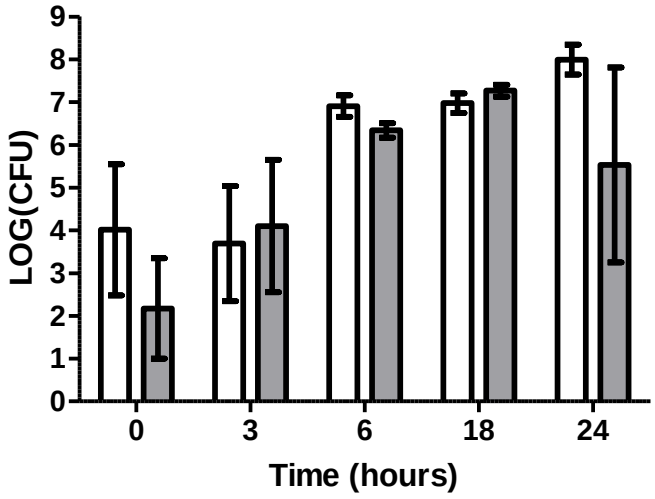

Fig. 4.12: Comparison of adhesion between *L. innocua* UC 8410 and *L. innocua* UC 7117 on stainless steel 1 cm^2 coupon immersed in BHI. Bars represent average of three replicates ± standard error mean.

The dynamic apparatus described in par. IV, sect. 2.5 of Chapter 2 was used to monitor adhesion of UC 8410 and UC 7117 in presence of 1 ml/min of milk. Results focused only to sessile cells are shown in Fig. 4.13. In presence of a turbulent flow of milk, *luxS* did not alter significantly density of planktonic cells (which were costant at 10^8 CFU/cm^2), although more than 10^8 CFU/cm^2 were found at 3 h. Differently from what observed in static adhesion on the same surface, obtained results showed clearly that gene disruption alter strongly the strain originary adhesive capacity: despite the high amounts of cells in the flowing medium, *luxS*-null mutant strain lost completely ability to adhere on all tested surfaces, including SS, which showed to be the most propitious support for establishment of sessile communities in testing conditions.

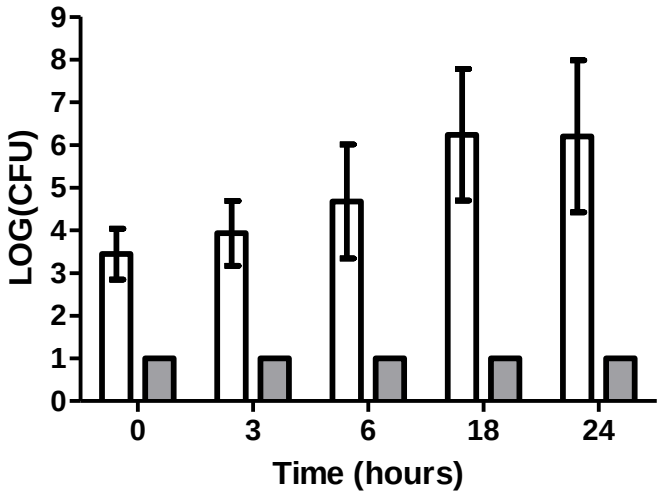

Fig. 4.13: Comparison of adhesion between *L. innocua* UC 8410 and *L. innocua* UC 7117 on a stainless steel wire immersed in 1 ml/min flow of milk within 24 h. Bars represent average of three replicates ± standard error mean. Data are expressed as LOG (CFU/cm^2). 1 LOG is to be intended as less than 10 CFU/ cm^2.

In presence of a turbulent flow of milk, *luxS* did not alter significantly density of planktonic cells (which were constant at 10^8 CFU/cm^2), although more than 10^8 CFU/cm^2 were found at 3 h. Differently from what observed in static adhesion on the same surface, obtained results showed clearly that gene disruption alter strongly the parental strain adhesive capacity: despite the high amounts of cells in the flowing medium, *luxS*-null mutant strain lost completely ability to adhere on SS, which showed to be the most propitious support for establishment of sessile communities in testing conditions.

The adhesion capacity of *L. innocua* UC 7117 on different surfaces in presence of a flow of milk was investigated and compared with the one of its parental strain (Fig. 4.14). Beside SS, also other materials like PET or C were investigated. While the amount of planktonic cells was not markedly modified, *L. innocua* UC 7117 was not detected even at the lowest dilution on all the tested materials, demonstrating that the genetic disruption abolished completely the adhesion capacity of the used *L. innocua* strain.

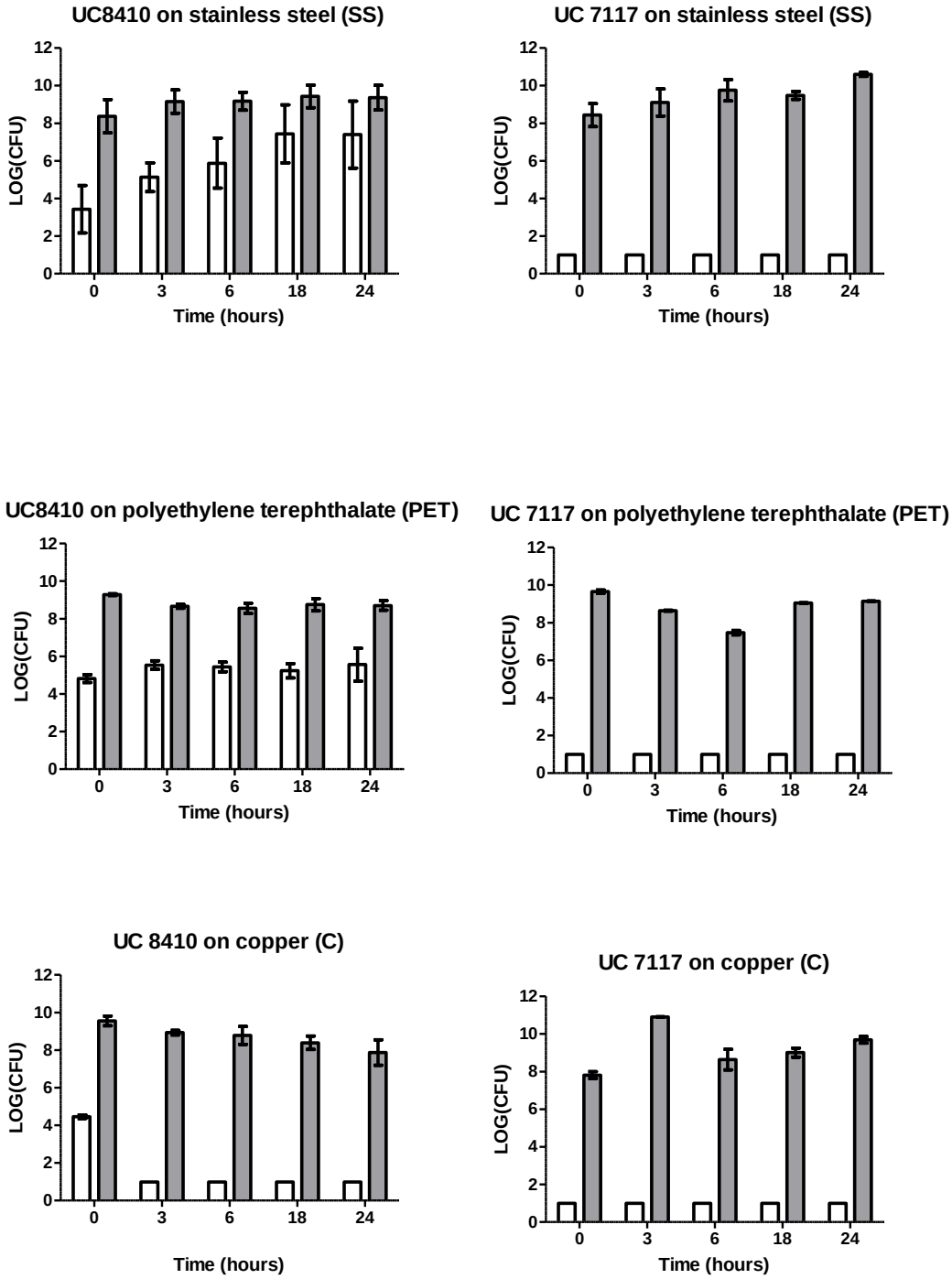

Fig. 4.14: Comparison of adhesion and biofilm formation of *L. innocua* UC 8410 (left images) and *L. innocua* UC 7117 (UC 8410 *ΔluxS*, images on the right side) on different materials in presence of shear forces due to flowing solution. All results arte express average of three replicates ± standard error mean. White bar show adhered cell on tested surfaces, while grey bars are referred to planktonic cells.

L. innocua ΔluxS strain UC 7117 and its adhrence ability on stainlees steel in dBHI was compared with its parental strain *L. innocua* UC 8410 in static conditions, as described in par. III of section 2.5, Chapter 2 (Fig. 4.15). A shared trend of behavior can be observed among the two examined strains: more than 10^7 CFU/cm^2 were found on statically incubated SS coupons after 24 h at 37°C, while, after 168 h adhered cells were reduced by 3 LOG for both the examined strains. After 24 h incubation under strong nutrient depletion, *luxS*-null was present in slightly higher amount on used surface than UC 8410 (1 LOG difference).

L. innocua UC 8410 was reduced by one logarithm and three logarithms at 72 h and 168 h, respectively, during starvation, while population density of UC 7117 decreased by two logarithms and four logarithms at 72 and 168 h, respectively, in same experimental conditions. These data demonstrated that *L. innocua* was susceptible to prolonged starvation in strictly limiting conditions. Moreover, *luxS* disruption made *L. innocua* slightly more susceptible to nutrient depletion for long period, although higher cellular densities could probably mask an increased sensitivity due to genetic modifications.

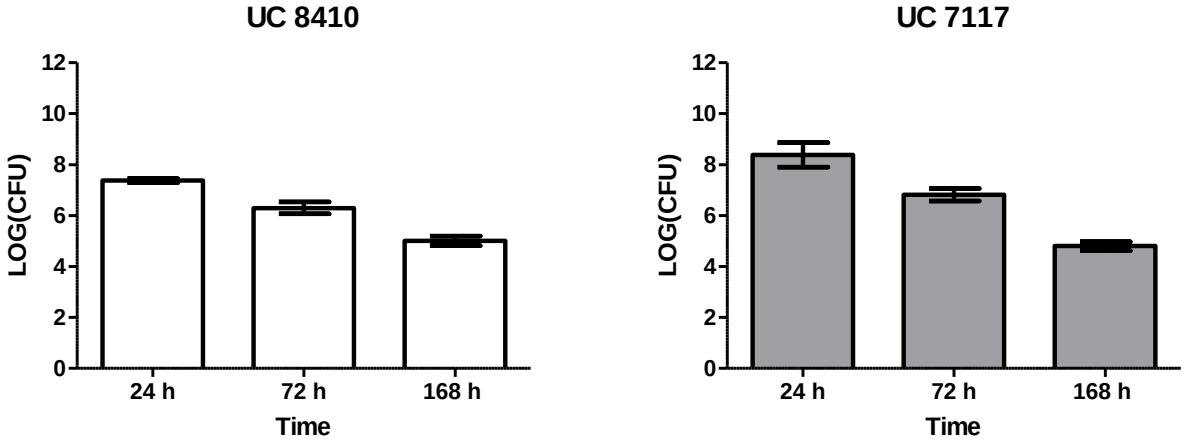

Fig. 4.15: Adheson of *L. innocua* UC 8410 (white bars) and *L. innocua* UC 7117 (grey bars) on stainless steel coupon. Data are expressed as average of replicates ± standard error mean.

IV. Influence of *luxS* on biocide resistance

In parallel to superficial adhesion on used wires, both *L. innocua* UC 8410 and UC 7117 were evaluated for their susceptibility against BC. From the above mentioned device for biofilm formation under dynamic conditions, aliquots of wire and milk, within a 24 h monitoring period, were exposed for 30 min at 200 μg/ml BC, as reported in the standard UNI EN 1040:2006, to evaluate bactericidal effectiveness on sessile and planktonic cells. This investigation was performed on different materials, such as SS, PET and C, to investigate possible effect of influence of tested surfaces.

In terms of adhered cells, UC 7117 showed a higher susceptibility to BC than UC 8410, probably because of its reduced capacity to adhere on SS (Fig. 4.16). BC was strongly effective against planktonic cells at 0 and 24 h, while, from 3 until 18 h, none quantitative difference was observed between two examined strains. In presence of PET no colony was detectable at all stages of sampling for both the investigated groups of cells, whereas BC acted efficiently only to sessile cells. In presence of copper, the antimicrobial effect of BC produced a reduction even stronger than the one observed for *L. innocua* UC 8410 in same experimental conditions. Statistical analysis revealed that wild-type and mutant significantly differs from each other, although test materials represent a further level of variability. Same conclusion was obtained from statistical analysis run on bacterial recoveries after disinfectant exposure.

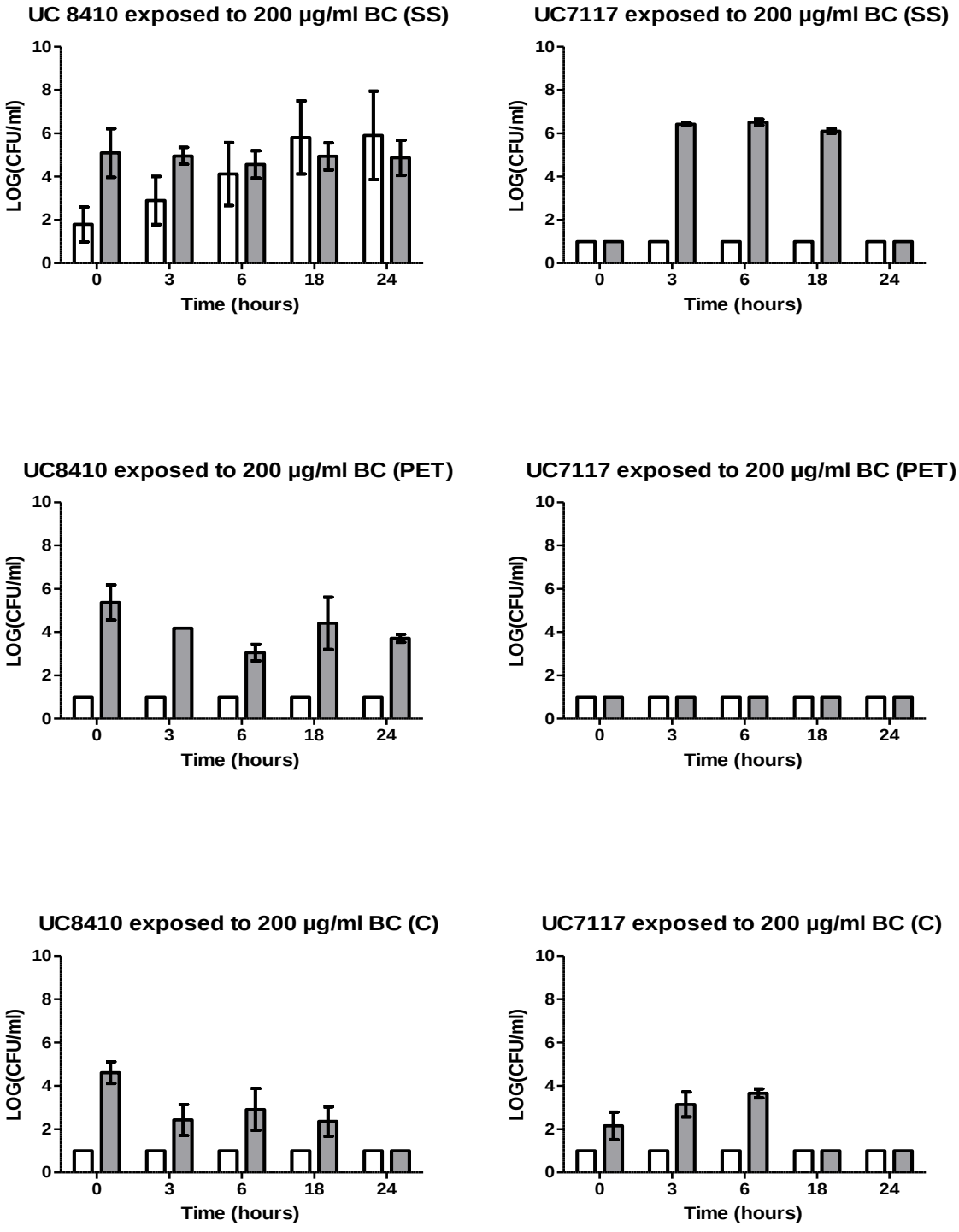

Fig. 4.16: Comparison of resistance to 200 µg/ml BC in *L. innocua* UC 8410 (left images) and UC 7117 (UC 8410 *ΔluxS*, images on the right side) on different materials in presence of shear forces due to flowing solution. All results arte express average of three replicates ± standard error mean. White bar show adhered cell on tested surfaces, while grey bars are referred to planktonic cells.

V. Evaluation of influence of *luxS* on growth kinetic and antimicrobial susceptibility through continuous monitoring methods

BioscreenC was used to compare *L. innocua luxS*-null mutant strain to its wild-type analogue, to demonstrate effect of *luxS* deletion on grow kinetics and susceptibility to pinosylvin and resveratrol, which were both dissolved in EtOH added at the final concentration of 3%. The experimental procedure was described in par. IV of sect. 3.8 (Chapter 3). Results are reported in Fig. 4.17 and Fig. 4.19. Mutant strain was less efficient to grow in comparison with wild-type: while UC 8410 started its log phase within the first few hours of incubation, more than 5 h of lag phase were observed in *L. innocua* UC 7117. While maximal growth of *L. innocua* UC 8410 was more than 1.3 units and was reached after 16 h, 0.8 units was the value of maximal growth reached in *L. innocua* UC 7117 after 18 h.

The interruption of the gene *luxS* increased markedly the sensitivity to 3% EtOH: the lag time of *L. innocua* UC 7117 was extended until 20 h and the maximal growth was reduced up 0.4 units, while *L. innocua* UC 8410 reached 1.1 units after 22 h. In comparison with its parental strain, *L. innocua* UC 7117 exhibited, in presence of 50 μM P, an slightly increased lag time (5h), while its parental strain grew after few hours) and a reduced maximal growth, which was equal to 0.9 and was reached after 22 h (Fig. 4.18Fig. 4.17). At 100 and 150 μM of P the lag time finished after 6 h and 8 h, respectively, and a reduced the maximal growth of *L. innocua* UC 7117 up to 0.6 units was observed, while the stationary growth was not reached within 24 h at both these concentrations. 200 μM of P extended the lag time until 11 h, while *L. innocua* UC 7117 reached 0.7 units after 22 h.

As observed in par. I, sect. 3.9 of Chapter 3with regards to *L. innocua* UC 8410, the concentrations ranging between 50 and 200 μM showed a protective effect of R towards *L. innocua* UC 7117 against EtOH (Fig. 4.19). In presence of 50 μM of R, the lag time lasted after 4 h and then the strain grew up to 0.8 units, which was kept constant after 22 h. 100 and 150 μM of R showed a similar effect on *L. innocua* UC 7117: both these two concentrations increased the lag time by 3 hours, while the strain grew up to be slightly less than 0.8 units. In presence of 200 μM the lag time of *L. innocua* UC 7117 was extended until 11 h and then the optical density of this strain was slightly higher than 0.8 units. 300 μM R led to a further increase of lag time until 15 h, while the maximal growth reach was 0.55 units.

In order to obtain a quantification of the inhibitory effect of these two polyphenols, the area under the curve was calculated through integral calculation for both the compounds and the *L. innocua* strains investigated.

Both P (Fig. 4.18) and R (Fig. 4.20) demonstrated a protective effect on *L. innocua* UC 7117 from EtOH at all the concentrations ranging between 50 and 200 μM, although the *luxS*-null mutant strain showed more reduced optical densities than its parental strain. Furthermore the protective effect on the *L. innocua* UC 7117 was more pronounced at 50 μM of P when compared with the same concentration of R. 300 μM of both P and R was not different from pure solvent.

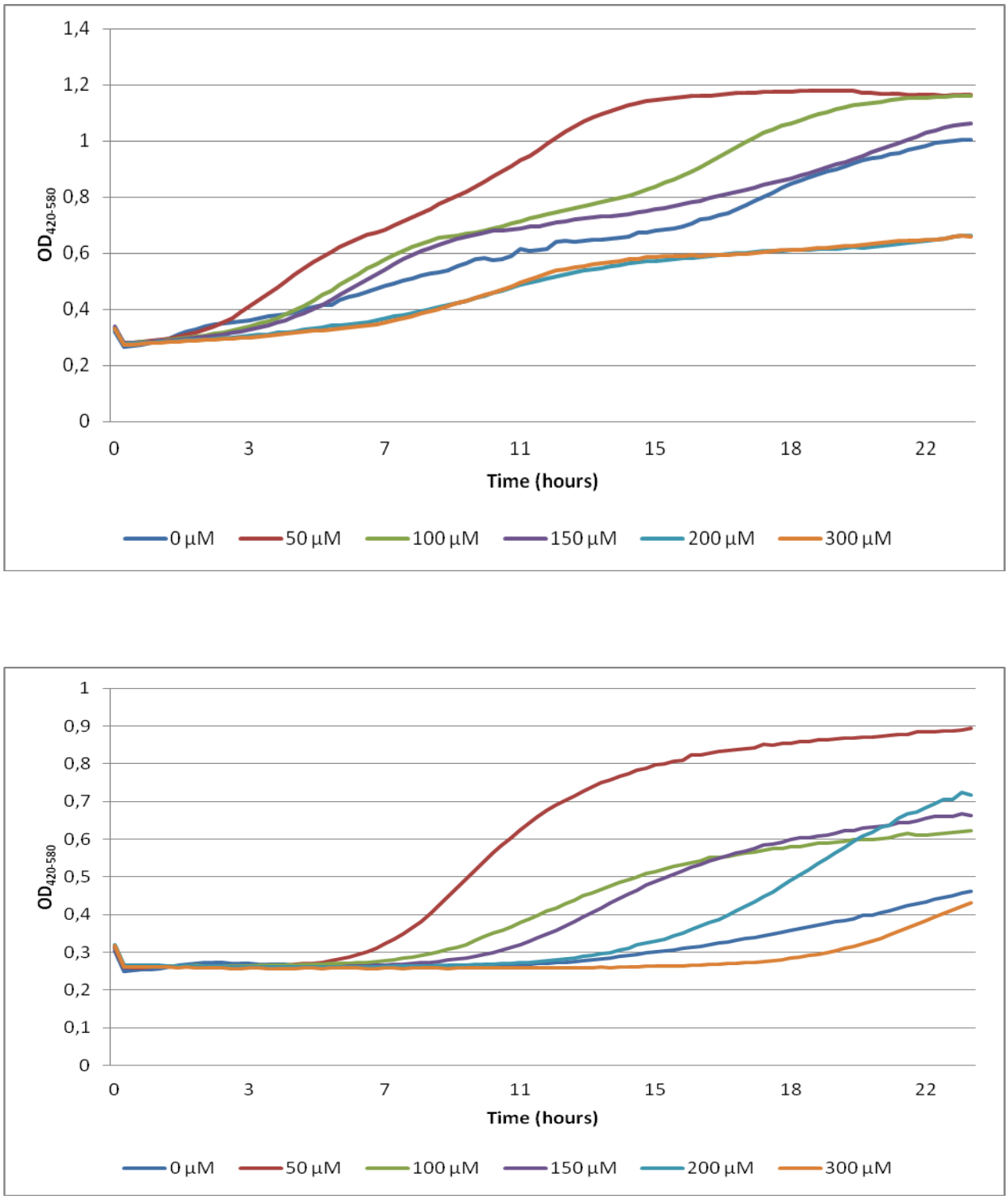

Fig. 4.17: Growth curve of *L. innocua* UC 8410 (above) and *L. innocua* UC 7117 (below) at different concentrations of pinosylvin. Data are expressed as average of five replicates of three independent experiments. Concentrations of pinosylvin are reported in µM. Error bars are omitted for clarity.

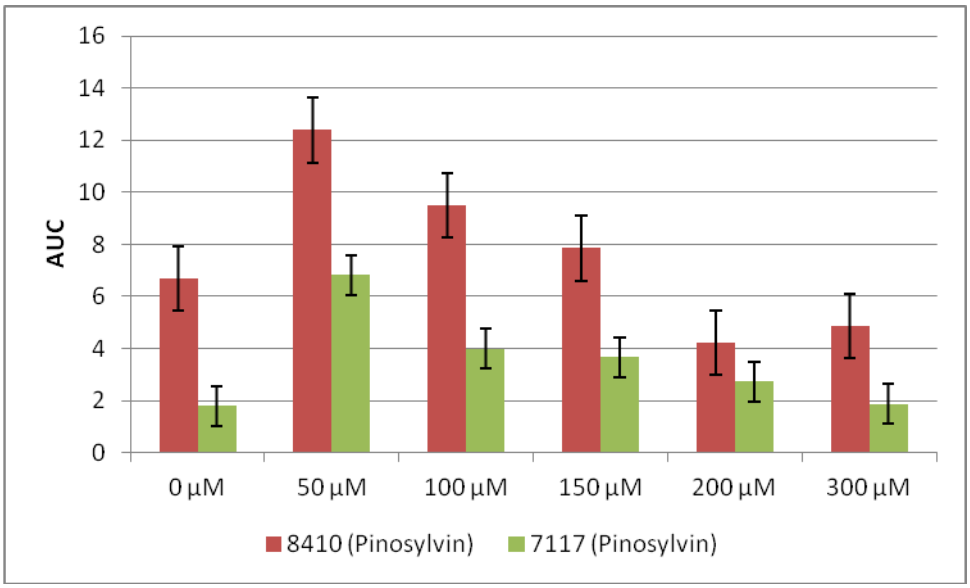

Fig. 4.18: Inhibitory effect of the stilbene pinosylvin on *L. innocua* UC 8410 (red bars) and *L. innocua* UC 7117 (green bars) at different concentrations in ethanol 99% through BioscreenC. Data were elaborated through GraphPad Prism 5 software to calculate the area under each curve (AUC). Measurement was performed on five replicates from three independent experiments. Bars represent average ± standard error mean.

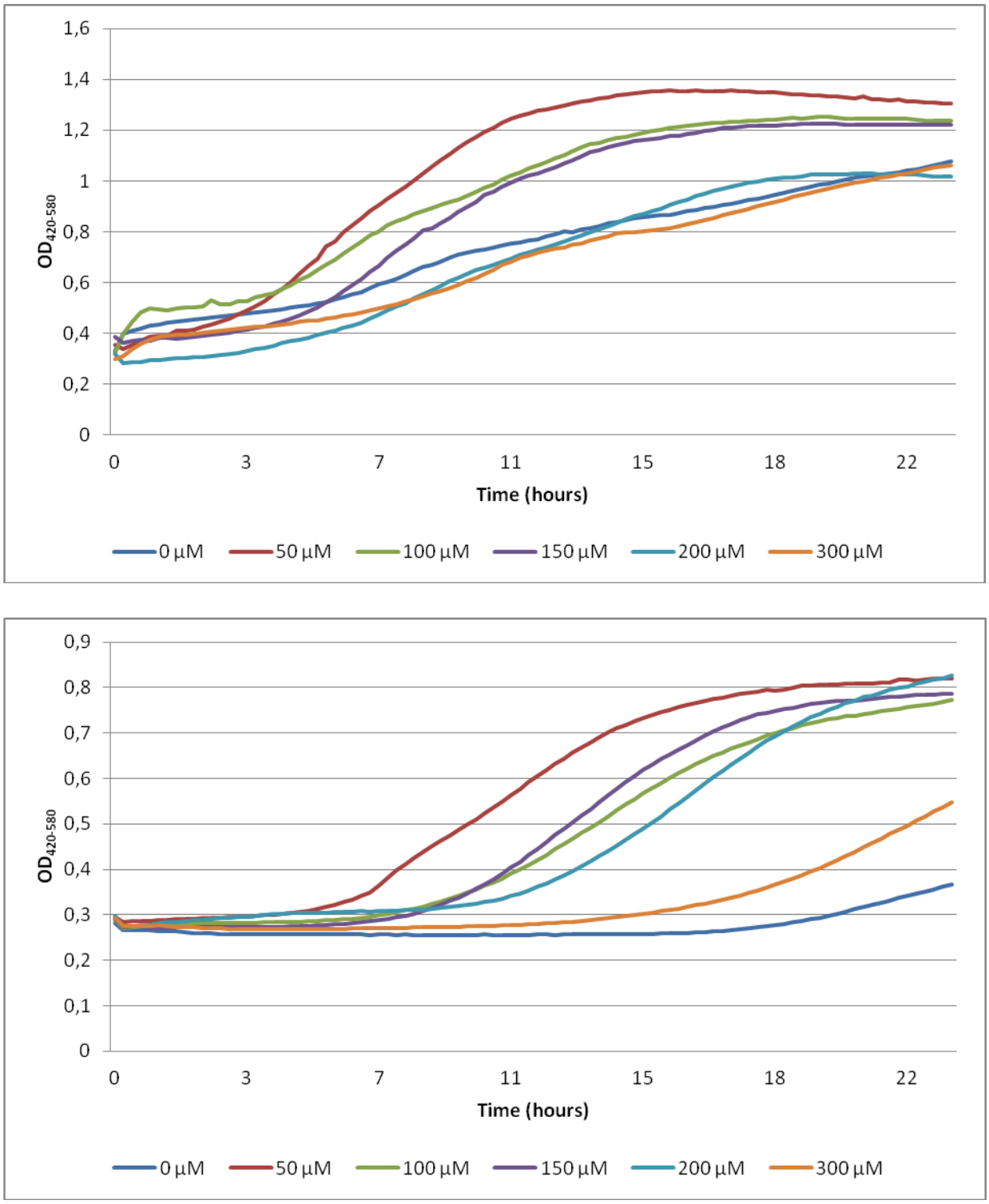

Fig. 4.19: Growth curve of *L. innocua* UC 8410 (above) and *L. innocua* UC 7117 (below) at different concentrations of resveratrol. Data are expressed as average of five replicates of three independent experiments. Concentrations of pinosylvin are reported in µM. Error bars are omitted for clarity.

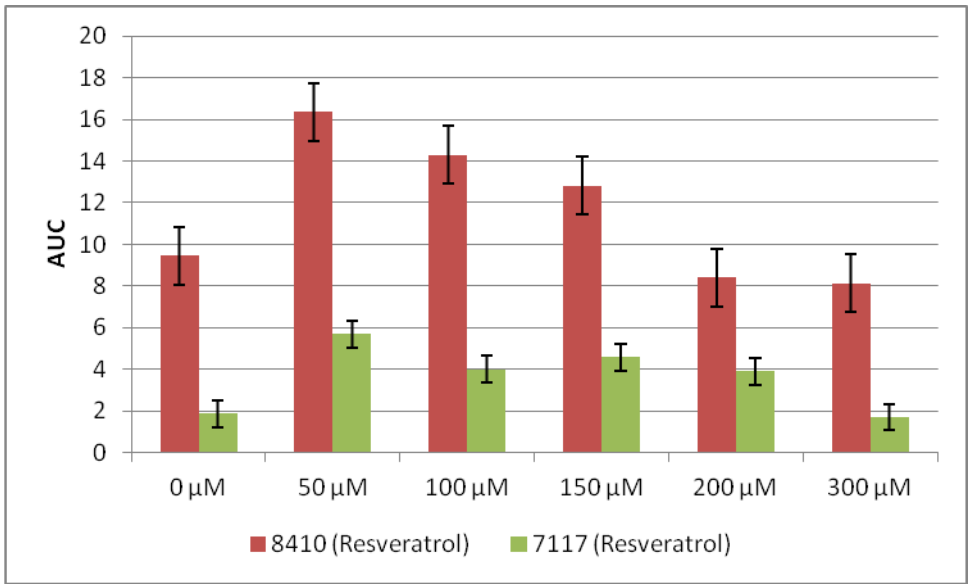

Fig. 4.20: Inhibitory effect of the stilbene resveratrol on on *L. innocua* UC 8410 (red bars) and *L. innocua* UC 7117 (green bars) at different concentrations (expressed in µM9 in ethanol 99% through BioscreenC. Data were elaborated through GraphPad Prism 5 software to calculate the area under each curve (AUC). Measurement was performed on five replicates from three independent experiments. Bars represent average ± standard error mean.

VI. Susceptibility of statically grown biofilms to polyphenols

Both UC 8410 and UC 7117 were also evaluated for their ability to adhere in static conditions on stainless steel and survival ability for prolonged time in presence of limited presence of nourishment and resistance to antimicrobial compounds like P. Data obtained from this experiment are shown in Fig. 4.21.

Despite the initial higher density, UC 7117 revealed increased susceptibility to both the two adverse conditions of growth: nutrient starvation and exposure to P. Cell density of UC 7117 was reduced by two logarithms and more than four logarithms after 72 h and 168 h, respectively, while nutrient depletion affected at a lesser extent UC 8410. While UC 8410 biofilm can tolerate concentration of 100 µM P, *luxS*-null mutant biofilm on SS is strongly decreased even at sub-inhibitory levels (four logarithmic reduction factor at 100 µM after 72 h). The inhibitory effect increases its effectiveness with correspondent increase of used concentration, confirming that inhibitory effect of P on biofilm formations was dose-dependent. Both the strains significantly differ both between each other and in comparison with other tested strains (Chapter II), demonstrating the above mentioned significant influence of QS.

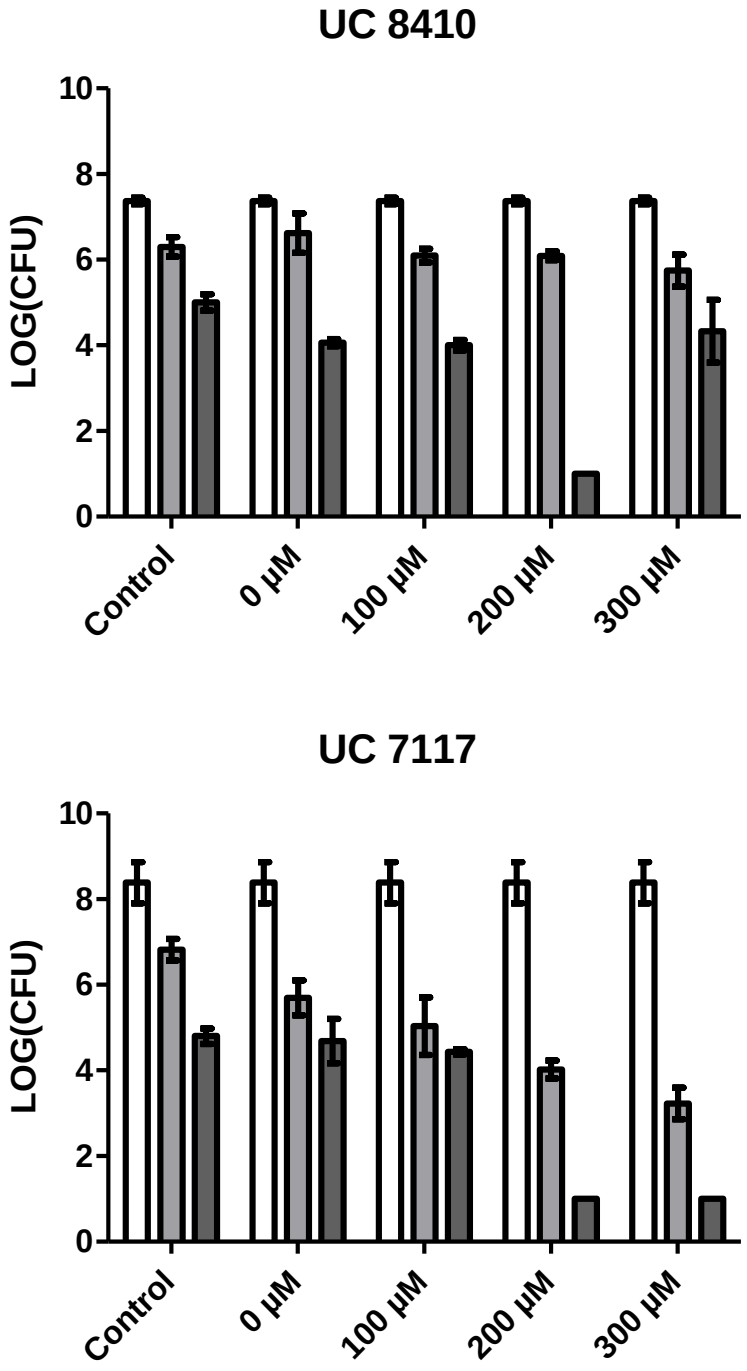

Fig. 4.21: Adhesion of *L. innocua* 8410 and *L. innocua* 7117 on stainless steel coupons immersed in dBHI and their exposure to different concentrations of pinosylvin for 7 days. Control refers to untreated sample, while added volume of ethanol with/without pinosylvin was kept constant. Data are expressed as mean of three replicates. Error bars are omitted for clarity.

[4.11]. Discussion

Listeria monocytogenes was often tested in former scientific papers for its aility to adhere on stainless steel. Moltz and Martin (2005) monitored adhesive capacity of *L. monocytogenes* strains on both microtiter plate and stainless steel chips, in order to exploit if material could be considered as a parameter significatively influencing this phenomenon. Tresse et al (2007) used a stainless steel microtiter plate to examine same phenomenon but on larger collection of *L. monocytogenes* strains, investigating also if genetic traits or source of origin could affect adhesivity observed in such surface. Silva et al. (2008) evaluated different materials for L. monocytogenes attachment and biofilm formation, observing that plastic surfaces supported markedly growth on surfaces in agreement with Oulahl et al. (2008). Mai and Conner (2007) demonstrated that rich growth medium allowed to obtain higher level of bacterial densities compared to minimal mediums, although also incubation temperature was described as a significantly discriminating parameter. Fuster-Valls et al. (2008) clearly stated that environmental condition could be also considered in experimental design as influencing factor.

All the above mentioned reports used static apparatus, while few papers reported adhesion under dynamic regimen (Perni et al. 2006, Perni et al. 2007). In this investigation a laboratory-sized system was used to monitor bacterial attachment on different materials in presence of a flowing solution, and obtained results were compared to those collected from stainless steel coupon. Both the system did not show any significative difference between each other, confirming often use of static deivice for evaluation of bacterial attachment o surfaces. But, as underlined by Perni et al. (2007), information collected by these two different approaches is completely different from a theoretical point of view. Actually, static system could give information about adhesive strength of a species and/or a strain, but, instead of biofilm development, cells deposition it is more likely to occurs in such devices. Presence of shear forces due to flowing liquids did not affect just bacterial densities found, but also alter structure, architecture and complexity of bacterial sessile communities (as can be noted comparing SEM images in Chapter II with picture from previous reports on this topic).

The recent research have been focusing on communication systems present within spatially organized and coordinated communities, with particular mention to quorum sensing processes. Among the diverse quorum sensing signal processes demonstrated in bacteria, LuxS-based signal processing have been found in several both Gram-positive and Gram-negative species and AI-2 (singal molecule indirectly derived by LuxS activity) was recognized as “interspecies communication signal”. Despite extensively exploitation in several articles (e.g. Xavier and Bassler, 2003; Vendeville, et al. 2005; De Keersmaecker, et al. 2006), Turovskiy et al. 2007) considered AI-2-based communication a weak hypothesis due to several conflicting theoretical aspects

concerning this topic, although further clarifying investigations focused on QS are were not excluded from the authors themselves. Analogue conclusion were reported by Garmin et al. (2009), who recognized *agr* as the "only effective communication system" present in the genus Listeria, although no experimental approach was proposed to support such affirmation. AI-2 were commented by these latter authors as not being able to fit with the definition of a signaling molecule, attributing importance to *agr*-based communication.

Apart from study of Belval et al. (2006), Sela et al. (2006) performed gentic deletion of *luxS* in *L. monocytogenes* EGD by mutagenesis through plasmid pKSV7::$luxS_{Lm}$ and evaluated biofilm formation on glass and in 24-well polystyrene plate in parallel to bioluminescence assay for detection of AI-2: mutant strain showed massively increased amounts of cells on glass slide, although more compromised growth was observed. In this research cloning procedure of *luxS* was performed by using pRV300 (Berthier et al. 1996, Leloup et al. 1997) for the construction of the knock-out vector.

pRV300 was originally designed for *Lactobacillus* spp. to integrate, within a certain locus of bacterial chromosome, through Campbell-like integration mechanism and to create a separation of selected gene from its natural regulation machinery, but here it was successfully demonstrated a possible application also for Gram-positive bacteria such *Listeria*. After verifying the correct construction of the knock-out vector harbouring a 240 bp internal fragment of *luxS*, the plasmid pPC 7051 was successfully electroporated into UC 8410. All the successfully constructed clones of the so treated *L. innocua* strain were designed UC 7117. MATS assay as well as bioluminesce assay for AI-2 detection, which were performed both on *L. innocua* UC 8410 and *L. innocua* UC 7117, successfully demonstrated the genetic disruption of *luxS*.

Bacterial attachment of wild-type *L. innocua* was examined in parallel to its *luxS*-null version both in static and dynamic approaches: obtained results on statically incubated stainless steel allowed to confirm results of both Belval et al. (2006) and Sela et al. (2006), while, in presence of shear forces, the *luxS*-null mutant strain was not able to adhere even to the most propitious surface, SS. Furthermore, when mutant was evaluated for its adhesion capacity n static devices, a sort of slime and limited growth during prolonged incubation under nutrient depletion were observed, suggesting that *luxS* could affect not only growth kinetic but also susceptibility under adverse conditions.

Another trait modified by genetic disruption was the sensitivity to biocides: BC was tested, through the European Standard 1040:2006, for its bactericidal effectiveness on UC 7117 in comparison with *L. innocua* UC 8410. The obtained results showed that the used biocide had an increased efficacy in reducing bacterial population up to 10^8CFU/ml for the planktonic cells, demonstrating that gene knock-out performed on *L. innocua* markedly modified the sensitivity to antimicrobials. Two natural stilbenes, P and R, were examined in

order to demonstrate any possible application of these compounds as antimicrobial. Lee et al. (2005) and Välimaa et al. (2007) demonstrated the bactericidal capacity of P, while none of study concerning bactericidal properties of wine and grapes investigated the antimicrobial properties of R, which is quantitatively relevant in those matrices.

For this purpose bacterial growth at different concentrations of these two compounds was monitored for 24 h: although they demonstrated a limited inhibitory capacity on the *luxS*-null mutant, pinosylvin demonstrated better performances than resveratrol and it could not represent promising antimicrobials, although there is still a gap of knowledge about its mechanism of action. By comparing the growth kinetic of on *L. innocua* UC 8410 with the one of *L. innocua* UC 7117, the genetic disruption of *luxS* was demonstrated to modify markedly the overall metabolism of the tested *L. innocua* strain. Furthermore, in comparison with *L. innocua* UC 8410, *L. innocua* UC 7117 has demonstrated an increased sensitivity to both the tested compounds at concentration higher than 200 μM as well as to EtOH, while concentrations ranging between 50 and 200 μM were demonstrated to have a protective effect towards *luxS*-null mutant strain from 3% EtOH. Further investigations should be focused to clarify the specific metabolic pathways affected by the genetic disruption of this gene.

[4.12]. Bibliography

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