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Isolation and Identification of Quorum Quenching Bacteria and Assessment of Their Inhibitory Effect on Quorum Sensing-Dependent Pathogenicity of Yersinia ruckeri

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Thesis submitted in fulfillment of the requirements for the joint degree of Doctor (Ph.D.) in Aquatic Animal Health / Applied Biological Sciences: Aquaculture

DEDICATION

This thesis is dedicated to my beloved parents who never stopped supporting and encouraging me. To my loving siblings, who are very special to me.

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AHL	*N*-acylhomoserine lactone
AI-2	Autoinducer-2
Ala	Alanine
ANOVA	Analysis of variance
Asp	Asparagine
CFU	Colony forming unit
Chlo	Chlorpromazine
CV026	*Chromobacterium violaceum* strain CV026
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DO	Dissolved oxygen
DOPA	Dopamine
ERMD	Enteric Redmouth Disease
FAO	Food and Agriculture Organization
GenBank	Genetic sequence database of the National Institute of Health, USA
Gly	Glycine
h	Hour
His	Histidine
IP	Intraperitoneal
L	Liter
Lab	Labetalol
LD50	Lethal dose, 50%
mg	Milligram
ml	Milliliter
MS222	tricaine methanesulfonate
NE	Norepinephrine
OD	Optical density
OHHL	*N*-3-oxohexanoyl homoserine lactone
P	Statistical p-value
PCR	Polymerase chain reaction
PCR	Polymerase chain reaction
Phenoxy	Phenoxybenzamine
Pro	Propranolol
QQ	Quorum quenching
QS	Quorum sensing
QSI	Quorum sensing inhibitor(y)
rpm	Rotations per minute
RR	Rifampicin resistant
SD	Standard deviation
sec	second
Ser	Serine
SPSS	Statistical Package for the Social Sciences
Cys	Cysteine
Thr	Threonine
Ti	Tumour inducing plasmid

Trp	Tryptophan
TSA	Tryptone soy agar
TSB	Tryptone soy broth
Tyr	Tyrosine
USD	US dollar
µg	Microgram
µl	Microliter

CHAPTER 1

INTRODUCTION

Introduction

Aquaculture

The global human population continues to increase quickly and is predicted to reach between 8.3 and 10.9 billion by 2050 (UN News Center, 2013), and feeding the population and simultaneously protecting the natural resources for future generations is regarded as a huge challenge.

The farming of aquatic organisms, such as fish, crustaceans, and mollusks, often known as aquaculture, is one of the fastest growing animal-food producing industries in the world. Currently, aquaculture supplies approximately half (approx. 37 %) of all global consumption of fish and in 2014 alone have produced 73.8 million tonnes with an estimated value of US$160.2 billion, consisting of 49.8 million tonnes of finfish (US$99.2 billion), 16.1 million tonnes of molluscs (US$19 billion), 6.9 million tonnes of crustaceans (US$36.2 billion), and 7.3 million tonnes of other aquatic animals including frogs (US$3.7 billion) (Food and Agricultural Organisation of the United Nations [FAO], 2016) **(Figure 1)**.

FAO estimates that “fish provides approximately 3.1 billion people worldwide with almost 20% of their intake of animal protein” (FAO, 2016). Overall, the world supply of fish for human consumption has kept ahead of population growth over the past five decades, growing at an average annual rate of 3.2 percent in the period 1961–2013, compared with 1.6 percent for world population growth. Hence, the average per capita availability has risen. World per capita apparent fish consumption increased from an average of 9.9 kg in the 1960s to 14.4 kg in the 1990s and 19.7 kg in 2013, with preliminary estimates for 2015 indicating further growth, exceeding 20 kg (FAO, 2016).

According to the latest statistics of FAO (2016), the diversity of aquaculture species has also increased. By 2014, a total of 580 species and/or species groups including 362 finfishes, 104 molluscs, 62 crustaceans, 6 frogs and reptiles, 9 aquatic invertebrates, and 37 aquatic plants, farmed around the world. At present,

it is thought that aquaculture has the potential to make a significant part to the increasing food demand and to supply safe and high-quality aquatic food.

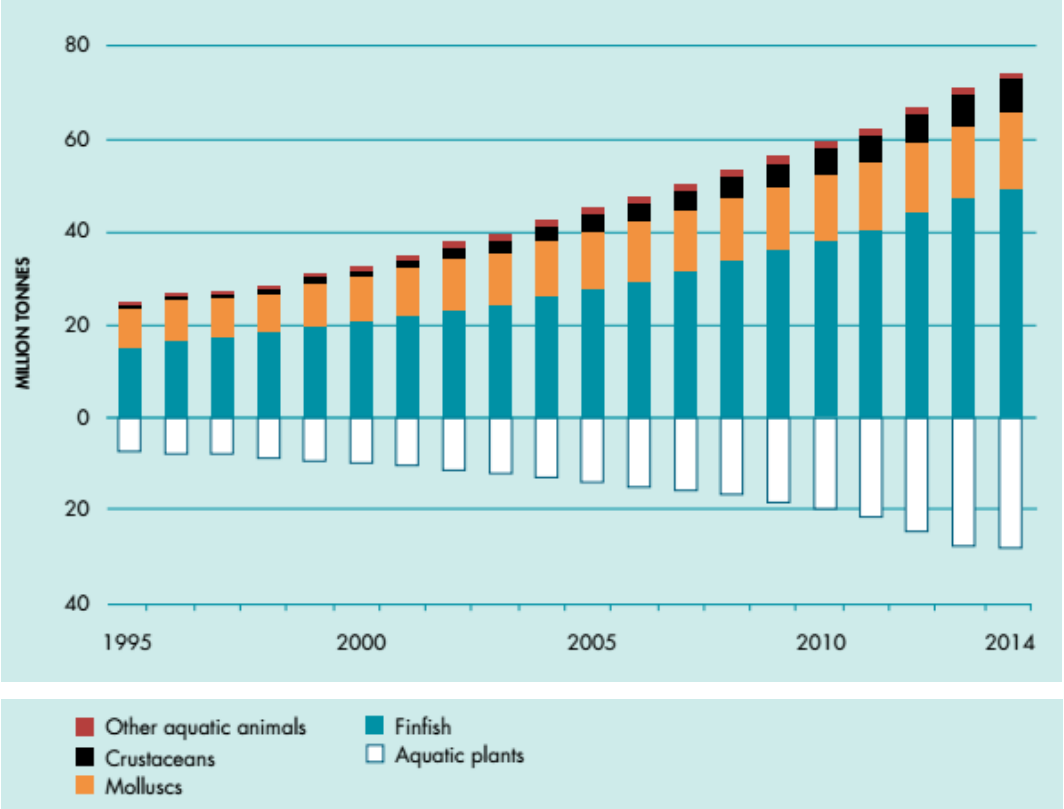


Figure 1. World aquaculture production volumes (1995–2014) (FAO, 2016.)

Infectious diseases and antibiotic use in aquaculture

In spite of the development of intensive aquaculture worldwide, infectious diseases are still an important challenge for this sector. Based on the estimates by the FAO, economic losses in farmed fish and shellfish caused by the diseases are around several billion USD per year, equals approximately 15% of the value of aquaculture production (Subasinghe et al., 2001). Bacteria and viruses are considered the major pathogenic agents in aquaculture however, it has been reported that bacterial pathogens cause more disease problems than other causes (Meyer,

1991). Some chemical compounds such as disinfectants, anthelmintic agents and importantly antibiotics are utilized in aquaculture in order to treat and prevent infectious diseases (Rawn et al., 2009). As a first-line therapy in aquaculture, antibiotics still play an important role to cure various bacterial infections (Defoirdt, 2013).

It is almost impossible to present a complete report of the type and the dosage of antimicrobial agents used in aquaculture due to the lack of information, excluding in some countries such as Northern Europe, North America and Japan with strict regulations on the use of chemical compounds (Smith, 2008; Heuer et al., 2009). For instance, according to a study in Thailand in the year 2000, 74% of seventy six interviewed farmers used antibiotics mostly prophylactically and daily. Additionally, the used antibiotics included at least thirteen various antibiotics such as chloramphenicol, trimethoprim, gentamycin, tiamulin, gentamycin, tetracyclines, sulfonamides and quinolones (Holmström et al., 2003). Since different countries have different regulations for using antibiotics, determining the amounts of used antibiotics is difficult as well (Burridge et al., 2010). With respect to the problems related to the antibiotic used in aquaculture, a drastic limitation in the use of antibiotics in aquaculture has been exerted in many countries (Cabello, 2006, Defoirdt et al., 2011a).

All the antibiotics legally used in aquaculture farms must be authorized by a government agency (as a responsible for veterinary medicine) such as the Food and Drug Administration (FDA) in the USA. Furthermore, different rules for antibiotic use, including dose forms, permissible routes of delivery, withdrawal times and tolerances, must be set by these regulatory agencies (Burridge et al., 2010). The increasing knowledge about consequences of the use of antibiotics has caused more strict regulations on the use of antibiotics in aquaculture as well as on the antibiotic residues in aquaculture products (Romero et al., 2014). As a notable case, the use of antibiotics as growth stimulants in animal productions was banned in Europe in the year 2006 (European Parliament and Council Regulation No 1831/2003).

Antimicrobial agents can be administered to aquatic animals through three methods including supplemented feed, immersion and injection (Smith, 2008). The mixing in the formulated feed is regarded as the most common route for delivery. This way is not as effective as injection because aquatic animals, particularly fish, do not effectively assimilate antibiotics and return them largely unused into the water. Seventy five percent of the antibiotics fed to aquaculture animals have been estimated to excrete into the environment (Burridge et al., 2010). It has been found that the residuals remain in the sediment, resulting in a change in the microflora composition of the sediment and selecting for antibiotic-resistant bacteria (Cabello, 2006).

The irregular use of antibiotics in the past has resulted in the development of multi-antibiotic resistances in bacterial pathogens. Consequently, at this moment, antibiotic therapy is becoming less effective in controlling bacterial diseases. For instance, mass mortality has been reported in *Penaeus monodon* larvae caused by *Vibrio harveyi* strains with multiple resistant to some antibiotics including chloramphenicol, cotrimoxazole, streptomycin and erythromycin (Karunasagar et al., 1994).

Since the determinants of antibiotic resistance have been found to locate on mobile genetic elements, the massive use of antibiotics in aquaculture can be also considered a threat to the human health and environment (Alderman & Hastings, 1998; Cabello, 2006). The transferable plasmids and integrons have been known as the mobile genetic elements in pathogenic *Aeromonas* spp., *Citrobacter* spp., *Edwardsiella* spp., *Photobacterium* spp. and *Vibrio* spp. (Sorum, 2006; Ishida et al., 2010). It has been revealed that a pathogenic *Vibrio parahaemolyticus* isolated from infected shrimp also showed resistance to oxytetracycline, indicating the transfer of antibiotic resistance through mobile genetic elements among bacterial strains (Tran et al., 2015). In the aquatic antibiotic-resistant bacteria, the resistance gene determinants have been found to be capable of being transmitted by horizontal gene transfer, not only to other aquatic bacteria, but also to human and animal pathogens. This phenomenon has been observed in *Salmonella enterica*

serotype Typhimurium and *Vibrio cholerae* (Cabello, 2006; Sørum, 2006; Defoirdt et al., 2011a).

The presence of residual antibiotics in aquaculture products is another concern in addition to selection for resistance. Undetected consumption of antibiotics by people who regularly consume aquaculture products is one of the consequences of the irregular use of antibiotics in aquaculture (Cabello, 2006). Due to the lack of information on the antibiotic content of the aquaculture product, the generated problems such as allergy and toxicity are difficult to diagnose (Alderman & Hastings, 1998). Some cases of antibiotics used in aquaculture and examples of (multi) resistant pathogenic bacteria are listed in **table 1**.

Furthermore, the application of disinfectants with broad-spectrum targets leads to not only an alternation in the pathogenic bacteria, but also in the normal host microflora. In this way, microflora is killed as well and this might generate a more problematic situation (De Schryver et al., 2014). Therefore, in order to more effectively treat bacterial diseases, it is necessary to promote the more prudent use of antibiotics in aquaculture and novel alternative strategies to antibiotics are also urgently required for the sustainable development of aquaculture.

Table 1 The different classes of antibiotics used in aquaculture, their importance for human medicine and examples of (multi) resistant pathogenic bacteria isolated from aquaculture settings (adapted from Bondad-Reantaso et al., 2005).

Drug classes	Importance for human medicine	Example	Resistant bacteria	Multiple resistance	Isolated from	Reference
Aminoglycosides	Critically important	Streptomycin	*Edwardsiella ictulari*	Yes	Diseased striped catfish (*Pangasianodon hypophthalmus*), Vietnam	Dung et al., 2008
Amphenicols	Important	Florfenicol.	*Enterobacter spp. and Pseudomonas spp*	Yes	Freshwater salmon farms, Chile	Fernández Alarcón et al., 2010
Beta-lactams	Critically important	Amoxicillin	*Vibrio spp., Aeromonas spp.* and *Edwardsiella tarda*	Yes	Different aquaculture settings, Australia	Akinbowale et al., 2006
Beta-lactams	Critically important	Ampicillin	*Vibrio harveyi*	Yes	Shrimp farms and coastal waters, Indonesia	Teo et al., 2000
Fluoroquinolones	Critically important	Enrofloxacin	*Tenacibaculum maritimum*	Yes	Diseased turbot (*Scophthalmus maximus*) and sole (*Solea senegalensis*), Spain and Portugal	Avendaño-Herrera et al., 2008
Macrolides	Critically important	Erythromycin	*Salmonella spp*	Yes	Marketed fish, China	Broughton & Walker, 2009
Nitrofurans	Critically important	Furazolidone	*Vibrio anguillarum*	Yes	Diseased sea bass and sea bream, Greece	Smith & Christofilogiannis, 2007

Nitrofurans	Important	Nitrofurantoin	*Vibrio harveyi*	Yes	Diseased penaeid shrimp,	Taiwan Liu et al., 1997
Quinolones	Critically important	Oxolinic acid	*Aeromonas spp., Pseudomonas spp.* and *Vibrio spp*	Yes	Pond water, pond sediment and tiger shrimp (*Penaeus monodon*), Philippines	Tendencia & la Peña, 2001
Sulphonamides	Important	Sulphadiazine	*Aeromonas spp.*	Yes	Diseased katla (*Catla catla*), mrigel (*Cirrhinus mrigala*) and punti (*Puntius spp*.), India	Das et al., 2009
Tetracyclines	Highly important	Tetracycline	*Aeromonas Hydrophila*	Yes	Water from mullet and tilapia farms, Egypt	Ishida et al., 2010
Tetracyclines	Highly important	Oxytetracycline	*Aeromonas Salmonicida*	Yes	Atlantic salmon (*Salmo salar*) culture facilities, Canada	McIntosh et al., 2008

Antivirulence therapy for aquaculture

In order to treat bacterial infection in aquaculture without using antibiotics, some alternative methods including bacteriophage (Nakai & Park, 2002), growth inhibition of pathogenic bacteria by using shortchain fatty acids and polyhydroxyalkanoates (Vasquez et al., 2005; Defoirdt et al., 2006b), inhibition of chromosome II replication in vibrios (Yamaichi et al., 2009) and inhibition of virulence gene expression have been developed (Defoirdt et al., 2011a). These alternative strategies are termed "antivirulence therapy" (Clatworthy et al., 2007). The inhibition of virulence factors can occur through two pathways: 1) interfering with the regulation of virulence factor expression (often influencing several virulence factors simultaneously) and 2) inhibition of one specific virulence factor (Defoirdt, 2013). Bacteria are able to communicate and sense concentration of their cell density via a "language" called quorum sensing (QS) (Swift et al., 1993; Fuqua et al., 2001; Reading & Sperandio, 2006). QS bacteria are able to produce, release and uptake small diffusible molecules, known as "signal molecules" or "autoinducers", through quorum sensing (Kaplan & Greenberg, 1985). Different coding genes are responsible to encode the signal molecules in negative and positive gram bacteria. The signal molecules show distinct structural characteristics and are found in different bacterial classes. Numerous Gram-negative bacteria produce N-Acyl Homoserine Lactone (AHL) as signal molecules in QS system. A generic AHL molecule contains an acyl chain with variable numbers of carbon and a homoserine lactone ring (Czajkowski & Jafra, 2009). It is thought that at a certain threshold density, a chain of biological functions will be generated inside the bacterial cells and their surroundings, leading to the regulation of expression of genes such as virulence factors (Fuqua et al., 1994; Eberl, 1999). In fact, it has been proven that some physiological actions in bacteria such as biofilm formation, swarming motility, bioluminescence and secretion of virulence factors, but not growth, are controlled by quorum sensing system (Miller & Bassler, 2001; Chen et al., 2013).

Quorum sensing interference (QSi) can be a putative remedial strategy with quorum quenching being at the basis of an anti-virulence therapy (Chen et al., 2013). In contrast

to antibiotic therapy, in QSi, pathogenic bacteria are not killed nor are the cell growth inhibited, but virulence factors gene expression can be suppressed. QSi can be accomplished through three main mechanisms: 1) interfering in synthase of signal molecules, 2) signal molecules degradation and 3) blocking of receptors of signal molecules (Geske et al*.,* 2008; Galloway et al*.,* 2011).

Many bacteria are able to produce different enzymes to degrade AHL molecules. These enzymes are divided into four main groups: AHL acylase, AHL lactonase, oxidoreductase and paraoxonase (Chen et al., 2013). AHL acylase can irreversibly hydrolyze the amide bunds between the homoserine ring and the acyl chain of AHL molecules (Leadbetter et al., 2000; Lin et al*.,* 2003). AHL lactonase enzyme cleaves the homoserine lactone ring and the remaining structure of AHL molecule reversibly loses its ability in a QS system (Dong et al*.,* 2000; Dong et al*.,* 2001). The hydrolysis of lactone ring also occurs at a pH above 7 and can be reversed at acidic pH (Yates et al*.,* 2002). In a few numbers of bacterial species, the oxidoreductase enzymes oxidize or reduce the acyl side chain without degradation of AHL structure (Chan et al*.,* 2011). The last QQ enzyme class, paraoxonase (PONs), exists in mammalian sera and possesses a function similar to AHL lactonase (Yang et al*.,* 2005).

AHLs are produced by many Gram-negative fish pathogenic bacteria including *Aeromonas salmonicida*, *A. hydrophila*, *Vibrio anguillarum*, *V. harveyi*, *V. salmonicida* and *Y. ruckeri* and play an important role in QS system as signal molecules (Cao & Meighen, 1989; Milton et al., 1997; Swift et al., 1997; Bruhn et al., 2005; Kastbjerg et al., 2007).

In the last years, many bacteria with AHL degradation ability isolated from a wide range of aquatic animals and environments have been identified (Tinh et al., 2007; Chu et al., 2010; Christiaen et al., 2011; Noorashikin et al., 2016). Moreover, there are many studies reporting successful results from *in vitro* and *in vivo* administration of these bacteria to inhibit quorum sensing and bacterial diseases (Tinh et al., 2007; 2008; Chu et al., 2010; Defoirdt et al., 2010; Christiaen et al., 2011). In the present study, isolation and characterization of QQ bacteria from rainbow trout was performed with the aim to explore and use them in the biocontrol of bacterial infections in aquaculture instead of

using antibiotics due to negative consequences of misuse of chemotherapy. As *Y. ruckeri* produces several AHLs, the control of ERMD through QQ could potentially be an effective alternative for conventional treatments such as antibiotic therapy. To date, few studies have been conducted regarding QS in *Y. ruckeri* and how to control through QS inhibition (Temprano et al., 2001; Kastbjerg et al., 2007). To our knowledge, this is the first attempt to study, *in vitro* and *in vivo,* the use of QQ bacteria (isolated from the rainbow trout), for inhibition of QS in *Y. ruckeri*.

Furthermore, previous works have indicated that catecholamine stress hormones can influence growth, motility, biofilm formation, and/or virulence of pathogens such as *Vibrio parahaemolyticus* (Nakano et al., 2007a) *Escherichia coli* and *Salmonella* spp. (Verbrugghe et al., 2012) and *V. campbellii* and *V. anguillarum* (Pande et al., 2014). Further, antagonists of adrenergic and dopaminergic receptors in mammals have been found to block catecholamine-induced effects in bacteria (Sharaff & Freestone, 2011). Hense, interfering with binding the catecholamines to their receptors by various antagonists and subsequently, reducing the virulence of bacteria can be regarded as an alternative method to antibiotics. Therefore, detecting and responding to the catecholamine hormones might also be important with respect to ERMD in aquaculture as aquatic animals also produce these compounds (Ottaviani & Franceschi, 1996).

Additionally, it has been revealed that glucocorticoids are also able to influence the growth of bacteria such as *Porphyromonas gingivalis*, *Fusobacterium nucleatum* and *Flavobacterium columnare* (Jentsch et al., 2013; Akcal et al., 2014; Declercq et al., 2016). So far, few studies have focused on the effects of the glucocorticoids on the growth and especially on virulence factors of bacteria.

However, little knowledge is available about the efficacy of stress hormones on the virulence of aquaculture pathogens. To our knowledge, no study has been yet performed to evaluate the effects of stress hormones (catecholamines and glucocorticoids) on the growth and virulence factors of *Y. ruckeri*. Therefore, In addition to quorum sensing, this study also focused on host-pathogen signaling through catecholamine and glucocorticoid stress hormones and their impact on the virulence factors of *Y. ruckeri*.

The general objective of this study was to evaluate the impact of quorum sensing, quorum quenching bacteria and sensing of host factors on the virulence of *Y. ruckeri* in rainbow trout. The specific objectives and the thesis outline were as follows:

- **Chapter 1** gives a general introduction to the problem and the thesis outline.
- **Chapter 2 (Literature review)** gives an overview of the current knowledge of *Y. ruckeri*, including the virulence, pathogenesis, control and treatments, as well as quorum sensing, quorum quenching, stress hormones such as catecholamine and glucocorticoid stress hormones.
- **Chapter 3 (Identification of N-Acyl Homoserine Lactone-Degrading Bacteria Isolated from Rainbow Trout)** aims at isolation and identification of bacteria with quorum quenching ability from fish. We further investigated enzymatic and also extracellular/ intracellular activity in the QQ isolates. In addition, the presence of an *aiiA* gene and its HXHXDH motif in the isolates were investigated. Phylogenic analysis was also performed on the QQ bacteria and AHL-lactonase as well.
- **Chapter 4 (Effect of Quorum Quenching Bacteria on Growth, Virulence Factors and Biofilm formation of *Yersinia ruckeri in vitro* and an *in vivo* Evaluation of Their Probiotic Effect in Rainbow Trout)** presents experimental data on the impact of QQ bacteria on virulence factors, motility and biofilm formation of *Y. ruckeri* (*in vitro*). Moreover, we evaluated the probiotic effect of the QQ bacteria in rainbow trout against *Y. ruckeri* (*in vivo*).
- **Chapter 5 (The Catecholamine (Norepinephrine and Dopamine) and the Glucocorticoid Stress Hormones Increase Growth and Virulence Factors of *Yersinia ruckeri in Vitro* and *in Vivo*)** deals with the effect of catecholamine and glucocorticoid stress hormones on the growth, virulence factors, motility and biofilm formation in *Y. ruckeri.* An *in vivo* evaluation also carried out to study the effect of the catecholamines and their antagonist receptors on the virulence of *Y. ruckeri* to rainbow trout.

- **Chapter 6 (General discussion and conclusion)** recapitulates the overall findings obtained in the entire thesis, conclusions are drawn and possibilities for future research are proposed.

CHAPTER 2

LITERATURE REVIEW

Literature review

Enteric redmouth disease

Enteric redmouth disease in aquaculture

Enteric redmouth disease (ERMD) or yersiniosis is a significant condition of farmed fish, notably salmonids, and is caused by *Yersinia ruckeri*. The pathogen was first isolated from rainbow trout, in the Hagerman Valley of Idaho, USA, in the 1950s (Rucker, 1966). *Y. ruckeri* is a Gram-negative and a member of the Enterobacteriaceae. *Y. ruckeri* is able to produce lysine decarboxylase which makes it different from all other members of the genus *Yersinia* (Bottone, 2005). The clinical signs of the disease appear as hemorrhagic septicemia. Salmonids, especially rainbow trout, are the most susceptible to ERMD (Furones et al*.,* 1993a). Presently, *Y. ruckeri* has been isolated in North-western USA, Canada, Europe, Australia, South Africa, and Asia. It is generally considered that the organism has been transmitted all over the world as a consequence of the spread of salmonid aquaculture. In Iran, Soltani et al. (1999) could isolate the pathogen from farmed rainbow trout for the first time. Nowadays, ERMD is regarded as the most important bacterial disease in rainbow trout farms after Streptococcusis in Iran.

Characteristics of *Y. ruckeri*

Taxonomic position of *Y. ruckeri*

The taxonomic position of *Y. ruckeri* has been subjected to discourse since its first classification in the genus *Yersinia* by Ewing et al*.* (1978). The authors indicated that *Y. ruckeri* isolates had a G + C ratio of 47.5 - 48.5%, and accordingly, it would be included in the genus *Yersinia*. However, investigations of the biochemical and serological reactions demonstrated that the conclusion was really not so perspicuous. *Y. ruckeri* is

typically recognizable from other members of the *Yersinia* by the ability to produce lysine decarboxylase. Other studies found that *Y. ruckeri* has biochemically similarities with *Serratia* and *Salmonella* (Llewellyn, 1980). Serological cross-reactions with *Hafnia alvei* and *Salmonella* sp. indicated that there is work to be done on the exact taxonomic status of the bacterium (Ross et al*.,* 1966, Stevenson & Daley, 1982). Recently, Kotetishvili et al*.* (2005) carried out multilocus sequence typing (MLST) and found that *Y. ruckeri* was genetically the most distant species within the genus *Yersinia*, and that the taxonomic position might need to be re-evaluated. According to the results of many researches and the existence of similarities with many other members of the Enterobacteriaceae, *Y. ruckeri* could possibly be included in a separate genus.

Growth characteristics

Y. ruckeri is a Gram-negative rod with 0.5-0.8 x 1.0-3.0 μm in size. Peritrichous flagella are seen in the motile bt 1 types of the microorganism (Davies & Frerichs, 1989). *Y. ruckeri* is not able to form endospores, and a capsule is not observed as well (Tobback et al., 2007). The pathogen may be isolated easily on different media from the internal organs of infected fish. Colonies are 1-1.5 mm in diameter, and glistening after 24-48 h incubation at 22ºC. The organism grows in a wide range of temperatures and its optimum temperature is 28ºC (Stevenson et al*.,* 1993). Early studies on the pathogen by Austin et al*.* (1982) revealed that making a change in formulations of culture media had an effect on general cell morphology of *Y. ruckeri*. Isolates appeared various in the number of flagella under transmission electron microscopy (TEM). Additionally, a marked difference in cell size, from 0.5 – 4 μm was observed for TSA media supplemented with 3% (w/v) NaCl.

Biochemical characteristics

It has been revealed that *Y. ruckeri* is catalase-positive, oxidase-negative, nitrate-reductive and glucose-fermentative similar to the other members of the

Enterobacteriaceae (Ross et al., 1966). As mentioned above, *Y. ruckeri* is differentiable from other members of the *Yersinia* by the production of lysine decarboxylase. Strains of *Y. ruckeri* display identical biochemical characteristics although there is a variation in the methyl red test, VP reaction, hydrolyzing Tween and ferment sorbitol. Up to the present, there is no standard method for distinguishing between bt 1 and bt 2 other than tests for motility and lipase activity. The main tests to differentiate between the biotypes concentrate on motility and the ability to hydrolyze Tween (Davies & Frerichs, 1989). The API 20E rapid identification system may also be used to identify *Y. ruckeri* (Coquet et al*.,* 2002b). Consequently, *Y. ruckeri* may be differentiated from *Hafnia alvei* by the inability to ferment xylose on the API 20E system (Coquet et al*.,* 2002b).

Furones et al. (1990) expressed that serotype O1 isolates have a heat sensitive factor (HSF) (virulence-associated), which was detectable using a selective medium containing bromophenol blue and Congo red. This HSF is considered to play a role in resistance to serum antimicrobial effect. Secades & Guijarro (1998) demonstrated that the composition of the culture medium had an impact on the production of the 47-kDa protease, which was optimum in peptone medium, implying that particular peptides are required for its induction.

Serological classification

Various procedures involved in biochemical, serological, and phenotypic methods have been applied to characterize *Y. ruckeri* strains (Busch, 1978; Green & Austin, 1982; De Grandis et al*.,* 1988; Davies, 1990; 1991a). Most of the typing procedures were done based on serological reactions in the whole cell. De Grandis et al*.* (1988) classified isolates into 5 serovars (I, II, III, V and VI). Romalde et al*.* (1993) modified this serological procedure by suggesting a new typing procedure by identifying four different O-serovars. The revision was performed in this way that serovar O1 was subdivided into two subgroups O1a (former serovar I) and O1b (former serovar III). Serovar O2 (serovar II) was divided into three subgroups O2a, O2b, and O2c. The remaining serovars are known as serovar O3 (serovar V) and serovar O4 (serovar VI). Davies (1990) has

identified five O-serotypes (O1, O2, O5, O6, O7) from 127 isolates of *Y. ruckeri* from Europe and North America. Given these results, biotype, O-serotype and outer membranes protein (OMP) type were considered the basis of a clonal complex method to group isolates (Davies, 1991a). The type of OMP is ascertained by the mobility on 1-D sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Isolates contain a peptidoglycan-associated protein (PAP) with a molecular weight of 36.5, 37.5 38.0, 39.5 or 40.5 kDa, and a 36.5 or 38 kDa heat modifiable protein (HMP) (Davies, 1990). A clonal group theory for *Y. ruckeri* O1 isolates was formed based on the combination of biotyping, OMP typing and serotyping (Davies, 1991a). *Escherichia coli* strains were the first to be described with the clonal concept of bacterial population structures in the 1970's (Ørskov et al*.,* 1976). It was suggested that certain O:H serotypes represent clones carrying necessary plasmids to cause diarrhea. This method of classification is usable in epidemiological studies as it has been shown that ERM occurrence in rainbow trout farms is influenced by *Y. ruckeri* serotype O1 strains. According to the clonal complex theory, serotype O1 may be subdivided based on bt and OMP-type into six clonal groups. There are two clonal groups associated with ERM in rainbow trout including clonal group 5 strains responsible for ERM in Europe and the USA, and the original Hagerman strain; clonal group 2 strains causing ERMD in the U.K. (bt 2). The other O1 clonal groups have not been reported to cause disease in rainbow trout. The degree of difference between bt 1 and bt 2 isolates is not yet clear serologically. As the O antigen used in the Davies method is the sero specific antigen, the molecules must be identical. As described by Romalde et al*.* (1993) some evidence shows that the O antigen within serotype O1 is different, but due to little biochemical or growth data, it was difficult to hypothesize if these were bt 2 isolates. Since the O antigens are different, hence, other same antigens are probably present within the O1 serogroups, such as OMPs, causing agglutination with specific antiserum. Therefore, the O antigen is regarded as the basis for the serogroups. Tinsley et al. (2011) revealed that there are phenotypic and antigenic differences in *Y. ruckeri* bt 1 and bt 2 with O antigen recognized as the dominant immunogenic molecule involved in the protection of rainbow trout against ERMD. Besides, *Y. ruckeri* in naive rainbow trout demonstrate that

both biotypes of serotype O1 are pathogenic to fish. Their results also indicated the absence of cross-protection afforded by the current monovalent vaccine, therefore suggesting a difference in protective antigens between bt 1 and bt 2 isolates of *Y. ruckeri*.

Epizootiology

Transmission and susceptible species

Y. ruckeri is transmittable from fish by direct contact with infected carriers. Initially, there was a thought that the disease occurs by transportation of asymptomatic carrier cases and eggs all over the world, but after isolation from mammals, a possibility was considered that wild animals, such as wild fish, invertebrates, birds and even humans could behave as vectors (Willumsen, 1989). The first report of ERM outbreak in Canada resulted from asymptomatic carrier fish (Wobeser, 1973). Hunter et al. (1980) indicated the importance of stress in the transmission of *Y. ruckeri* from asymptomatic carriers to uninfected fishes. The pathogen was re-isolated from the faeces of the carriers after two months. In addition, it was observed that water temperature, along with density, handling, and other stressful conditions resulted in transmission of *Y. ruckeri* to healthy fishes. Interestingly, this was not the case for the unstressed fishes. The organism has been recovered from the aquatic environment, including water, faeces and sediment (Willumsen, 1989) and is able to form biofilms (Coquet et al*.,* 2002a). *Y. ruckeri* was recovered from Chinook salmon *(Oncorhynchus tshawytscha)* eggs by Sauter et al*.* (1985), suggesting that transmission could occur vertically. Recently, Glenn et al. (2015) could detect the bacterium in ovarian fluid from spawning female adults, unfertilized eggs, eyed eggs, sac fry, and juvenile Chinook salmon using a nested polymerase chain reaction (nPCR) assay and a real-time quantitative PCR (qPCR) assay.

Although the disease has been a problem for rainbow trout in aquaculture, all salmonids may be affected. A detailed report was provided by McDaniel (1971) on different

salmonids that could be potentially sensitive to the disease. In particular, isolation of the pathogen has been reported from non-salmonid wild fish, such as pike (*Esox lucius*), eels (Fuhrmann et al*.,* 1984), gudgeon (*Gobio gobio*) and sturgeon (Vuillaume et al*.,* 1987). The pathogen has also been seen in various marine fish such as turbot, coalfish, sole and gilthead sea bream (Michel et al*.,* 1986). At present, there is no any publication regarding isolation of *Y. ruckeri* from Atlantic salmon (*Salmo salar*) in sea cages, although there is some evidence indicating that *Y. ruckeri* is able to affect farmed specimens in freshwater (Wheeler et al., 2009). Currently, there are not any species-specific strains of *Y. ruckeri*. Moreover, it is not clear whether other fish species are susceptible to bt 2 isolates. A list of species susceptible to *Y. ruckeri* is represented in **Table 2.**

Table 2 Fish and other species susceptible to *Y. ruckeri* infections, adapted from Tinsley (2010).

Common name	**Scientific name**	**Reference**
Rainbow trout	*Oncorhynchus mykiss*	Rucker (1955)
Atlantic salmon	*Salmo salar*	Rintamäki et al. (1986)
Coalfish	*Pollachius virens*	Michel et al. (1986)
Sole	*Soleidae sp.*	Michel et al. (1986)
Sturgeon	*Acipenser sturio*	Vuillaume et al. (1987)
Brown trout	*Salmo trutta*	McDaniel (1971)
Gudgeon	*Gobio gobio*	McDaniel (1971)
Turbot	*Scophthalmus maximus*	Michel et al. (1986)
Goldfish	*Carassius auratus*	McArdle & Dooley-Martyn (1985)
Common carp	*Cyprinus carpio*	Fuhrmann et al. (1987)
Eel	*Anguilla anguilla*	Fuhrmann et al. (1987)
Pike	*Esox lucius*	McDaniel (1971)
Other species:		
Muskrat	*Ondatra zibethica*	Stevenson & Daley (1982)
Sea gulls	*Larus argentatus*	Willumsen (1989)
Human	*Homo sapiens*	Farmer et al. (1985)

Clinical signs of infection

The gross external signs caused by infection with *Y. ruckeri* were first reported by Rucker (1966). It was stated that infected rainbow trout show lethargy, darkening of the skin and congestion of the vessels of the oral region. Additionally, it has been observed hemorrhages on the body surface, with reddening in the head area, along the lateral line and at the base of the fins. The presence of hemorrhages in and around the oral cavity has caused the name 'redmouth' disease (**Figure 2**). Clinical signs differ as cases have been found without the principle "red mouth" trait (Tobback et al*.,* 2007). Both bt 1 and bt 2 infections are characterized as hemorrhagic septicemia. The disease leads to hemorrhaging on the internal organs, along with kidney and spleen swelling. The watery and yellow fluid may be seen in the stomach and intestine, respectively (Stevenson et al*.,* 1993; Tobbck et al*.,* 2009). In chronic conditions, granulomas may be found in the kidney, but in acute cases, the spleen, liver, and kidney are infiltrated with leucocytes, and observed with necrotic foci and hemorrhaging. Obvious changes in density and structure of the hematopoietic tissue have been found in the anterior and posterior kidney of infected fish (Wobeser, 1973). Haematoxylin and eosin (H & E) staining of the tissue sections revealed congestion of the posterior kidney, degeneration of renal tubules and a notable increase in the number of melano-macrophages; coagulative necrosis may also be observed in spleen sections (Tobback et al., 2009). These findings were previously highlighted by Wobeser (1973) that packed cell volume (PCV) and total blood protein (TBP) were reduced by 50%, indicating degradation of hematopoietic tissue in the anterior kidney.

Figure 2. Typical clinical signs of Enteric redmouth disease caused by *Y. ruckeri*.

Pathogenicity of *Y. ruckeri*

There are limited studies on the pathogenicity mechanisms of *Y. ruckeri*. The present knowledge is regarding other *Yersinia* species such as *Y. pestis*, *Y. pseudotuberculosis* and *Y. enterocolitica,* causing disease in terrestrial animals. *Y. pestis* is known as the aetiological agent of the bubonic and pneumonic plague, which are lethal diseases in humans through affecting the lymph nodes and the respiratory tract. *Y. enterocolitica* is an enteric pathogen and cause of yersiniosis in humans, which transmitted through contaminated food or water (Bottone, 1997). Infections with *Y. enterocolitica* are known by symptoms including gastro-enteritis, extraintestinal manifestations and postinfectious sequelae, such as reactive arthritis (Bottone, 1997). *Y. pseudotuberculosis*, another enteric microorganism, is known by causing various clinical signs, fever, scarlatiniform rash, arthritis, diarrhea and vomiting (Abe et al*.,* 1993).

The virulence of *Y. ruckeri* isolates is variable with location and experimental procedure used. Avci & Birincioğlu (2005) observed that mortality occurred 5 to 10 days following I.P. injection with *Y. ruckeri*; these results are in a line with those of other authors who investigated I.P. administration of bt 1 isolates. LD_{50} reported from the challenge experiments was 5×10^5 CFU fish^{-1} for I.P. challenge, and 1×10^7 CFU ml^{-1} *Y. ruckeri* in water for bath challenge (Raida & Buchmann, 2008b). Studies conducted by Fouz et al. (2006) demonstrated that bt 2 isolates indicated an LD_{50} dose of 5 x 10^2 CFU fish^{-1} upon I.P. injection.

Immune response to *Y. ruckeri* infections

Research into fish immunology has been increased over the last years (Bebak et al., 2009; Forlenza et al., 2009). Understanding the immune defense mechanisms of fish against bacteria is important in order to control and prevent disease (Tobback et al., 2007). Like mammals, the immune system of fish may be included in two separate parts, i.e. the innate or specific immune systems. Each one has been separated into further subgroups between the humoral and cellular components (Ellis, 1999). Even though these two systems are often described separately, they may act together. The innate component can eradicate or restrain the infection until the adaptive immune response develops and eventually takes over (Raida et al., 2011). A pathogen is able to infect a host when it can overcome these two systems. In order to make resistant the fish, vaccination may prime the immune responses of fish against a pathogen.

The non-specific (innate immune) system in fish plays a key role in defense against invading pathogens. It has been expressed that the non-specific immune response is of paramount importance for defending the host from *Y. ruckeri* infections (Raida & Buchmann, 2008b). The system is composed of humoral factors, such as inhibitors and lysins, and cellular factors, specifically neutrophils and macrophages. It has been characterized ladderlectin, a non-specific humoral factor, binding to *Y. ruckeri* (Young et al., 2007). It was thought that lectins are able to bind to the pathogen and enhance complement activity and phagocytosis. The second is very important when protecting a host from bacterial infection (Ellis, 1999). Neutrophils and macrophages contribute to killing the bacteria by the engulfing and internalization of them through the process of the respiratory burst (Neumann et al., 2001). Afonso et al. (1998) have shown that vaccination, with a formalin-killed whole cell of *Y. ruckeri* by injection resulted in an increased number of macrophages and neutrophils. Phagocytic cells, such as neutrophils and macrophages are able to produce molecules of a variety of reactive oxygen species such as H_2O_2, OH^- and superoxide anion (O^{2-}). Nitric oxide is another molecule produced by macrophages, which has been revealed to possess an important

antibacterial activity to *Y. ruckeri* (Campos-Pérez et al*.,* 2000). Certainly, much more research into the immune response against *Y. ruckeri* has been using motile bt 1 isolates; there are no data about interaction between bt 2 isolates and the fish immune system. It has been reported that flagellin is an important molecule contributing to the stimulation of the innate immune system; therefore, non-motile isolates could have an obvious advantage in evading detection. Sallum & Chen (2008) described the lack of flagella in non-motile *Vibrio* sp. to benefit the pathogen by evading detection by host molecular pattern receptors, such as Toll-like receptors (TLR).

Both humoral and cellular parts are also involved in adaptive immunity system with their components such as antibodies and activated macrophages, respectively (Ellis, 1999). There is limited evidence that circulating antibodies are able to protect the fish against *Y. ruckeri* infections (Cossarini-Dunier, 1986). This view was supported by the findings reported by Raida & Buchmann (1998a), who revealed that passive immunization with antibodies associated with *Y. ruckeri* did not cause any protection. Cipriano & Ruppenthal (1987) previously indicated the same results using brook trout. Indeed, it might be concluded that the conferred protection is caused by circulating cellular factors excluding IgM. Antibodies present in the blood could be contributed to opsonizing bacteria and activating the complement pathway in *Y. ruckeri* infections (Crossarini-Dunier, 1986). Moreover, Raida & Buchmann (2008a) highlighted that cellular specific immune responses were active in secondary *Y. ruckeri* infections. They discussed the up-regulation of CD8α, a cytotoxic T cell, during secondary infection, using RT-PCR, proposing a potential role of the cellular adaptive immune mechanisms against *Y. ruckeri*. Although there is a newer study indicating that the specific IgM antibody titers increase after vaccination through immersion against ERM (Raida et al., 2011). Deshmukh et al. (2013) demonstrated that innate immune factors such as SAA, complement factors, lysozyme, and macrophage activity were detected in vaccinated fish (with two commercial vaccines) at higher levels than were found in control group. Their results indicated that the low levels of circulating antibody in infected groups in the early stage of infection show that the protection at the beginning of infection is not only caused by an adaptive humoral response but might include innate immunity factors,

such as lysozyme and SAA until the antibody production has been upgraded. There are some researches into the importance of immunoregulatory gene expression induced by *Y. ruckeri* infections as well as the role of antibacterial immunity (Rodriguez et al*.,* 2005; Wiens et al*.,* 2006). These works found that numerous cytokines were expressed during infection, such as IL-1*β*2, Toll-like receptor 2, and *CXCd* (Rodriguez et al*.,* 2005; Wiens et al*.,* 2006). Bonn et al*.* (1998) showed that interleukin 12 (IL-12) was involved in the protection of mice against *Y. enterocolitica* infections. As it was observed that administration of IL-12 induced interferon-gamma (IFN-γ), a pro-inflammatory cytokine involved with activation of macrophages.

Moreover, the gene expression of immune-related genes was investigated by Raida & Buchmann (2007) upon I.P. injection of a *Y. ruckeri* bacterin in rainbow trout. It was indicated that the pro-inflammatory cytokines IL-1β and IFN-γ and the anti-inflammatory cytokine IL-10 were significantly up-regulated after immunization. The results also showed the level of this expression was higher in the spleen in comparison with the head kidney. Although the head kidney of fish is considered to have a major role in the clearance of bacteria by macrophages, recruitment and activation of lymphocytes have been thought to occur in the spleen (Raida & Buchmann, 2008b). IgM was very strongly expressed compared to IgT; in addition, C5aR expression was enhanced following I.P. vaccination (Raida & Buchmann, 2007). In another study, a primary infection with a *Y. ruckeri* O1 strain led to a significant upregulation of different genes encoding cytokines in the spleen (Raida & Buchmann, 2008b).

Diagnostics

Quick diagnosis of any disease in aquaculture is of paramount importance to control disease occurrence. Traditional (clinical signs), immunological and molecular technique have been considered as the reported methods to detect and identify *Y. ruckeri*. Clinical signs diagnostic tests are still applicable and the most cost-effective ways of diagnosing *Y. ruckeri* infections. One of the rapid methods is the API 20E rapid identification system based on biochemical analysis, which has been utilized for the identification purposes

(De Grandis et al*.,* 1988). Although, there are still some problems with interpreting the results of the API 20E systems for analyzing the bt 2 as well as new biogroups. For instance, the obtained profile might be confused with *Hafnia alvei* (a member of the Enterobacteriaceae) (Austin et al., 2003). However, as the biochemical characteristics between different *Y. ruckeri* isolates are not always similar, the tests require reproducibility (Ross et al*.,* 1966; De Grandis et al*.,* 1988; Austin & Austin, 2007). The lack of available antisera against the different serotypes and bt of *Y. ruckeri* is one of the problems to do the serological assay. Immunological methods, including the enzyme linked immunosorbent assay (ELISA), have been developed for the detection of *Y. ruckeri* (Cossarini-Dunier, 1985). Thus, rabbit antiserum was determined with more specific in detecting antigens using the ELISA but it was seen no difference in agglutination reactions (Cossarini-Dunier, 1985). In spite the fact that immunological methods have been useful in the identification of *Y. ruckeri*, because of the heterogeneity of isolates it might only be possible to identify particular isolates/serotypes with the specific antibodies (Romalde et al., 1993). The presence of antibodies in the serum may imply their role in protection (Tobback et al., 2007). Monoclonal antibodies (Mabs) have widely been used in the diagnosis of many bacterial fish pathogens (Adams et al*.* 1995; Wagner et al*.* 1999). However, until now, there are very few specific monoclonal antibodies developed for the detection or characterization of *Y. ruckeri* (Furones et al., 1993b). Molecular techniques, such as PCR and restriction fragment-length polymorphism (RFLP), have been set up for diagnostic aims. PCR based method has been well developed for detecting *Y. ruckeri* in the tissues of artificially and naturally infected fish using different specific primers (Gibello et al., 1999; LeJeune & Rurangirwa, 2000). A loop-mediated isothermal amplification (LAMP) assay was introduced by Saleh et al*.* (2008) for the detection of *Y. ruckeri*. This method possesses a high sensitivity, as it is able to detect up to six copies of DNA per sample (Notomi et al*.,* 2000). The LAMP has a certain advantage compared to standard PCR, being simple to exert only by a set of primers and a water bath.

Virulence factors

Extracellular toxins: Yrp1 and YhlA

In a study, it has been demonstrated that some characteristic signs of ERM, such as haemorrhage in the mouth and the intestine were reproduced in fish after injection of extracellular products (ECPs) of *Y. ruckeri*, including lipases, proteases, and hemolysins (Romalde & Toranzo, 1993). Therefore, apparently, these ECPs are involved in the pathogenesis of *Y. ruckeri* infection. The 47-kDa metalloprotease, a molecule playing role in virulence, is produced at the end of the exponential growth phase of *Y. ruckeri* (Secades & Guijarro, 1999). The protease is another EPC, secreted by a type I Gram-negative bacterial ABC exporter protein secretion system. This system is composed of three genes, *yrpD*, *yrpE* and *yrpF*, and a protease inhibitor *inh* (Fernández et al*.,* 2002). The presence of Yrp1 is not considered a general trait for *Y. ruckeri* strains and it is not associated with the serovar. It has been found that protease activity was not present in all *Y. ruckeri* strains, as in a research some strains of serovar I, the most virulent serovar, possessed protease activity and other strains of the same serovar did not (Secades & Guijarro, 1999). Therefore, two groups of strains were described, i.e. Azo^+ and Azo^-, based on the ability of the Yrp1 proteolytic activity with azocasein (as substrate) (Secades & Guijarro, 1999). Upon analysis of both groups, Fernández et al. (2003) indicated that all strains carried the *yrp1* operon, but the operon is regulated at the transcriptional level. A transcriptionally inactive *yrp1* operon or a very low transcriptional level of the operon lead to the Azo^- phenotype, hence it is not sufficient to detect the proteolytic activity (Fernández et al., 2003). Yrp1 protease is involved in the virulence of the pathogen and contributes to the colonization and invasion of different tissues. Indeed, invasion of tissues by Yrp1 protease takes place through digesting the muscle proteins and extracellular matrix and may lead to membrane changes and pores in the capillary vessels. This may result in the leaking of blood from the hurt vessels and consequently, cause the typical hemorrhages especially around the mouth and intestine (Fernández et al., 2003). The expression of the protease depends on the environmental

condition, as the production of Yrp1 may be repressed by carbon and nitrogen sources. For instance, it has been revealed that glucose and fructose might be the greatest production inhibitors, whereas glycerol, mannitol, and maltose also had a potent repressive effect. The adding of ammonium to the culture medium also led to a decrease in protease production (Secades & Guijarro, 1999). The osmolarity and temperature play a significant role in the regulation of *yrp1* expression, whereas pH does not. The increase in the osmotic pressure of the medium causes a decrease in the production of the protease (Fernández et al., 2003). *Yrp1* expression increased at 18°C and repressed at 28°C (Fernández et al., 2003), suggesting that this is an adaptation to the optimal temperature for effective infection as well as colonization. Further characterization of the protease also demonstrated that Mg^{2+} and Ca^{2+} cations are required for maximal activity (Secades & Guijarro, 1999). Sequence alignment revealed that there is a high degree of homology between Yrp1and metalloproteases from *Erwinia chrysanthemi* (Fernández et al*.,* 2002). A hemolysin, YhlA, has been observed to be involved in the pathogenicity of *Y. ruckeri* (Fernández et al., 2007b). Two genes are thought to be required for the production of YhlA. The upstream genes *yhlB* and *yhlA* encode the secretion and activation of the hemolysin, respectively. Both genes showed a high homology to genes encoding hemolysins of the pore-forming toxins of *Serratia* which are secreted by a two-component secretion system, type V secretion system. Genomic analysis indicated that similar hemolysins are present in human pathogenic yersiniae; however, their function is not yet clear out (Fernández et al., 2007b). The hemolysin YhlA is found to lyse erythrocytes as well as cultured fish cells. The evaluation of fifty percent lethal dose (LD_{50}) using *yhlB* and *yhlA* insertional mutant strains showed the involvement of the toxin in the virulence of *Y. ruckeri* (Fernández et al., 2007b). As it has been seen in the *yrp1* operon, *yhlA* expression was found significantly higher at 18°C, the infection temperature, compared to 28°C, the optimal growth temperature of *Y. ruckeri*. Fernández et al. (2007b) revealed that haemolysin production also enhanced in a low concentration of iron, suggesting being important in the acquisition of iron from the host cells (Fernández et al., 2007b).

Adhesins and invasins

Adhesion is considered as an important factor in the pathogenisity of bacteria as it is important for transfer of the bacterial toxins into target cells (Ben Hamed et al., 2018). In aquaculture pathogenic bacteria initial attachment occurs to the outer surface of fish such as mucus. It has been revealed that external mucus of fish can immobilize the pathogen before they can contact epithelial surface (Ben Hamed et al., 2018). A hydrated matrix of exopolymerase, capsulare material such as exopolysaccharides (EPSs) has been found to stabilize the osmotic changes in biochemical intractions between the bacteria and mucus, cells and tissues (Logan & Hun, 1987; Decho, 1990). Such a stable environment is essential for localization of the secreted exo-enzymes required for the adhesion mechanisms (Decho, 1990; Decho & Herndl, 1995).The adhesin, a surface structure in the bacteria, mediates binding to the epithelial extracellulare matrix following recognizing specific carbohydrate structures (Bavington & Page, 2005). It is believed that bacterial exo-products including outer membrane proteins or extracellular polysaccharides are involved in the adhesion (Walker et al., 2004; Abu- Lail & Camesano, 2003). In the members of the Enterobacteriaceae family, several mechanisms have been found to allow the bacterium to use host mechanisms to gain entry into the host cells. “Zipper mechanism” is one of these strategies and has been observed in *Yersinia enterocolitica* and *Yersinia pseudotuberculosis*. In these species, adhesion molecules on the surface of the bacteria join with surface receptors of the host cell. For example, both *Yersinia* invasins (Leong et al., 1990) and adhesin A (YadA) (Eitel & Dersch, 2002; Eitel et al., 2005) proteins interacts with the β1-integrins. The interactions between these molecules initiate a signalling cascade including the proteins Rac1 and CDC42 as key molecules. This signalling cascade leads to the activation of extra receptors, especially focal adhesion kinase (FAK) (Grassl et al., 2003; Cossart, 2004) and these additional receptors bind to the surface of the bacterium, folding the membrane of the host cell to engulf the bacterium (Cossart, 2004). The other major strategy of entry found in Enterobacteriaceae is termed the trigger mechanism, which is based on the type III secretion system (TTSS) to inject

effector proteins into the cytoplasm of the host cell where they activate different proteins from the Rho family, including CDC42 and Rac1. These proteins are responsible for regulation of the activity of actin filaments and the formation of filopodial and lamellipodial structures that are essential for cell migration as well as the cytoskeletal deformation required for phagocytosis. Effector proteins secreted through the TTSS can initiate the uptake of the bacterium by the host cells (Van Nhieu & Sansonetti, 1999). TTSSs belonging to the Ssa-Esc family (the major family of TTSSs) have also been seen in the *Yersinia* genus (Parkhill et al., 2001). A TTSS (belonging to the Ssa-Esc family) has also been described in *Y. ruckeri* (Gunasena et al., 2003; Liu et al., 2016), suggesting that the trigger mechanism is possibly important in the invasiveness of *Y. ruckeri*. It has been shown that *Y. enterocolitica* and *Y. pseudotuberculosis* release three invasion proteins: invasin and ail, which are encoded on the chromosome and the YadA protein on a 70-kb virulence plasmid. Invasin and YadA are known as two outer membrane adhesins and both are able to bind to β1 integrin receptors. Attachment of the bacteria exerts via these adhesins, suggesting that it is important for promoting internalization into macrophages, neutrophils and dendritic cells (Hudson et al*.,* 2005). Ail is another outer membrane protein, playing a role in cell invasion, although its binding receptor is not yet proved. Kawula et al. (1996) investigated the presence of the genes *inv* and *ail* in *Y. ruckeri* by using southern blot analysis. No evidence was found for *inv* or *ail* homologues in *Y. ruckeri*, although it should be noted that only one strain was examined (Kawula et al., 1996). Fernández et al*.* (2007a) showed the presence of an *inv* homologous gene, involved in bacterial adherence and invasion into host cells, in *Y. ruckeri* using PCR and sequencing analysis. The expression of invasin, Ail or YadA do not exert in *Y. pestis* as the genes encoding these proteins are disrupted by frameshift mutation or transposon insertion (Cowan et al*.,* 2000). Cowan et al., (2000) has found that *Y. pestis* is invasive for epithelial cells similar to the way for the enteropathogenic *Yersinia* species. Pla (plasminogen activator protease), which is encoded by the *pla* gene placed on a 9.6-kb plasmid pPCP1, activates the process of plasminogen and induces the attaching to epithelial cells and macrophages. However, no study has yet been carried out on the possible presence of Pla in *Y. ruckeri*. Most of

bacteria have been found to replicate in an extracellular phase. It has also been observed that all three human pathogenic yersiniae can survive and multiply in macrophages (Pujol & Bliska, 2005). Intracellular survival and multiplication in macrophages may appear during the *Yersinia* infection but is very important in the earlier stages of infection. There is also some evidence showing that the normal functions of macrophages were disrupted by *Y. pestis*, *Y. pseudotuberculosis* and *Y. enterocolitica* while replicating in phagosomes. Tsukano et al. (1999) found that *Y. pseudotuberculosis* inhibits acidification of its phagosomes to levels lower than the level that is important in the destruction of intracellular pathogens and in phagosome maturation. A pigmentation segment (102-kb pathogenicity island), which is necessary for replication inside activated macrophages, has been found in *Y. pestis* and *Y. pseudotuberculosis*. This segment is not present in *Y. enterocolitica* and it causes phagocytosed *Y. enterocolitica* to be killed in macrophages after adding IFN-γ, while *Y. pestis* and *Y. pseudotuberculosis* were still able to replicate (Pujol & Bliska, 2005). The pigmentation segment can reduce the production of nitric oxide in macrophages when they infected with *Y. pestis* or *Y. pseudotuberculosis* (Pujol & Bliska, 2005). The macrophages exposed to LPS and IFN-γ expressed an inducible nitric oxide synthase, a key protein killing intracellular pathogens via the production of nitric oxide. Intracellular *Y. pestis* and *Y. pseudotuberculosis* are able to interfere with macrophage activity and the production of nitric oxide. In a research, few intracellular bacteria were detected in kidney, spleen and peripheral blood phagocytes of rainbow trout when infected with *Y. ruckeri* through immersion and I.P. injection using a green fluorescent protein (GFP) (Welch & Wiens, 2005). There is also some evidence reporting that intracellular survival time of *Y. ruckeri* in cultured cells is very short and this microorganism is not able to survive inside of the cells (García et al., 2007; Tobback et al., 2010; Menanteau-Ledouble et al., 2018). However, Ryckaert et al. (2010) could find that *Y. ruckeri* is capable of showing an intracellular phase in macrophages in the infected rainbow trout. The bacteria were detected inside the macrophages *in vitro* as well as *in vivo* assay immediately after infection and for at least 24 h, indicating that *Y. ruckeri* can be considered an intracellular pathogen.

Ruckerbactin

The iron uptake system ruckerbactin is another molecule involved in virulence of *Y. ruckeri* (Fernández et al*.,* 2004). Iron acquisition is required in many microbial pathogens for sufficient colonization and invasion and hence iron transport systems have been developed for high affinity for the metal (Faraldo-Gomez & Sanson, 2003). Siderophores, low molecular-weight Fe^{3+}-chelating compounds, may be considered as virulence factors because of the availability of iron that causes the bacteria to be able to multiply in the host. Siderophores can be grouped into three main classes including catecholates, hydroxamates and heterocyclic compounds. Siderophores bind to host iron using their high affinity for the metal and transport it to the bacteria. In bacteria, the siderophore-Fe^{3+} complex is recognized by a specific outer membrane receptor and translocated into the cytosol where the iron is separated from its siderophore and utilized in other metabolic pathways (Faraldo-Gomez & Sanson, 2003). It has been shown that up-regulation of genes involved in the siderophore pathway of *Y. ruckeri* are induced when the fish infected (Fernández et al., 2004). This regulation was temperature-dependent, being higher at 18°C (the infection temperature) in comparison with 28°C, the optimal growth temperature. The chemical structure and biosynthetic route of this catechol siderophore, known ruckerbactin, are not yet clear. The highest homology was found between the ruckerbactin receptor and the ferrichrysobactin receptor from the plant pathogen *Erwinia chrysanthemi* using sequencing analysis (Fernández et al*.,* 2004). The human pathogenic yersiniae only produce yersiniabactin, a heterocyclic compound, used for their pathogenicity (Fernández et al., 2004). According to the findings, Fernández et al. (2004) suggested that *Y. ruckeri* is more similar to *E. chrysanthemi* than to other yersiniae in terms of pathogenic mechanisms.

Plasmids

Different authors described the presence of plasmids in *Y. ruckeri* strains. The function of these plasmids is not determined, although they may be important in the virulence of

the pathogen. In a study, eight different plasmid profiles were detected in 183 isolates from a wide variety of sources (Garcia et al., 1998). Additionally, a large plasmid of approximately 75 MDa was found in most of the *Y. ruckeri* strains in agreement with other reports (Guilvout et al., 1988; Romalde et al., 1993). Apparently, the geographical origin of the isolates was significantly important to the existence of the large plasmid. Large plasmids have only been observed in serovar O1 strains. Different serovars (e.g. serovar O1) contain the small plasmids. The human pathogenic *Yersinia* species carries a 70-kb virulence plasmid, encoding a TTSS, required for counteracting the immune response of the host. A TTSS is able to inject effector proteins into the cytosol, providing direct communication between bacteria and host and indeed the bacterium controls the cell functions to its advantage as explained before (Menanteau-Ledouble et al., 2018). A TTSS or 'injectisome' utilizes two pairs of rings to span the membranes of two bacteria, linked by a rod and a needle protruding outside the bacterial body. Injection is performed using translocator proteins forming a pore into the target cell (Troisfontaines & Cornelis, 2005). This type of connection between the bacterial cytosol and the plasma membrane of the host explains how effectors can be efficiently injected into the host cytosol without 'leaking' and how the integrity of the plasma membrane can be still maintained. In human pathogenic *Yersinia* species, the Ysc (Yop secretion) injectisome, which is encoded by the 70-kb virulence plasmid, releases Yop (*Yersinia* outer protein) effectors in a low concentration of calcium. Upon bacterial attachment, extracellular calcium locally decreases and consequently activates the Ysc TTSS (Allen, 2003). The needle formed by translocators is used to insert into the host plasma membrane and to transport six effectors (YpkA/YopO, YopH, YopM, YopT, YopP/J and YopE) into the cytosol of the host cell. Yops acts as a protein kinase or phosphatase and interferes with signaling pathways by phosphorylation or dephosphorylation of involved proteins. Several reviews discussed these Yops in terms of their biochemical function, the signaling pathways modulated by them and their importance in *Yersinia* pathogenesis (Cornelis, 1998; Fallman & Gustavsson, 2005; Viboud & Bliska, 2005). In general, inhibition of bacterial uptake and killing by phagocytes, suppression of proinflammatory cytokine production and induction of macrophage apoptosis may be

regarded as the main functions of Yops. The results from a comparison between the plasmid profile in one American and 18 French *Y. ruckeri* strains and the patterns of *Y. pestis*, *Y. pseudotuberculosis* and *Y. enterocolitica* indicated that the large plasmid of *Y. ruckeri* is significantly different from the virulence plasmid of human pathogenic *Yersinia* species (Guilvout et al., 1988)

Type three secretion system

Gunasena et al. (2003) and Liu et al. (2016) found a TTSS in *Y. ruckeri*, apart from that common to human pathogenic *Yersinia* species. These researchers performed a sequencing analysis on the gene encoding the type three ATPase, the conserved proteins of TTSS in fish pathogens, as well as adjacent genes. Their findings showed a significant homology with the chromosomally encoded Ysa (*Yersinia* secretion apparatus) TTSS genes of *Y. enterocolitica* biovar 1B (Gunasena et al., 2003). This may propose the presence of a Ysa-like TTSS in *Y. ruckeri*. The Ysa TTSS of *Y. enterocolitica* biovar 1B was found to be involved in infection stages in the gastrointestinal tract and the delivery of effector proteins, called Ysps (*Yersinia* secreted proteins) (Venecia & Young, 2005). It is thought that some of the Ysps appear homologous to other virulence factors (Matsumoto & Young, 2006), but further investigations on the accurate function are needed to understand how *Y. enterocolitica* biovar 1B interacts with host cells. Moreover, more research is required to determine the presence and the function of the Ysa TTSS in *Y. ruckeri*.

Type four secretion system

Recently, Méndez et al. (2009) detected a chromosomal cluster of eight encoding genes, *traHIJKCLMN* in short, the *tra* operon or *tra* cluster in *Y. ruckeri*, forming a part of a virulence-related type four secretion system (TFSS). This operon was observed in *Y. ruckeri* strains with different origins, suggesting that these genes are conserved and important in the pathogenesis of the microorganism. TFSS have been characterized in

intracellular pathogens to contribute to the transfer of various effector molecules into target cells and play a key role in their survival within macrophages. Hence, the *tra* operon found in *Y. ruckeri* may be involved in the intracellular stage (Méndez et al*.,* 2009). It has been seen that expression of the operon depends on temperature, as it has been seen for other virulence factors it is upregulated at 18°C in comparison with 28°C and is also higher in low nutrient conditions (Méndez et al*.,* 2009). Moreover, there is a high similarity between the *tra* operon and the virulence plasmid pADAP from *Serratia entomophila* in sequence and genetic analysis; although no similar *tra* operon has been yet detected in human pathogenic yersiniae (Méndez et al*.,* 2009).

Treatments and control

Generally, a strong health management is always considered important in preventing disease (Bondad-Reantaso et al., 2005). A good farm management may include disinfection of tanks, control of water quality, the reduction of potential stress conditions and the use of vaccines. Ignoring any of these factors could lead to disease outbreaks (Ashley, 2007).

Antibiotics

The antibiotics have been employed for the control of ERMD since the bacterium was first isolated from diseased fish (Rucker, 1966). Compounds, such as chloramphenicol or oxytetracycline, have been utilized to control infections (Rodgers, 1990). However, the misuse of antimicrobial agents has led to the emergence of drug-resistant strains (Coquet et al., 2002b). In addition, exposing bacteria to inadequate dosage, failure to complete the recommended course or repeated short-term treatment probably result in drug resistance (Rodgers, 2001). This researcher indicated that repeated exposure to inhibitory compound such as oxolinic acid, oxytetracycline, and a potentiated sulphonamide how to lead to resistance of bt 1 isolates to these antimicrobial agents.

Certainly, the use of antibiotics in aquaculture should be minimized, and if necessary, the administration should be practiced with good management.

Immunostimulants and probiotics

Fish can become more resistant to bacterial pathogens by administration of immunostimulants to enhance the effectiveness of vaccines. For example, β-hydroxy-β-methylbutyrate (HMB) is a product from the breakdown of the amino acid leucine, an essential building unit of proteins in all tissues and has been observed to show a positive immunostimulatory effect upon *in vivo* vaccination of rainbow trout with anti-*Y. ruckeri* vaccine (Siwicki et al*.,* 2001). Indeed, HMB added in the diet, caused activating cellular and humoral defense mechanisms and consequently, protection against ERMD in rainbow trout. Immunostimulating effects against *Y. ruckeri* have also been investigated in rainbow trout using levamisole immersion (Ispir & Yonar, 2007). Levamisole has been widely used in both human and veterinary medicine as an antihelmintic compound and found to have an effect on the immune system of different fish species (Ispir & Yonar, 2007). Further investigations are required on the scheduling, timing and regimens of administering immunomodulators to provide efficient protection against yersiniosis.

The studies for controlling diseases e.g. ERMD have mainly centered on the use of antimicrobial agents and on vaccination. However, some research has regarded the application of probiotics (Raida et al*.,* 2003; Kim & Austin, 2006; Capkin & Altinok, 2009). The use of probiotics has resulted in increasing the survival when cultured trout exposed to *Y. ruckeri*. The mode of actions of these live microbial feed supplements may be defined as beneficially effects on the host by the production of inhibitory compounds, competition for chemicals and adhesion sites, immune modulation and stimulation, and improving the microbial balance. In a study, Raida et al. (2003) revealed that feed supplemented with spores of *Bacillus subtilis* and *B. licheniformis* (with dose: 4 x 10^4 spores g^{-1} feed) improved survival in rainbow trout against infection with *Y. ruckeri*. This result was in agreement with another work indicating that

administration of feed supplemented with *Carnobacterium maltaromaticum* or *C. divergens* also induced protection against *Y. ruckeri* through enhancing cellular and humoral immune responses (Kim & Austin, 2006). Rainbow trout fed with a diet supplemented with *Enterobacter cloacae* and *Bacillus mojavensis* for 60 days, showed a significantly increased survival when they exposed to *Y. ruckeri* by a bath challenge (Capkin & Altinok, 2009). Such organisms may be useful in aquaculture to control bacterial disease.

Vaccination

Vaccines against ERMD are one of the successful vaccines produced for control of bacterial diseases in aquaculture, released in the 1970's. The purpose of vaccination is to induce memory in T and/or B lymphocytes via the administration of a non-virulent antigen (Lydyard et al*.,* 2004). Therefore, during an infection, the pathogen is encountered by a secondary response or specific immune system rather than non – specific immune system. Vaccination of fish is regarded to be an effective method in reducing economic losses caused by disease. The protective mechanisms of vaccination may stimulate cell-mediated immunoprotection and humoral immunity. Certainly, there are many advantages compared to conventional drug treatment. Therefore, a few commercial vaccines are available to aquaculture. The present vaccination procedure for ERMD exerts by immersion or injection at the fry stage. The protection from bathing vaccination falls short compared to injection vaccination due to the nature of immersion vaccination. Hence, an oral booster vaccine is used 6 months later. The booster vaccines against *Y. ruckeri* were found to provide an enhanced protection in rainbow trout (Tatner & Horne, 1985). The route administered for vaccine can confer a different effect on the immune response (Palm et al*.,* 1998). Vaccination via I.P. injection provides the best levels of protection (Palm et al*.,*1998). Although considering that fish are most susceptible to ERMD at a size around 4 g, immersion vaccination is more practicable (Hastein et al*.,* 2005). Clearly, the historical disease data such as an endemic infection or a seasonal outbreak should be considered for

vaccination strategy (Rodgers, 1990). Seasonal outbreaks of the disease usually coincide with low water temperatures at the beginning of the season and stressful conditions, such as handling and sorting (Rodgers, 1990). Bruno & Munro (1989) revealed that vaccination prevents clinical disease but does not necessarily affect the carrier fish (Bruno & Munro, 1989). At present, one bivalent vaccine is available (RELERA, SP Aquaculture) against bt 1 and bt 2 isolates of *Y. ruckeri*, released in 2008. The vaccine contains a formalin-killed whole-cell product of both biotypes. Tinsley et al. (2011) performed a study to investigate for the first time the induction of cross-protection and the antigenic cross-reactivity between bt 1 and bt 2 isolates of *Y. ruckeri* using a commercial monovalent (AquaVac ERM; Intervet International B.V.) and a bivalent vaccine (AquaVac RELERA). The results demonstrated that AquaVac RELERA significantly protected rainbow trout against bt 1 and bt 2 isolates of *Y. ruckeri*. Moreover, whole cells of the non-motile *Y. ruckeri* (new biogroup) inactivated by formalin have been found to provide protection when I.P. injection was performed with a virulent strain (Austin et al*.,* 2003). Antigen dosage and the nature of the antigen are factors affecting the immune response in fish. In this connection, it should be noted that the immune response depends on dosage, route and water temperature. The antigen has been found to be directly correlated with immunological memory in fish (Lamers et al., 1995). There are many studies regarding the nature of the antigens in vaccines (Ross and Klontz, 1965; Anderson & Nelson, 1974). Amend et al*.* (1983) demonstrated that the conditions of culture, such as water temperature, and inactivation methods can influence the potency of *Y. ruckeri* whole cell vaccines. These authors suggested that whole cell vaccines should be used as opposed to specific antigens (e.g. OMPs and LPS). Certainly, the quantity and quality of the immune response can be determined by the nature of the antigen, and chemical modifications of the vaccine may lead to differences in expression of the immune response (Amend et al., 1983). Adjuvants are considered immunological stimulants without any specific antigenic effect. Freund's Complete Adjuvant (FCA) is used in fish immunization and results in a stronger and more prolonged antibody response (Jaio et al., 2010). Despite defects such as some granulomatous side-effects (Secombes et al., 1996), FCA is widely applied in

commercial vaccines especially in Norway. Generally, oil-adjuvanted vaccines induce an inflammation at the injection site, leading to migration of phagocytes and possibly an increase in the leucocytes present in the circulation (Mutoloki et al., 2006). However, there is some evidence indicating that adjuvants cannot be always useful, as a report by Horne et al. (1984) expressed that administration of the aluminum salts and potassium alum adjuvant led to a decrease in growth rates along with an enhanced chronic peritonitis. It was thought that protection induced by the vaccine can be failed due to poor water quality. Similarly, Rodgers (1990) was observed that the disease condition in previously vaccinated Atlantic salmon and rainbow trout was dependent on the poor quality of the farm and low water temperature. The findings indicated the importance of good husbandry conditions in the success of vaccination. For instance, it has been revealed that stress caused by intensive vaccination could change phenotype and immunogenic characteristics in formerly vaccinated fish (Bachrach et al., 2001). Subunit vaccines can be potentially used in the future. Subunit vaccine contains a porin of a pathogen (e.g. peptides) (Winton, 1998). The *Yrp1* protease was suggested as an important virulence factor in *Y. ruckeri* by Fernández et al. (2003) who reported a significant protection in fish upon I.M. injection of the *Yrp1* protease. Recently, the use of live attenuated vaccines against bacterial fish pathogens is increasing. In mammals, it has been proven that live vaccines have numerous advantages compared to preparations, as they effectively stimulate cell-mediated immunity (Marsden et al., 1996). In this connection, a mutant of *Y. ruckeri* with a dysfunction of the *aroA* gene as a live vaccine was investigated by Temprano et al. (2005). The *aroA* gene, encoding the enzyme 5-enolpyruvylshikimate-3-phosphatesynthase, is involved in the biosynthesis of aromatic amino acids. A dysfunction in this gene causes the pathogen to be unable to proliferate within fish. A 90% relative percentage survival (RPS) obtained through vaccination using this mutant against *Y. ruckeri* bt 1 (Temprano et al., 2005).

Quorum sensing

Introduction

At the beginning of the previous century, the plant pathologist E.F. Smith suggested that “a number of bacteria are stronger than a few and thus are able to overcome obstacles too great for the few” (Smith, 1905). However, the first reports on intraspecies communication were published in the 1970s (Nealson & Hastings, 1979). They described that bioluminescence in *Vibrio fischeri* only appears at high cell density and that the compounds so-called autoinducers in the supernatant of a luminescent culture could confer luminescence in a non-luminescent culture (Kempner & Hanson, 1968; Nealson et al., 1970). In the early 1990s, Fuqua and Greenberg proposed the term quorum sensing (QS), by the definition of that a quorum as a minimal unit of bacteria to perform a certain function (Fuqua et al., 1994). At present, it is well known that bacteria are able to communicate with each other, which allows them to organize the expression of various phenotypes.

N-acyl homoserine lactone meditated QS

As mentioned above, bacterial cell-to-cell communication was first described in *V. fischeri* (Nealson et al., 1970; Eberhard, 1972; Nealson & Hastings, 1979; Eberhard et al., 1981). An N-acyl-homoserine lactone (AHL), produced by a synthase called LuxI, was introduced as the autoinducer responsible for this phenomenon. Further biochemical investigations revealed that this signal molecule binds to LuxR, a cytoplasmic receptor protein and activator of transcription (Engebrecht et al., 1983; Engebrecht & Silverman, 1984) **(Figure 3)**. This type of three-component regulatory network is widely used by numerous Gram-negative bacteria, which many of them are pathogenic and employ QS to produce virulence factors (de Kievit & Iglewski, 2000; Williams et al., 2000; Fuqua & Greenberg, 2002).

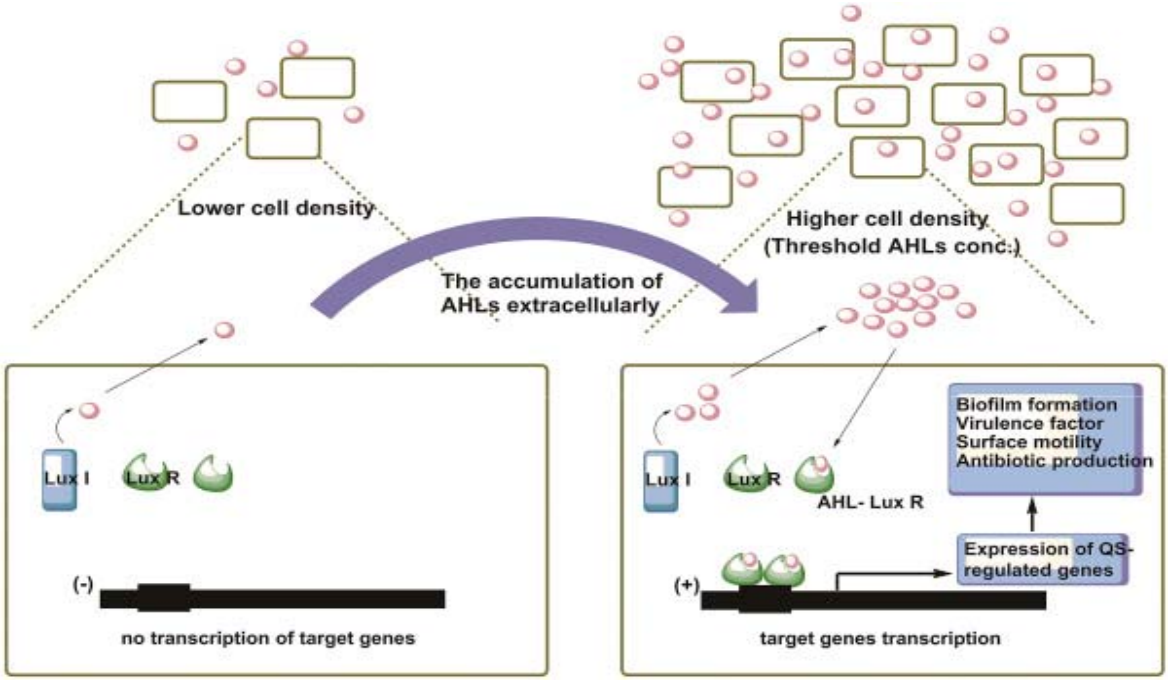


Figure 3. The acyl-homoserine lactone (AHL)- mediated quorum sensing mechanism. LuxI and LuxR are enzymes involved in the synthesis and reception of AHLs, respectively. AHL-LuxR complex acts as a transcriptional activator for some target genes e.g. virulence factors (adapted from Huang et al., 2016).

AHL biosynthesis

The signal molecules most commonly recognized in Gram-negative bacteria are AHLs. They are known as diffusible, low molecular weight molecules and characterized by a variable acyl side chain and a conserved lactone moiety, connected together by an amide bond. It is found that AHLs produced by different bacterial species vary in side chain length, saturation and substitution at the third carbon atom, as well as the production of a variety of AHLs within one species is commonplace. Acyl side chains with from 4 to 18 carbon atoms have been reported in the literature, and they are found to ramify or be (un)saturated at different positions. Furthermore, they may or may not carry a hydroxy or oxo group at the C3 position (Brelles-Marino & Bedmar, 2001; Withers et al., 2001; Marketon et al., 2002). Moreover, an intact lactonized homoserine is necessary for biological performance. LuxI homologues are AHL synthases that catalyze two reactions. They contribute to both the acylation of the amino group of S-

adenosyl-L-methionine (SAM) and the intramolecular nucleophilic substitution and loss of methylthioadenosine (Dickschat, 2010). The acyl chain is made from fatty-acid precursors conjugated to the acyl carrier protein (acyl-ACP), and the HSL segment is derived from SAM (Fuqua & Greenberg, 2002) **(Figure 4)**.

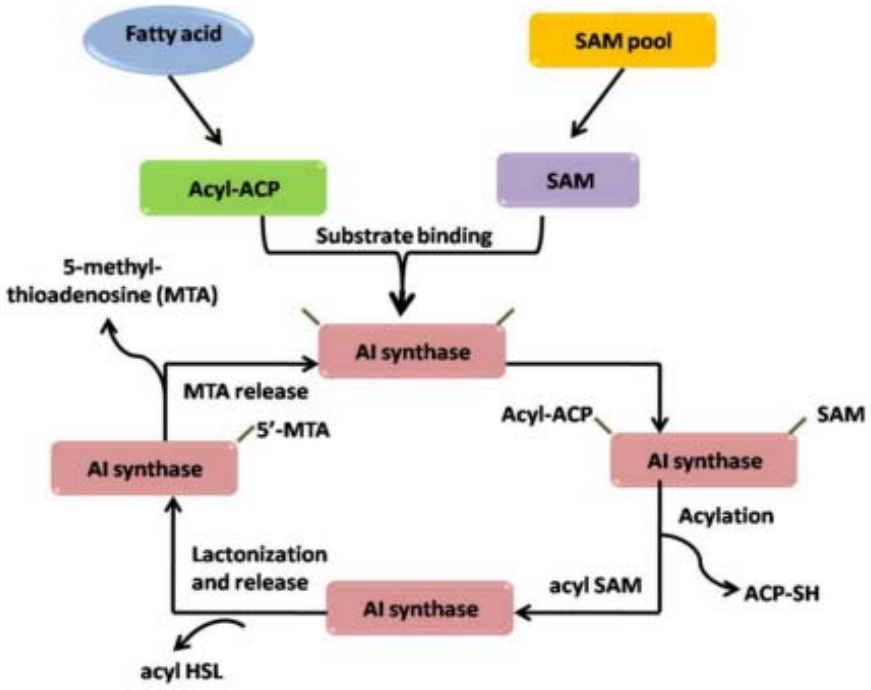


Figure 4. Acyl-HSL (homoserine lactone) synthesis by LuxI-type protein. S-adenosyl-L-methionine (SAM) and the acyl-acyl carrier protein (acyl-ACP) act as substrates for LuxI type -enzymes (autoinducer (AI) synthase). The LuxI proteins bind a specific acyl-ACP to SAM through the formation of an amide bond between the acyl side chain of the acyl-ACP and the amino group of homo cysteine moiety of SAM. HSL is formed due to the lactonization of the ligated intermediate in the reaction along with the release of methylthioadenosine

The chemical reactions previously catalyzed by LuxI determine acyl chain length. The reactions include the transfer of acetyl-CoA to the ACP by malonyl-acyltransferase enzyme (MAT) and elongation of the obtained chain through the repeated incorporation of malonyl-CoA units that are loaded onto the ACP by MAT (Dickschat, 2010). This function leads to the production of fatty acyl derivatives, which have even-numbered

carbon atoms. Nevertheless, in the rare case that propionyl-CoA is used as a starter unit, AHLs with the odd number of carbon atoms can also be found in trace amounts (Gould et al., 2006). Three different types of AHL synthases include LuxI (*V. fischeri*), AinS/LuxM (*V. fischeri / Vibrio harveyi*) and HdtS (*Pseudomonas fluorescens* / *Acidothiobacillus ferrooxidans*). There is necessarily no significant sequence similarity among these enzyme types. For instance, there is no significant similarity between the AinS protein with LuxI family members, but a similarity is seen with the *V. harveyi* LuxM protein (Bassler et al., 1993; Hanzelka et al., 1999). HdtS is not related to either of the AHL synthase families (LuxI or LuxM) and may hence belong to the third type capable of AHL biosynthesis (Laue et al., 2000).

Transport of AHLs across cell membranes

Upon synthesis, in most cases, AHLs diffuse freely in and out of the cell due to their amphipathic property. As cell density increases, the concentration of AHL in the extracellular environment enhances as well (Waters & Bassler, 2005). It was shown that within few seconds after adding 3-oxo-C6-homoserine lactone (3OC6HSL) labeled by tritium to suspensions of *V. fischeri* or *Escherichia coli*, internal concentrations equaled external concentrations in both bacteria. Given that homologues of the *V. fischeri* QS genes are absent in *E. coli*, this demonstrates that no specific system for transfer of the signal molecules was involved for this AHL (Kaplan & Greenberg, 1985). In contrast, another study showed that this does not occur for 3H-labeled 3-oxo-C12-homoserine lactone (3OC12HSL) in *Pseudomonas aeruginosa* (Pearson et al., 1999). Although a steady state was reached within 30 seconds after the addition of [3H]C4-homoserine lactone (C4HSL), with cellular and extracellular concentrations nearly equal, it took about 5 minutes to reach a steady state for [3H]3OC12HSL, and the internal concentration was 3 times higher than the external level. In a study, inhibiting the cytoplasmic membrane proton gradient with azide causes a highly increase in the internal level of [3H]3OC12HSL, proposing the role of active efflux systems. Indeed, a mutant lacking the *mexA-mexBoprM*-encoded active-efflux pump was evaluated and the

results indicated that [3H]3OC12HSL accumulated to a density similar to those in the wild-type cells treated by azide (Pearson et al., 1999). Moreover, it was demonstrated that virulence factors known to be regulated by QS were produced in lower levels by *P. aeruginosa* mutants hyperexpressing the MexAB-OprM multidrug efflux system, again confirming the presence of active transport of AHLs (Evans et al., 1998). Other cases of this mechanism are also found in *Burkholderia pseudomallei*, a mutant lacking the BpeAB-OprB efflux pump was impaired in the export of several types of AHLs; and in *Bacteroides fragilis*, a BmeB pump was suggested to play a role in AHL transfer (Evans et al., 1998; Pumbwe et al., 2008). It can be summarized that AHLs with short chain are able to diffuse freely and rapidly across cell membranes, whereas the more long-chain AHLs are exported out of the cell slowly and gradually through efflux pumps. This slow efflux can be justified by partitioning of the long chain AHL because of interactions with the lipophilic cytoplasmic membrane (de Kievit & Iglewski, 2000). The specific mechanisms for cellular AHL uptake it is not clear yet, but it is generally thought to take place by passive diffusion.

AHL signal reception and signal transduction

Signal molecule concentration increases with increasing cell density. Upon reaching a particular threshold level, the signals join in the LuxR-type receptor to form a LuxR-AHL complex, in turn, acting as a transcriptional activator. LuxR homologues are located in the cytoplasm or loosely connected to the inner layer of the cytoplasmic membrane (Ni et al., 2009). Three classes of LuxR-type receptors have been defined based on the type of interaction with their respective signals (**Table 3**). The first class, including LasR (*P. aeruginosa*), TraR (*Agrobacterium tumefaciens*) and CepR (*Burkholderia cenocepacia*), requires AHL for folding and binds AHLs irreversibly when folded .The second class of proteins, containing QscR (*P. aeruginosa*) and LuxR (*V. fischeri*), also needs AHL for folding, but the mature protein can attach to AHL reversibly. EsaR (*Pantoea stewartii*) and ExpR (*Erwinia chrysanthemi*) constitute members of the third class of LuxR-type receptors. In this class, AHL is not required for folding, and the

binding of AHL with the mature protein is reversible (Schuster & Greenberg, 2008). Sequence conservation, biochemical analysis of LuxR, TraR and other family members, and the TraR crystal structure revealed that LuxR-type proteins consist of two important functional domains: an activator domain located at the C-terminal end of the protein, and a regulator domain located at the N-terminal end. The regulator domain provides the AHL-binding site. It comprises a conserved cluster of residues, and mutations in this region lead to the stopping of the binding of 3OC6HSL to LuxR (Hanzelka & Greenberg, 1995; Stevens & Greenberg, 1999). Sequence alignment of AhyR (*Aeromonas hydrophila*), LasR and RhlR (*P. aeruginosa*), LuxR (*V. fischeri*) and TraR (*Ag. tumefaciens*) indicated that conserved amino acid residues required for the interaction with the AHL: Ala38 (Gly in LasR), Tyr53 (Cys in AhyR), Trp57, Asp70 and Ser129 (Thr in TraR) (Whitehead et al., 2001). The crystal structure of the transcriptional regulator TraR bound to its signal molecule and to target DNA was determined by Vannini et al. (2002). The findings revealed a- multitude of intermolecular interactions between the autoinducer and the protein. The AHL was observed to be wholly embedded in an enclosed cavity, formed by a cluster of aromatic and hydrophobic residues. Additionally, two hydrogen bonds between the amide nitrogen and Asp70, and between the carbonyl oxygen of the lactone moiety and Trp57 are used to stabilize the AHL (Vannini et al., 2002). The second functional domain, located in the C-terminal site, carries a helix-turn-helix motif necessary for DNA binding (Stevens & Greenberg, 1999). The central region of the protein is required for multimerization, an important consequence of AHL binding. It has indeed been shown *in vivo* with LuxR (*V. fischeri*) and LasR (*P. aeruginosa*), and *in vitro* with TraR (*A. tumefaciens*) and CarR (*Erwinia carotovora*) that oligomeric LuxR homologues are formed following binding of the signal. The active, ligand-bound form of TraR is a dimer, for instance; whereas the CarR protein appears as a dimer without signal molecule and is altered towards a multimer in the presence of AHL (Welch et al., 2000; Zhu & Winans, 2001). Results with RhlR (*P. aeruginosa*) are more eristic. In one study it was demonstrated that RhlR dimerizes *in vitro* in the absence of the signal molecule, C4HSL (Ventre et al., 2003), however, some evidence shows that C4HSL confers multimerization *in vivo* (Lamb et al., 2003; Schuster & Greenberg, 2008).

Although there is only an end-to-end sequence similarity of 18- 23% among LuxR homologues, they all show several conserved amino acids and a similar structure. Moreover, LuxR-type proteins of various bacteria can be found in size from 70 up to 300 amino acids (Fuqua et al., 2001). Upon binding the AHL to the LuxR homologue, the signal-receptor complex binds to DNA promoter elements similar to the *lux* box in *V. fischeri*. This *lux* box, a 20 bp inverted repeat, is located in the *luxI* promotor region and essential for LuxR-related induction of the luminescence genes. The presence of many *lux*-type boxes upstream of the -35 sequences of regulated promoters indicates that LuxR homologues can interact directly with RNA polymerase. A decreased target gene expression has been found following null mutations in genes encoding LuxR, indicating that most of the LuxR-type regulators play a key role as a transcriptional activator (Stevens & Greenberg, 1997; Fuqua et al., 2001). In contrast, several other LuxR-type proteins, from different species and subspecies of *Erwinia* function as transcriptional repressors. For example, EsaR (*P. stewartii*, formerly *E. stewartii*) binds to DNA in the absence of AHLs. The EsaR-AHL complex shows a reduced affinity for DNA and terminates the repression (von Bodman et al., 1998; Minogue et al., 2002). A last and very strange type of LuxR homologues are orphan LuxR regulators (Patankar & Gonzalez, 2009). These LuxR solos act without the corresponding LuxI homologue and may detect AHLs produced by other bacterial species, or may be involved in interkingdom signaling (Smith & Ahmer, 2003; Subramoni & Venturi, 2009). These orphan proteins are both found in bacteria which possess (a) complete AHL QS system(s) and in bacteria that do not. ExpR of *Sinorhizobium meliloti*, BisR of *Rhizobium leguminarosum*, CepR2 of *B. cenocepacia* and QscR of *P. aeruginosa* are examples of LuxR solos in AHL producing bacteria (Malott et al., 2009; Patankar & Gonzalez, 2009); whereas SdiA is an example of a LuxR solo known in non-AHL producing bacteria (*Escherichia*, *Salmonella* and *Klebsiella* species) (Michael et al., 2001).

Biosensors for the detection of AHLs

Biosensors for the detection of AHLs are bacteria with an easily detectable phenotypic trait regulated by QS. They generally respond to the presence of exogenously added AHLs but do not possess the respective AHL synthase. Thay act by binding the activated LuxR homologue to a QS-related promoter region (usually the promoter of the cognate *luxI* synthase) of a reporter operon, leading to the production of a detectable signal (Steindler & Venturi, 2007). Most biosensors contain a recombinant plasmid in which *lacZ*, *gfp* or *luxCDABE* genes inserted into a QS-responsive promoter.
The *lacZ* gene is responsible for encoding β-galactosidase, an exoglycosidase used as a known specific indicator of gene expression. It affects the synthetic substrate 5-bromo-4- chloro-3-indolyl-β-D-galactopyranoside (X-gal), resulting in the production of a visually or spectroscopically detectable blue pigmentation. Binding *lacZ* to QS -dependent genes allows detecting the exogenous AHLs through measurement of β-galactosidase activity (Pearson et al., 1994).
Another commonly used gene in QS biosensors is *gfp* from the jellyfish (*Aequorea Victoria*), which encodes green fluorescent protein (GFP) (Chalfie et al., 1994). Considering high stability of GFP, a number of *gfp* genes encoding proteins with different half-lives were constructed by Andersen et al. (1998). They constructed a manipulated *gfp* producing a GFPs carried C-terminal peptide tags, which are detectable and degradable by specific proteases (Andersen et al., 1998). The construction of the produced *gfp* variants allowed AHL detection at the single-cell level and suitable for real-time measurements of changes in AHL amounts (Andersen et al., 2001). The third system employed in QS measurements is a plasmid structure containing the *luxCDABE* operon from *Photorhabdus luminescens*, which results in bioluminescence (Winson et al., 1998). Each AHL biosensor is related to a certain LuxR-type protein, thus showing a particular specificity for its cognate AHL. Many biosensors are capable of detection of only a limited range of signal molecules, resulting in the necessity of the use of the right type of biosensor for experimental procedures (Steindler & Venturi, 2007).

Table 3 Some Gram-negative bacteria possessing LuxI/LuxR homologues (adapted from Miller & Bassler, 2001; Tay & Yew, 2013)

Organism	LuxI/LuxR Homologue(s)	Reference
V. harveyi	LuxN / LuxM	Freeman et al. (2000)
	LuxP / LuxS	Galloway et al. (2010)
	CqsS / CqsA	Ng et al. (2011)
P. aeruginosa	RhlR / RhlI	Gilbert et al. (2009)
	LasR / LasI	Gilbert et al. (2009)
	QscR / NA	Chugani et al. (2001); Lee et al. (2006)
A. baumannii	AbaR / AbaI	Bhargava et al. (2010)
***Enterobacter* spp.**	SdiA / NA	Shankar et al. (2013)
	LsrB/ LuxS	Rezzonico et al. (2012)
Vibrio fischeri	LuxI / LuxR	Eberhard et al. (1981); Engebrecht et al. (1983)
Aeromonas hydrophila	AhyI / AhyR	Swift et al. (1997)
Aeromonas salmonicida	AsaI / AsaR	Swift er al. (1999)
Agrobacterium tumefaciens	TraI / TraR	Piper et al. (1993); Zhang et al. (1993)
Burkholderia cepacia	CepI / CepR	Lewenza et al. (1999)
Chromobacterium violaceum	CviI / CviR	Chernin et al. (1998); McClean et al. (1997)
Enterobacter agglomerans	EagI / EagR	Swift et al. (1993)
Erwinia carotovora	ExpI / ExpR	Jones et al. (1993); Pirhonen et al. (1993)
	CarI / CarR	Bainton et al. (1992)
Ralstonia solanacearum	SolI / SolR	Flavier et al. (1997)
Vibrio anguillarum	VanI / VanR	Milton et al. (1997)
Yersinia ruckeri	YruI / YruR	Temprano et al. (2001)

Based on the specificity for certain AHLs, the biosensors have been grouped in the following. *Chromobacterium violaceum,* a Gram-negative bacterium, uses the CviI/R AHL system to induce violacein (a purple pigment) production. An AHL-negative mutant, *C. violaceum* CV026 was constructed by manipulating *C. violaceum* through a mini-Tn5 transposon mutagenesis, (McClean et al., 1997). Adding exogenous signaling

molecules leads to violacein production in this strain. In addition to C6-homoserine lactone (C6HSL), which is the natural *C. violaceum* AHL, this biosensor is also able to detect C4HSL, C8HSL, 3OC6HSL, 3-oxo-C8-homoserine lactone (3OC8HSL) and 3-oxo-C4-homoserine lactone (3OC4HSL). AHLs with acyl side chains longer than 8 carbon atoms are not recognized by this biosensor strain. Furthermore, CV026 is not capable of detecting any 3-hydroxy-AHLs. Adding short chain AHLs with long chain AHLs caused a decrease in induction by the short chain AHLs, suggesting that this mutant could be applied to detect AHLs consisting longer acyl side chains (C10 to C14) as well (McClean et al., 1997).

Several bioluminescence-relevant biosensors are also found to detect AHLs with short and medium acyl chain. *E. coli* (pSB401) and *E. coli* (pHV200I) both contain LuxR of *V. fischeri* and the related *luxI* promoter, which controls the expression of *luxCDABE*. They show the most sensitivity to 3OC6HSL and are good sensitive towards C6HSL, 3OC8HSL and C8HSL. Both strains can be used for measurement of AHL in low concentrations. However, certainly, a luminometer is required to measure luminescence (Pearson et al., 1994; Winson et al., 1998). Finally, *E. coli* strains (pSB536 and pAL101) are biosensors with sensitivity to C4HSL. The former plasmid was produced using the *ahyR* from *A. hydrophila* and the cognate *ahyI* gene promoter inserted into *luxCDABE*. In order to construct the latter, RhlI/R QS system of *P. aeruginosa* was used in a similar way (Swift et al., 1997; Lindsay & Ahmer, 2005). The LasI/R system from *P. aeruginosa,* producing and detecting 3OC12HSL, is based to construct biosensors for the specific detection of long-chain AHLs. *E. coli* pSB1075 carries a plasmid harboring the *lasR* gene and cognate *lasI* promotor, which controls *luxCDABE* expression. This biosensor was found to respond well to 3OC12HSL, 3-oxo-C10-homoserine lactone (3OC10HSL) and C12-homoserine lactone (C12HSL) (Winson et al., 1998). Moreover, *E. coli* pKDT17 and *P. aeruginosa* PAO1 M71LZ are known as two biosensors for long-chain AHLs, producing β-galactosidase. The plasmid pKDT17 possesses *lasR* controlled by the *lac* promoter and a *lasB*::*lacZ* translational fusion. *lasB* is a gene encoding an elastase, which is under control of the LasI/R QS system (Pearson et al., 1994). The *E. coli* strain containing this plasmid is highly sensitive to C12HSL, C10HSL and their 3-

oxo derivatives (Cha et al., 1998). *P. aeruginosa* PAO1 M71LZ is a *lasI* knock-out mutant, which contains a transcriptional fusion of the promotor of *rsaL,* a gene under directly control of the LasI/R QS system, and reporter gene *lacZ* (de Kievit et al., 1999; Dong et al., 2005; Rampioni et al., 2007). Adding 3OC12HSL to this strain will hence lead to *rsaL* transcription via LasR. This biosensor also responds well to 3OC10HSL.

Biosensors that can detect a broad range of AHLs

The specificity of the LuxR homologues used in AHL biosensors is always a restricting factor, hence, most AHL biosensors are limited in the range of AHLs which they can respond. However, biosensors containing the TraI/R system of *A. tumefaciens* are capable of detecting a broad range of signaling molecules (Cha et al., 1998; Farrand et al., 2002; Zhu et al., 2003). *A. tumefaciens* NT1 pZLR4 is known as a biosensor for broad range AHL. In this strain, the Ti plasmid, responsible for AHL production, has been cured and instead the pZLR4 plasmid has been provided, which harbors the *traR* gene and one of the *tra* operons, containing a *traG*::*lacZ* reporter fusion, and the transcription is known to be regulated by the TraI/R QS system (Cha et al., 1998; Farrand et al., 2002). This β-galactosidase producing biosensor displays a sensitivity to a remarkable range of AHLs, as it detects 3-oxo-substituted AHLs (from 4 to 12 carbons), and also 3-unsubstituted AHLs excluding C4HSL. Additionally, it is able to detect some hydroxy-substituted signal molecules specifically 3-hydroxy-C6-homoserine lactone (3OHC6HSL), 3-hydroxy-C8-homoserine lactone (3OHC8HSL) and 3-hydroxyC10-homoserine lactone (3OHC10HSL) (Cha et al., 1998; Shaw et al., 1997). A similar biosensor, *A. tumefaciens* WCF47 (pCF218) (pCF372) (Zhu et al., 1998), recently has been modified for even more sensitivity. This was carried out by overexpressing the TraR protein and increasing amounts of the regulator in the cytoplasm, resulting in a higher sensitivity and broader specificity towards AHLs (Zhu et al., 2003).

The role of QS in virulence and biofilm formation

Many traits such as the expression of virulence factors are controlled by QS in pathogenic bacteria. Some of the pathogens using QS in such a way are *P. aeruginosa, S. aureus*, *Bacillus cereus* and *V. cholerae*. In the following, it is described how QS is involved in virulence production and biofilm formation in a few known pathogens.

In *P. aeruginosa*, binding 3OC12HSL to the receptor protein LasR at a high cell density results in the transcription of target genes encoding virulence factors such as elastase, proteases, and exotoxin A (Schuster et al., 2003). Some other targets include *lasI*, causing the establishment of a positive autoinducing feedback (Seed et al.,1995), as well as *rhlI*, a second *luxI* homologue. Genes regulated by the *rhlI* QS system encode elastase, proteases, pyocyanin, and siderophores as well (Schuster & Greenberg, 2007). It has been found that the PQS system also plays a key role in pyocyanin production. Additionally, other virulence factors such as type IV pili biosynthesis and type III secretion systems showed to be linked to QS in *P. aeruginosa* (de Kievit & Iglewski, 2000; Wagner et al., 2003). In addition to the mentioned *in vitro* evidence, the importance of QS in the pathogenicity of *P. aeruginosa* has been indicated in several *in vivo* experiments, as strains with mutations in the QS system showed significantly less pathogenicity in comparison with the wild pathogen (Tang et al., 1996; Rumbaugh et al., 1999; Pearson et al., 2000; Christensen et al., 2007; Kim et al., 2010). Detection of PQS, C4HSL, and 3OC12HSL in the sputum, bronchoalveolar fluid and mucopurulent fluid from cystic fibrosis patients colonized with *P. aeruginosa* provided further evidence for the role of QS in virulence (Geisenberger et al., 2000; Singh et al., 2000).

Biofilm formation is another QS-regulated process in *P. aeruginosa*. This fact has been proven that not only additional environmental signals are involved in *P. aeruginosa* biofilm development, but also microarray analyses have indicated that QS regulation of genes responsible for the production of rhamnolipids, siderophores and swarming motility participate in biofilm formation (Davies et al., 1998; Parsek & Greenberg, 1999; De Kievit et al., 2001; Hentzer & Givskov, 2003). Besides, it has been found that these genes are involved in all stages of biofilm formation (Wagner & Iglewski, 2008). *lasI*

mutants of *P. aeruginosa* have been observed to form unstructured instead of mushroom-shaped biofilms in a flow cell, and that this mutant biofilm was more sensitive to sodium dodecyl sulfate, tobramycin, and phagocytosis (Davies et al., 1998; Hentzer & Givskov, 2003; Bjarnsholt et al., 2010). Furthermore, gene clusters encoding the production of important components such as PEL and PSL polysaccharides are also controlled by QS (Wagner et al., 2003; Sakuragi & Kolter, 2007). QS is also involved in biofilm resistance against antimicrobials. In a study, it was investigated the effect of inhibition of QS on the susceptibility of bacterial biofilms to treatment with some conventional antimicrobial compounds (Brackman et al., 2011). The authors used AHL-based QS inhibitors in *P. aeruginosa* and *Burkholderia cepacia* as well as peptide-based QS inhibitors in *S. aureus*. *In vitro* and *in vivo* biofilm model systems, including two invertebrate models (*G. mellonella* and *C. elegans*) and one mouse pulmonary infection model, were used to evaluate the impact of treatment with tobramycin (*P. aeruginosa, B. cepacia* complex) and clindamycin or vancomycin (*S. aureus*), alone or in combination with QSI. *In vitro* experiment, an increased killing was observed when a combination of an antibiotic and a QS inhibitor was used in comparison with killing by an antibiotic alone. The observations from the *in vivo* assays confirmed the results from *in vitro* showing a higher survival for infected organisms receiving a combined treatment compared to those only received the antibiotic. Besides, it was found that the combined use of tobramycin and baicalin hydrate (a QS inhibitor targeting AHL-based QS) led to a reduction in the microbial load in the lungs of BALB/c mice infected with *B. cenocepacia* more than alone treatment with tobramycin (Brackman et al., 2011). Finally, it has also been investigated the synergistic antimicrobial efficacy of the combined use of a QSI and tobramycin on mice, resulting in an increased clearance of *P. aeruginosa* in BALB/c mice (Christensen et al., 2012).

The importance of QS systems in the pathogenicity of *Vibrio* spp. has been well studied. For instance, two parallel QS systems are used in *V.cholerae* to control the expression of virulence factors and biofilm formation. One of the signal molecules, CAI-1, is synthesized by CqsA and the second signal is AI-2. *V. cholerae* activates the expression of virulence factors and forms biofilms at low cell density. However, the high

concentration of the two signals results in a reduction in these phenotypes at high cell density. Indeed, the signals synergistically control the gene expression. CAI-1 showed the stronger activity (Higgins et al., 2007). Interestingly, the expression of virulence factors and biofilm forming capacity in *V. cholerae* occurs at low cell density. This low cell-density lifestyle makes *V. cholerae* capable of remaining attached to host tissue during the expression of virulence factors, whereas a high cell-density facilitates dispersal of the population (Nadell & Bassler, 2011). Although, in most cases, a positive regulation of virulence factors by QS is observed in this genus. The pathogenic phenotypic properties QS-controlled in *V. cholerae* include the production of cholera toxin (an enterotoxin causing severe diarrhea) and toxin-coregulated-pili production (Novick et al., 1995). QS is also used in Gram-positive bacteria to control virulence factor production and biofilm formation. By turning on QS in *S. aureus*, the phosphorylated response regulator activates the transcription of two *agr* promoter regions P2 and P3, and the RNAIII regulatory RNA. RNAIII posttranscriptionally causes the production of virulence factors and inhibition of the expression of *rot*, fibronectin binding proteins A and B and coagulase. Repression of *rot*, encoding a repressor of toxins, results in de-repression of toxins, enterotoxins, proteases, lipases, and urease. This regulatory system leads to a downregulation of surface virulence factors and upregulation of secreted virulence factors (Janzon & Arvidson, 1990; Novick et al., 1993; Morfeldt et al., 1995; Novick et al., 1995; Dunman et al., 2001; Said-Salim et al., 2003; Geisinger et al., 2006; Queck et al., 2008). Biofilm formation is known as another important component of the *S. aureus* virulence properties. *S. aureus* uses a similar way to *V. cholerae* for biofilm formation, as the *agr* system inhibits biofilm formation, it is the reason for establishing a biofilm at low cell density, causing *S. aureus* to grow to high cell density and, at that point, release virulence factors. At high cell density, *S. aureus* terminates biofilm development and reduces surface proteins and adhesions to facilitate dispersal (Boles & Horswill, 2008).

Quorum sensing in *Y. ruckeri*

The ability of regulation of virulence gene expression based on bacterial cell density through quorum sensing (QS) has subjected to study in various pathogenic bacteria, including those harmful for fish (Natrah et al., 2011). Indeed, QS is a form of cell-to-cell communication, detecting the signaling molecules such as acylated homoserine lactones (AHLs), which regulates some bacterial phenotypes (e.g. those associated with virulence). Besides understanding virulence phenotypes related to bacterial density/AHL concentrations, identifying QS inhibitors (QSIs) may prevent disease outbreaks and is considered an alternative to antimicrobial therapy. This is an attractive technique for controlling *Y. ruckeri* infections since resistance to antimicrobials in this pathogen is rising. Preliminary analysis by Bruhn et al. (2005) and Kastbjerg et al. (2007) revealed that *Y. ruckeri* can produce a wide range of AHLs, specifically an *N*-3- oxo-octanoyl homoserine lactone (OOHL). These AHLs have been recognized in fish tissues infected with *Y. ruckeri*, proposing an *in vivo* role for QS. 3-oxo-C8-HSL is the most predominant AHL produced by *Y. ruckeri* (Kastbjerg et al., 2007). In a research by Kastbjerg et al. (2007) to determine the role of QS in regulating virulence, the relation of Yrp1 protease production with different AHLs and QSIs was investigated. However, testing different AHLs or QSIs at different concentrations under different culturing conditions revealed that production of the extracellular protease was generally not regulated by QS. At present, this is the only published study focused on the correlation of QS with virulence phenotypes of *Y. ruckeri*. Genes involved in QS were identified by constructing a genomic library from a *Y. ruckeri* strain in *E. coli* and using *Chromobacterium violaceum* to test for the production of violacein (Temprano et al., 2001). One of the *E. coli* transformants inducing violacein production in CV026 was found to carry a genomic *Y. ruckeri* fragment containing two open reading frames (ORFs) homologous to genes which encode the LuxR/LuxI protein class. The first ORF, *yruR*, encodes a protein (of 247 amino acids (aa)) and displays remarkable similarity to a transcriptional activator described in *V. fischeri*. The second ORF (*yrul*), encoding a protein (of 217 aa) which plays a key role in AHL synthesis. Moreover, the importance of these genes has been

proved in detecting *Y. ruckeri* in a PCR reaction. Constructing mutations within the *yruR*/*yruI* genes might be useful for determination of any effect on pathogenicity *in vivo*. However, the identification of AHL produced by *Y. ruckeri in vivo* indicates that QS is involved in virulence (Bruhn et al., 2005).

Quorum quenching (QQ)

Since bacterial resistance to antibacterial agents develops quickly, treatment of bacterial diseases is becoming increasingly more difficult. Several opportunistic pathogens employ biofilm formation and QS-regulated expression of virulence factors to spread their infections in humans and animals. Inhibition of QS has been suggested as potentially novel therapeutics for the treatment and control of bacterial diseases (Martin et al., 2008).

Strategies to interfere with QS

Basically, five key processes can be considered in Gram-negative QS mechanism: (1) signal generation, (2) signal transportation, (3) signal accumulation, (4) signal recognition, and (5) signal autoinduction (Wang et al., 2008). Interfering with each of these pathways can be suggested as a potential QQ mechanism **(Figure 5)**.

Blocking signal generation

Blocking signal molecule generation, as the most straightforward QQ strategies, can occur by preventing the signal molecules from being synthesized by the *luxI*-encoded AHL synthase (Wang et al., 2008). Thus, AHLs will not be detected by the bacteria and consequently, QS-regulated genes will not be induced. Triclosan, a commonly used biocide, was found to inhibit enoyl-acyl carrier protein reductase, producing a compound required in AHL biosynthesis (Hoang & Schweizer, 1999).

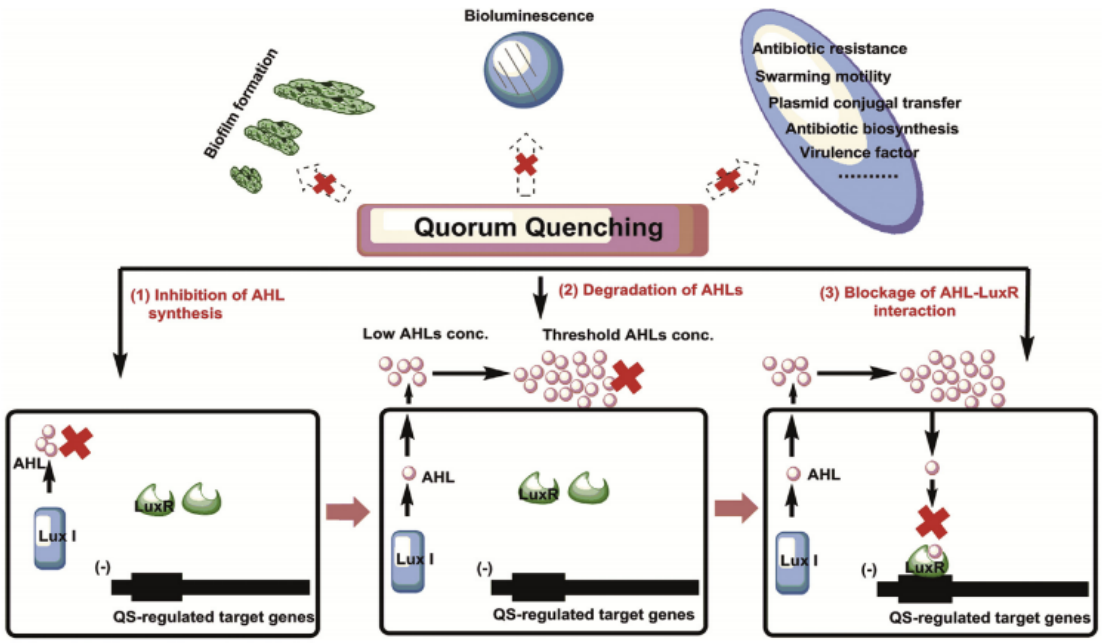


Figure 5. External intervention of three major components within bacterial LuxI/LuxR-type QS system. In the figure, AHL demonstrates acyl-homoserine lactone (as a signal molecule). LuxI indicates the LuxI-type synthase which catalyzes AHL synthesis and LuxR represents a receptor for AHL (adapted from Huang et al., 2016).

Analogues of SAM such as Holo-ACP, L/D-*S*-adenosylhomocysteine, sinefungin and butyryl-SAM have been suggested to be inhibitors of the *P. aeruginosa* AHL synthase, RhlI. L-S-adenosylhomocysteine showed the most efficiency with a decrease in the activity of RhlI by 97% in *P. aeruginosa* (Parsek et al., 1999) **(Figure 5)**.

Disturbing signal exchange

Although the short-chain AHLs are able to diffuse passively across bacterial membranes, efflux of AHLs with long chain takes place through active transportation mechanisms (Pearson et al., 1999; Dong et al., 2005). Active transportation of AHL is important for some bacterial pathogens. Pearson et al. (1995) reported that there are

the nonspecific multidrug efflux pumps MexAB-OprM, involved in the active transport of C12-oxo- HSL, in *P. aeruginosa*. It has been demonstrated that making a mutation in these pumps results in a reduction in AHL production and virulence factors (Aendekerk et al., 2002). Furthermore, several chemicals, such as pyridopyrimidine and quinazolinone derivatives, were reported to inhibit ABC-type efflux pumps (Nakayama et al., 2004; Watkins et al., 2007).

Preventing signal recognition

The use of an AHL analogue to block the AHL receptor is considered a classical pharmacological strategy and probably is the most investigated technique for QS inhibition (Wang et al., 2008). QS can be inhibited using the halogenated furanones, a structural mimic of AHL signals, by reducing the half-life of LuxR receptor protein (Martinelli et al., 2004, Defoirdt et al., 2006a). In a study, the carbonyl group of AHLs was replaced with a sulfonyl group in the acyl side chain by Castang et al. (2004). They were able to generate some inhibitors for LuxR-dependent QS using this molecule with variation in side chains and substituents at the end of the side chain. Rasmussen et al. (2005) could identify various nonrelated compounds able to block both LuxR- and LasR-based QS, such as para-benzoquinone, 2,4,5-tri-bromo-imidazole, indole, 3-nitro-benzene-sulfonamide, and 4-nitro-pyridine-N-oxide (4-NPO). DNA array-based analysis revealed that 4-NPO, with the most QSI efficacy, significantly was able to downregulate 37% of the QS-controlled genes in *P. aeruginosa*. However, these QSI compounds possess different structure from the AHLs and their analogues, suggesting that further studies are required to clarify their mode of action (Rasmussen et al., 2005).

Signal trapping

There are two types of signal-trapping mechanism. The first one relies on signal-specific antibodies. It has been found that C12-oxo-HSL at the low concentration can confer the production of immunoglobins IgG1 and IgE (Telford et al., 1998), which can be

employed for trapping QS signals. Van Delden & Iglewski (1998) treated the bacterial pathogen with the C12-oxo-HSL-specific antibody, resulting in a decrease in pyocyanin, a cytotoxin, by up to 50%, and a survival benefit in mice infected with *P. aeruginosa*. The second type is polymer cyclodextrins which used to mimic a receptor and trap QS signal. The AHL-dependent production of pigment prodigiosin by *Serratia marcescens* partially is reduced, upon the adding 2-hydroxypropyl-β-cyclodextrin (Wang et al., 2008). However, it seems a high number of polymer or antibodies may be required to inhibit the QS signal molecules constantly produced by a pathogen.

Inactivating QS signal

The signal molecules can be targeted with metabolic, chemical or enzymatic degradation if the signal production cannot be prevented (Tateda et al., 2003). Alkaline conditions (pH>7) and high temperature (>37°C) can affect the stability of the lactone ring of AHL (Yates et al., 2002). Four types of QQ enzymes, degrading or modifying the AHL-type QS signals, are widely found in both prokaryotic and eukaryotic kingdoms, including AHL-lactonase, AHL-acylase, paraoxonase (PONs), and AHL-oxidoreductase (Draganov et al., 2005; Uroz et al., 2005). Quorum quenching by AHL-degrading enzymes has been explored and suggested as novel antimicrobial agents against various pathogens (Dong et al., 2007). A strong resistance to a bacterial pathogen *Erwinia carotovora*, which needs AHLs for the expression of virulence genes, was conferred by expression of the AHL-lactonase encoded by the *aiiA* gene from *Bacillus* sp. in transgenic potato and tobacco plants (Dong & Zhang, 2005; Zhang, 2003).

Several studies have investigated the effects of QQ bacteria on some pathogenic bacteria in aquatic animals. Some studies have previously isolated AHL-degrading enrichment cultures (mixed cultures resulted by subsequent culturing intestinal microbiota in a medium supplemented with AHLs as the sole carbon source) from the intestine of healthy shrimp and fish (Tinh et al., 2007; Cam et al., 2009). It has been demonstrated that the use of these mixed cultures increases survival of different aquatic

species, such as turbot (*Scophthalmus maximus*) larvae (Tinh et al., 2008) and larvae of the giant freshwater prawn (*Macrobrachium rosenbergii*) (Nhan et al., 2010).

Chen et al. (2010) could produce a recombinant AHL-lactonase (AiiAB546) enzyme from *Bacillus* sp. B546 in *P. pastoris.* After purification, the recombinant AiiA B546 was injected along with *A*. *hydrophila* in common carp and caused a reduction in the mortality rate and delayed the mortality time. In another study, a QS inhibitor strain, (QSI)-1, was isolated from the intestine of *Carassius auratus gibelio* and identified as the genus *Bacillus* spp. *In vitro* coculture of QSI-1 with *A. hydrophila* significantly decreased the concentration of AHLs. Additionally, fish fed diet supplemented with QSI-1 showed good survival rate, suggesting that QSI-1 induced protection against *A. hydrophila* infection (Chu et al., 2010).

In another study by the same authors, the effect of *Bacillus* sp. QSI-1 on virulence factors and biofilm formation of *A. hydrophila* was investigated. QSI-1 reduced the amounts of AHLs but did not affect the growth of *A. hydrophila* YJ-1 when cocultured. The supernatant of QSI-1 showed a significant inhibitory effect on protease production, hemolytic activity and biofilm formation in YJ-1. *In vivo* assay, QSI-1 significantly reduced the pathogenicity of *A. hydrophila* strain YJ-1 in zebrafish (*Danio rerio*). The fish fed with QSI-1 was observed to have a relative percentage survival of 80.8% (Chu et al., 2014).

Romero et al. (2014) in a screening for bacterial strains with QQ activity in different marine environments identified *Tenacibaculum* sp. strain 20J and selected it for its high degradation activity against a wide range of AHLs. In this study, the QQ activity of live cells and crude cell extracts (CCEs) of strain 20J was determined and revealed that CCEs of this strain is capable of quenching the AHL production in cultures of *Edwardsiella tarda* ACC35.1.

Molecular mechanisms of QQ enzymes

The function of interference with QS by QQ enzymes has been distributed among prokaryotes and a few eukaryotic organisms (Dong et al., 2007; Turovskiy et al., 2007).

AHL-degrading enzymes such as AHL-lactonases, AHL-acylases and AHL-oxidoreductases are produced by many different bacteria belonging to various genera; the AHL lactonase-like paraoxonases (PONs) have been found in mammalian cells as well (Czajkowski & Jafra 2009; Wang et al., 2008) **(Figure 6A, 6B)**.

AHL lactonases

Lactonases affect the AHLs by hydrolyzing the lactone ring and are found in numerous *Bacillus* spp. (Dong et al., 2002; Zhang, 2003). It has been indicated that heterologous expression of *aiiA*, encoding AHL lactonase from *Bacillus* spp., in *Pseudomonas, Burkholderia* and *Erwinia* lead to a notable decrease in the amount of their QS signal molecules (Dong et al., 2000; Reimmann et al., 2002; Ulrich, 2004).

Phylogenetic analyses revealed that the AHL-lactonases can be divided into two clusters with homology at about 30%, including the AiiA-like cluster and the AttM-like cluster (Dong & Zhang, 2005). AiiA lactonase homologues were explored in numerous bacteria belonging to the *Bacillus* genus. A high nucleotide sequence similarity, greater than 90%, is observed in all AiiA lactonase. It has been demonstrated that there is a conserved motif 'His106-X-Asp108-His109-59X-His169-21x-Asp191', essential for the enzyme activity, according to sequence alignment of the AiiA lactonase homologues (Dong et al., 2002; Lee et al., 2002; Kim et al., 2005). The AHL-lactonases in AttM cluster from Gram-negative bacteria, including *A. tumefaciens* (Zhang et al., 2002; Carlier et al., 2003), *Klebsiella pneumonia* (Park et al., 2003), *Arthrobacter* sp. and *Rhodococcus* sp. (Park et al., 2003; 2006) and *R. erythropolis* (Uroz et al., 2008), are less investigated compared to AiiA cluster, and the peptide similarity among these AHL-lactonases are found in the range of 21-26%. However, the mentioned 'His-X-Asp-His~His~Asp' motif is well conserved in these AHL-lactonases as well, indicating that the AHL-lactonases could share the same catalytic mechanism similar to the AHL-lactonases in AiiA cluster (Wang et al., 2008).

AHL acylases

The second QQ enzyme group is AHL-acylase, which hydrolyzes the amide bond between the acyl side chain and the homoserine lactone moiety in the AHL, resulting in the homoserine lactone and the free fatty acid, when are further metabolized finally in most cases used as carbon, nitrogen or energy sources by the bacteria (Leadbetter & Greenberg, 2000). The first described AHL acylase was from Gram-negative bacterium *V. paradoxus* VAI-C strain, showing a wide range of AHLs degradation ability (Leadbetter & Greenberg, 2000). Until present, five different AHL acylases have been characterized including AiiD from *Ralstonia eutropha* (Lin et al., 2003), PvdQ and QuiP from *P. aeruginosa* (Huang et al., 2003; 2006; Sio et al., 2006), AhlM from *Streptomyces* sp. (Park et al., 2005) and AiiC from *Anabaena* sp. PCC7120 (Romero et al., 2008). However, AHL acylases are found with remarkable differences in the substrate specificities. AiiD is effectively able to degrade long-chain AHLs as well as short-chain AHLs (with less efficiency) (Lin et al., 2003). PvdQ and AhlM are capable of degradation of the AHLs with acyl chains in a range of four to eight carbons (Huang et al., 2003; Park et al., 2005). Moreover, AiiD is unable to degrade penicillin G and ampicillin (Lin et al., 2003); the AhlM acylase degrades not only AHLs but also penicillin G, suggesting broader substrate specificity (Czajkowski & Jafra, 2009). The evaluation of molecular mechanisms of AHL acylases indicates a substrate specificity and further mutagenesis and crystal structure analysis will assist to unveil catalytic mechanism (wang et al., 2008).

Paraoxonases (PONs)

Mammalian cells such as human, rabbit, mouse, horse, sheep and bovine have shown a strong AHL inactivation capacity (Yang et al., 2005). The QQ function in eukaryotic lactonases-like enzymes, termed paraoxonases (PONs), differs from the former described bacterial enzymes (Draganov et al., 2005). It has been proven that PONs, including PON1, PON2 and PON3, participate in a variety of physiologically hydrolytic activities, such as organophosphate detoxification and drug metabolism (Draganov & La Du 2004; Ng et al., 2005). The PON enzymes exhibit the most activity with long-chain

AHLs, such as C12-oxo- HSL, but less efficient with short-chain AHL molecules (Chun et al., 2004; Yang et al., 2005).

These PON enzymes are similar to each other up to 60% in amino-acid sequence identity and contain the catalytically important conserved residues Glu53, His115, His134, Asn168, Asn224, Asp269 and Asn270, suggesting that these enzymes use the same catalytic mechanism (Dong et al., 2007). Nevertheless, these three enzymes differ in substrate specificities as well as catalytic efficiencies against AHLs (Draganov et al., 2005). The PON1 from human displayed a hydrolytic activity against C12-oxo-HSL of *P. aeruginosa*, indicating the role of these enzymes in host cell protection against bacterial infection (Czajkowski & Jafra, 2009).

AHL oxidoreductases

It was observed an AHL degradation activity in *R. erythropolis* strain W2 with the most efficacy on 3-oxo-substituted AHLs by Uroz et al. (2003). The further studies have demonstrated that *R. erythropolis* strain W2 is able to convert 3-oxo-AHLs to 3-hydroxy-derivatives through an oxidoreductase activity (Uroz et al., 2005). The oxidoreductase enzymes can affect not only 3-oxo-substituent AHLs but also derivative AHLs such as N-(3-oxo-6-phenylhexanoyl) homoserine lactone (with an aromatic acyl chain substituent) or 3-oxododecanamide (without the homoserine lactone ring). Furthermore, other two types of enzymatic activities have been observed in *R. erythropolis* W2, including AHL acylase and AHL lactonase, and it is considered a unique bacterial species which shows three different types of AHL degradation (Uroz et al., 2005; Park et al., 2006).

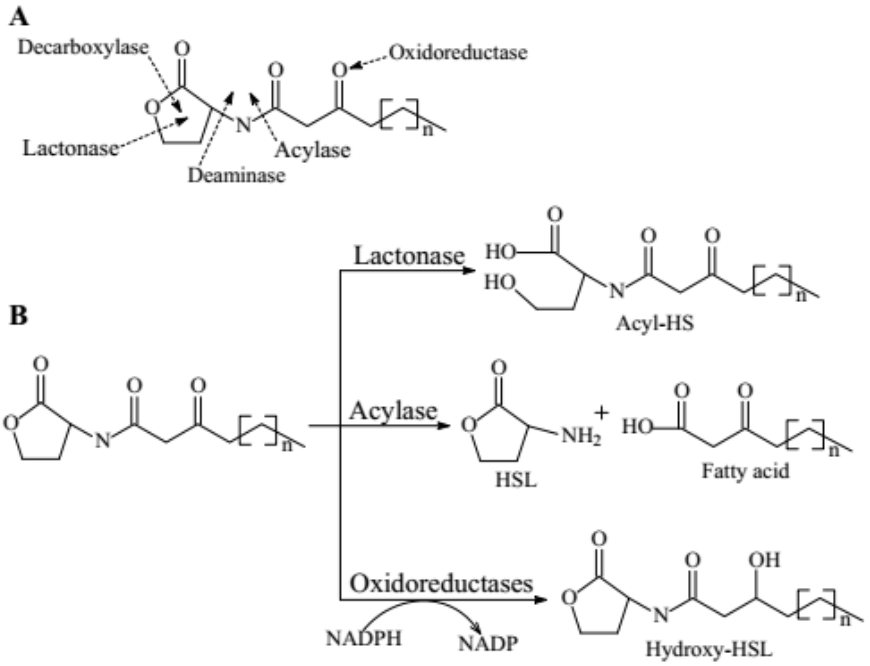


Figure 6. Possible linkage degraded by quorum quenching enzymes in quorum sensing molecule N-acyl homoserine lactone (**A**) and corresponding degradation mechanism of quorum quenching enzymes (**B**) In the figure the abbreviations represent: homoserine lactone (HSL), the amino radical or amide (NH_2), Nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) and Nicotinamide adenine dinucleotide phosphate (NADP) (adapted from Chen et al., 2013).

Sensing of host factors

The success of bacterial infection may be influenced by the metabolic condition of the host and some metabolic products. The environmental cues in the host are continuously monitored by bacteria during infection and consequently, the expression of virulence is coordinated at an appropriate timing (Mekalanos, 1992). Therefore, bacteria have developed several signal transduction systems to detect the presence of host cues such as mucus (Hsiao et al., 2006), catecholamine and lipid hormones (Hughes & Sperandio, 2008), bicarbonate (Abuaita & Withey, 2009) and bile (Gotoh et al., 2010). For instance, it has been reported that mucus and bile are able to increase production of various

virulence factors in *V. anguillarum*, such as flagellar motility, protease activity, exopolysaccharide production and biofilm formation (Li et al., 2014). Moreover, production of several virulence factors in *V. parahaemolyticus* such as proteins related to type III secretion system, hemolysins, and capsular polysaccharide is induced by bile (Hsieh et al., 2003). In the following, we will focus on the sensing of host stress hormones by bacteria.

Stress is considered the basis of innate response, a fundamental survival mechanism, which can support the host to challenge with a threat (Dhabhar, 2009). Several neurotransmitters, hormones, cytokines, peptides and other factors are activated and thus release into the circulation or locally in tissues by triggering the innate response (Verbrugghe et al., 2012). Among them, catecholamines and glucocorticoids are regarded as the major intermediates of the stress response. The catecholamines norepinephrine and epinephrine behave as fast-acting hormones which are released by the sympathetic nervous system; and glucocorticoids (cortisol and corticosterone) are slow-acting mediators secreted by the adrenal gland, upon activation of the hypothalamic–pituitary–adrenal (HPA) axis (Lundberg, 2005). In mammals, the nerve terminals, containing norepinephrine and dopamine, are found throughout the body including the intestine. It has been shown that more than 50% of the total amount of norepinephrine in the body is produced by the mesenteric organs (Lyte, 2004). The equivalent of all the organs and tissues involved in the stress response in mammals are anatomically found in fish. Particularly, interrenal tissue and chromaffin cells in fish are regarded as the equivalent of the adrenal gland in mammals (Ottaviani & Franceschi, 1996). For a long time, host stress was found as a determinant factor in the host-microbe interactions during an infection, exclusively involved in the increased susceptibility to infections or the suppression of the host immune system (Verbrugghe et al., 2012). However, further research has indicated that specific sensing systems have been evolved in infectious bacteria for recognizing the stress hormones released by the host and the growth and virulence of the pathogens can be directly affected by the sensing of these stress hormones (Lyte, 2004). Based on these findings, a new

research area termed microbial endocrinology has been developed, providing evidence of understanding the interaction between microbes and their host during stress time.

Catecholamine stress hormones

Catecholamines are derived from tyrosine and contain a catecholate moiety (a benzene ring with two hydroxyl groups) and an amine side chain. Catecholamines are effective compounds on different neuroendocrine signaling pathways and considered an important part of the acute stress response in multicellular organisms (Reiche et al., 2004). Epinephrine and norepinephrine are observed among both vertebrates, including fish, and invertebrates, including crustaceans and mollusks (Ottaviani & Franceschi, 1996). Most studies on sensing of host stress hormones by bacteria have been focused on the effects of the catecholamine stress hormones including epinephrine (adrenaline), norepinephrine (noradrenaline) and dopamine on bacteria such as *Escherichia coli* and *Salmonella* spp. (Freestone et al., 2008). Catecholamines have been demonstrated that are capable of induction of the growth in many Gram-negative and Gram-positive bacteria, including *Escherichia coli*, *Yersinia enterocolitica*, *Listeria monocytogenes*, *Aeromonas hydrophila*, *Pseudomonas aeruginosa*, *Vibrio parahaemolyticus*, *V. campbellii* and *V. anguillarum* in minimal medium containing serum (Lyte & Ernst, 1992; Coulanges et al., 1997; Kinney et al., 1999; Belay et al., 2003; Nakano et al., 2007a, Pande et al., 2014). Because of the presence of the high-affinity iron-binding proteins such as transferrin and lactoferrin in serum and subsequently chelation of free iron, such media are iron-limited. Sandrini et al. (2010) demonstrated that the catecholamines are able to remove iron from iron-binding proteins of the host through binding to transferrin and lactoferrin, resulting in a reduction of the Fe^{+3} to Fe^{+2}, which has a reduced binding affinity for the iron-binding proteins. Hence, catecholamines cause bacteria to receive the iron via iron uptake systems and subsequently, the enhanced availability of iron results in an increase in bacterial growth (Sandrini et al., 2010; Sharaff & Freestone, 2011). Bacteria possibly detect the increased amounts of stress hormones and respond with an enhanced growth (Freestone et al., 2008).

There are some evidence that catecholamines increase the production of virulence factors in bacteria, including biofilm formation, production of Shiga toxin, chemotaxis, attachment to epithelial cells in *E. coli*, motility and invasiveness in *Campylobacter jejuni*, motility and type III secretion by *Salmonella typhimurium*, and cytotoxic production by *V. parahaemolyticus* (Bansal et al., 2007; Cogan et al., 2007; Nakano et al., 2007a; Nakano et al., 2007b; Lyte et al., 2011; Sharaff & Freestone, 2011).

In animals, catecholamines exert their effects by binding with specific adrenergic and dopaminergic receptors. The eukaryotes possess adrenergic receptors (with two major families: α and β; several subtypes), which are used to bind to epinephrine and norepinephrine, while dopamine binds to dopaminergic receptors with at least 5 receptor subtypes. Antagonists specific to the catecholamine receptors have been reported to prevent catecholamine binding (Freestone et al., 2007). It has been indicated that these antagonists can also inhibit the effects induced by catecholamine in bacteria (Sharaff & Freestone, 2011). Clarke et al. (2006) revealed that epinephrine and norepinephrine are able to bind to the regulator sensor kinase QseC in *Escherichia coli* O157:H7, suggesting that this could be considered a prokaryotic adrenergic receptor for these hormones. In the presence of catecholamines, the QseC receptor localized in the inner membrane can autophosphorylate and delivers phosphate to the intracellular response regulator QseB. The function of QseBC has been well determined in several aquaculture pathogens, such as *Aeromonas hydrophila* (Khajanchi et al., 2011) and *Edwardsiella tarda* (Wang et al., 2011). Moreover, the reports of alternative receptors for catecholamines are increasing, as the notable examples QseE of the QseEF in enterohemorrhagic *E. coli* (Reading et al., 2007), BasS of the BasSR system in *Salmonella typhimurium* (Karavolos et al., 2008), and CpxA of the CpxAR system in *S. typhimurium* (Karavolos et al., 2011). Nevertheless, it is needed to be clarified whether norepinephrine or epinephrine directly binds to BasS and CpxA (Karavolos et al., 2013).

Glucocorticoid stress hormones

Glucocorticoids (cortisol, corticosterone) and mineralocorticoids are regarded as two constitutive subgroups of corticosteroids (Gwynne et al., 1982). Cortisol as the main stress hormone participates to the regulation of a wide range of physiological activities such as gluconeogenesis stimulation in the liver (Vijayan et al., 1997; Mommsen et al., 1999), lipolysis in the adipose tissues and proteolytic activities in the muscle (Mommsen et al., 1999), Control of osmoregulation (McCormick, 2001) and regulation of immune response, growth and behavior (Mommsen et al., 1999; Barton, 2002). An important role of cortisol in the stress response is the activation of catabolic processes to provide the energy demand required for appropriate stress response and recovery of homeostasis (Seyle, 1973). The synthesis and release of cortisol are coordinated through the Hypothalamus-Pituitary-Interrenal axis (HPI), in a similar way to function of the HPA axis in mammals (Mommsen et al., 1999). Cholesterol is known as the precursor for all glucocorticoids (Gwynne et al., 1982), however many enzymes and chemicals are responsible in biosynthesis and metabolism of this hormones. Pathways and enzymes involved in the synthesis of glucocorticoids are illustrated in **figure 7**.
Shortly, cholesterol is converted to progesterone and this product is also able to be hydroxylated to 17OH-progesterone (John et al., 1984). 21-Hydroxylation of either progesterone or 17-OHprogesterone results in the production of 11-deoxycorticosterone or 11-deoxycortisol, respectively (White et al., 1984). In the final step, the conversion of 11-deoxycorticosterone to corticosterone and 11-deoxycortisol to cortisol take place by the enzyme 11β-hydroxylase (Chua et al., 1987). Glucocorticoids are metabolized in the liver through many routes, but the major pathways include the interconversion of cortisol to cortisone through the activity of 11β- hydroxysteroid dehydrogenase (11β-HSD) or reduction of the C4-5 double bond, resulting respectively 5b-tetrahydrocortisol (THF) or 5a-THF (allo-THF). Additionally, tetrahydrocortisone directly results from the conversion of cortisone (Arlt & Stewart, 2005). Cortisone is convertible to 20β-hydroxycortisone as a final metabolite through an enzymatic reaction by 20β-HSD (Tokarz et al., 2012).

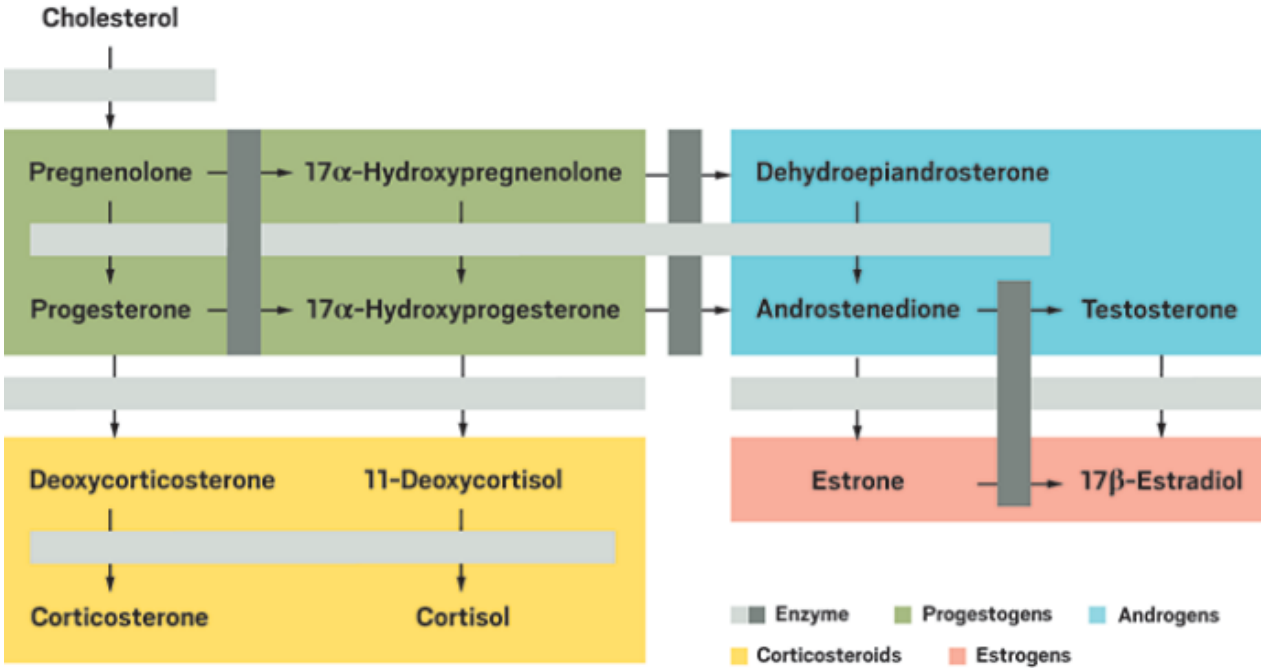


Figure 7. Pathways of steroidogenesis. Cholesterol is converted to progesterone and this product is also able to be hydroxylated to 17OH-progesterone. 21-Hydroxylation of either progesterone or 17-OHprogesterone results in the production of 11-deoxycorticosterone or 11-deoxycortisol, respectively. In the final step, the conversion of 11-deoxycorticosterone to corticosterone and 11-deoxycortisol to cortisol take place by the enzyme 11β-hydroxylase (adapted from Haggard et al., 2018).

As it is previously described, several compounds contribute to the synthesis and metabolism of glucocorticoids (Arlt et al., 2005). 17OH-progesterone and 11-deoxycortisol can be considered as the precursor for cortisol as active hormonal form. Alternatively, in case of active hormone corticosterone, 11-deoxycorticosterone acts as a precursor. Finally, tetrahydro metabolites such as tetrahydrocortisol and tetrahydrocortisone as well as 20β-hydroxycortisone, a final metabolite, are conjugated rapidly with glucuronic acid and eliminated via the exit routes. The hepato-biliary system is the major path to excrete cortisol, although there is some evidence indicating that renal and branchial routes are also involved (Vermeirssen & Scott, 1996; Mommsen et al., 1999).

As it was mentioned in catecholamines section, bacteria are able to detect the enhanced level of stress hormones and respond with an increased growth and virulence

factors (Freestone et al., 2008). Possibly, bacteria can show some response to the concentrations of glucocorticoids similar to how pathogens act to catecholamines. So far, few studies have focused on the effects of the glucocorticoids on the growth and virulence factors of bacteria. Based on these researches, cortisol is able to influence the growth of bacteria such as *Porphyromonas gingivalis*, *Fusobacterium nucleatum* and *Flavobacterium columnare* (Jentsch et al., 2013; Akcal et al., 2014; Declercq et al., 2016). However, to our knowledge, no study has been yet performed to evaluate the effects of stress hormones (catecholamines and glucocorticoids) on the growth and virulence factors of *Y. ruckeri*.

CHAPTER 3

Identification of N-Acyl Homoserine Lactone-Degrading Bacteria Isolated from Rainbow Trout (*Oncorhynchus mykiss*)

Adapted from:

Torabi Delshad, S., Soltanian, S., Sharifiyazdi, H., Haghkhah, M. & Bossier, P. (2018). Identification of N-Acyl Homoserine Lactone-Degrading Bacteria Isolated from Rainbow Trout (*Oncorhynchus mykiss*). *Journal of Applied Microbiology*, 125(2), 356-369.

Abstract

A variety of pathogens use quorum sensing (QS) to control the expression of their virulence factors. QS interference has hence been proposed as a promising anti-virulence strategy. The specific aim of this study was to isolate bacteria from trout tissue able to degrade N-Acyl Homoserine Lactones (AHL), a QS molecule family.

In total 132 isolates were screened for AHL degradation using *Chromobacterium violaceum* CV026 as a biosensor. Twenty four quorum quenching (QQ) isolates were identified biochemically and characterized using 16S rDNA sequencing. They belong to *Bacillus*, *Enterobacter*, *Citrobacter*, *Acinetobacter*, *Agrobacterium*, *Pseudomonas* and *Stentrophomonas* genera. Four *Bacillus* spp. showed the highest and fastest QQ activity. AHL degradation proved to be enzymatic in most isolates (except for *Stentrophomonas* spp. and *Pseudomonas* sp) as QQ activity could be destroyed by heat and/or proteinase K treatments. All QQ activity proved to be cell-bound except for *Pseudomonas* sp., where it could be detected in the supernatant. The results of *aiiA* gene homology analysis revealed the presence of *aiiA* gene encoding AHL lactonase in all examined isolates except *P. syringae* and *E. cloacae*. The HXHXDH motif conserved in all AHL lactonases and considered to be essential for AHL degradation was detected in all AiiAs after sequence alignment.

Some known and novel QQ bacteria were isolated from trouts and characterized in terms of enzymatic or non-enzymatic AHL degradation activity and their extracellular or intracellular location. In addition, an *aiiA* gene and its HXHXDH motif were detected in most isolates.

We could isolate and identify some novel QQ bacteria including *E. hormachea*, *A. radioresistens,* and *C. gillenii*. The *aiiA* gene was detected for the first time in these strains as well as in *S. maltophilia*. Our QQ isolates could be used for biocontrol of bacterial infections in aquaculture.

Keywords:

Aquaculture; Biocontrol; Degradation; Identification; Quorum sensing

Introduction

Bacteria are able to communicate and sense population density via a diverse set of signals. The process is generally called quorum sensing (QS) (Swift et al., 1993; Fuqua et al., 2001; Reading & Sperandio 2006). Through the QS system, they produce, release and sense small diffusible molecules (Kaplan & Greenberg, 1985). These signal molecules have distinct structural features and are applied by different microbial classes. A wide range of Gram-negative bacteria produces N-Acyl Homoserine Lactone (AHL) as signal molecules in cell to cell communication. A generic AHL molecule contains an acyl chain with variable numbers of carbon and a consistent homoserine lactone ring (Czajkowski & Jafra, 2009). It is assumed that at a critical threshold density, a cascade of biological events will be initiated inside the bacterial cells, resulting in regulated expression of specific genes such as virulence factors (Fuqua et al., 1994; Eberl 1999). Indeed, some physiological behaviors such as biofilm formation, swarming motility, bioluminescence, and secretion of virulence factors, but not growth, are regulated by QS mechanism (Miller & Bassler 2001; Chen et al., 2013).

Since antibiotic resistance has become a major concern in human and animal health care, quorum sensing interference (QSi) has been suggested to be an alternative strategy, with quorum quenching (QQ) being at the basis of an anti-virulence therapy (Chen et al., 2013). In contrast to antibiotic therapy, in QSi, pathogenic bacteria are not killed nor is cell growth inhibited; rather virulence factor production can be suppressed. QSi can be accomplished through three mechanisms: 1) interference in synthase of signal molecules, 2) signal molecule degradation and 3) blockage of receptors of signal molecules (Geske et al*.,* 2008; Galloway et al*.,* 2011).

Many bacteria are able to produce different enzymes to degrade AHL molecules. These enzymes are divided into four main groups: AHL acylase, AHL lactonase, oxidoreductase and paraoxonase (Chen et al., 2013). AHL acylase can irreversibly hydrolyze the amide bonds between the homoserine lactone ring and the acyl chain (Leadbetter et al., 2000; Lin et al*.,* 2003). AHL lactonase enzymes cleave the homoserine lactone ring deactivating the QS molecules (Dong et al., 2000; Dong et al.,

2001). The hydrolysis of lactone ring also occurs at a pH above 7 and can be reversed at acidic pH (Yates et al., 2002). In a few numbers of bacterial species, oxidoreductase enzymes oxidize or reduce the acyl side chain without degrading the AHL structure (Chowdhary et al., 2007; Bijtenhoorn et al., 2011). The last QQ enzyme class, paraoxonase (PONs), exists in mammalian sera and possesses a function similar to AHL lactonase (Yang et al., 2005).

In previous years, many bacteria with AHL degrading ability, isolated from a wide range of aquatic animals and environments, have been identified (Tinh et al., 2007; Chu et al., 2010; Christiaen et al., 2011; Noorashikin et al., 2016). In the present study, QQ bacteria from rainbow trout were isolated and characterized with the aim to use them in the biocontrol of bacterial infections in rainbow trout aquaculture.

MATERIALS AND METHODS

Bacterial strains, media and culture conditions

Chromobacterium violaceum strain CV026, a mini-Tn5 mutant derived from the *C. violaceum* strain ATCC31532 (McClean et al., 1997; Chu et al., 2010) was used to detect QS signal molecules. This reporter is able to synthesize the purple pigment violacein in the presence of exogenous acyl homoserine lactone (AHL) molecules with acyl side chain of four to eight carbons. P3/pME6863 and P3/pME6000 strains were used as positive and negative controls in AHL degradation assays, respectively (Reimmann et al., 2002; Maurhofer et al., 1998). P3/pME6863 is a recombinant *Pseudomonas fluorescence* carrying the *aiiA* gene from *Bacillus* sp. A24 encoding a lactonase enzyme (Molina et al., 2003). *Yersinia ruckeri* strain CCUG14190, a pathogenic bacterium in aquaculture was provided by the Laboratory of Aquaculture and Artemia Reference Center (ARC), Belgium. Standard HHL, N-hexanoyl L-homoserine lactone [C6-HSL], used in this study, was purchased from Sigma (Sigma-Aldrich, Germany). Tryptone Soy (TS) agar or broth was used as a medium to grow all strains at 28 °C. The CV026 strain was grown in TS medium supplemented with

kanamycin (20 mg l^{-1}) to maintain the plasmid harboring the gene responsible for violacein production. 3-[N-morpholino] propane sulfonic acid (MOPS) was added to all media used in AHL assays to buffer the medium at pH 6.8 preventing chemical degradation (Yates et al*.,* 2002).

Sample collection and preparation

A total of 35 healthy rainbow trouts (*Oncorhynchus mykiss*) (76 ± 10 g) were collected from five aquaculture farms in Fars province, Iran. Upon transfer to the Aquatic Animals Health and Diseases Department, Shiraz University, ten fish were randomly inspected by bacteriological, parasites and fungal examinations to ensure they were healthy. All fish were euthanized using a high dose of tricaine methanesulfonate (200 mg L^{-1}) (MS222; Sigma Aldrich, Germany). Mucosal samples taken by swabbing gills and skin were directly inoculated onto tryptone soy agar (TSA) plates. Subsequently, the ventral surface of the fish was disinfected with alcohol (70%) and then their intestines were taken out aseptically and rinsed with physiological saline (0.9% NaCl solution, pH 7.4). One gram of the intestine was homogenized with sterilized 0.9% NaCl solution (10:1, v/ w). Upon serial dilution up to 10^{-6}, 0.1 ml of the dilutions 10^{-4} - 10^{-6} were spread onto TSA plates in triplicates. The plates were incubated for 48 h at 28 °C. Subsequently, colonies with different morphologies were separated, sub-cultured on TSA plates, grown in TSB, supplemented with sterile glycerol (30%, v/v) and stored at -80 °C.

Screening for QQ isolates

In order to verify for a QQ ability in the isolated strains, *Y. ruckeri* as a natural AHL producer and CV026 strain were separately cultured on TSA plates and streaked as two parallel lines on TSA plate. Each isolate was then placed as a short streak between CV026 and *Y. ruckeri*, maintaining a 6-7 mm distance between them. The plates were checked for QS inhibition after 48 h incubation at 28 °C. P3/pME6863 and P3/pME6000 strains were used as positive and negative controls, respectively. The isolates capable

to inhibit purple pigment production were selected and stored in 30% sterile glycerol (v/v) and kept at -80 °C for further examinations.

AHL degradation assays

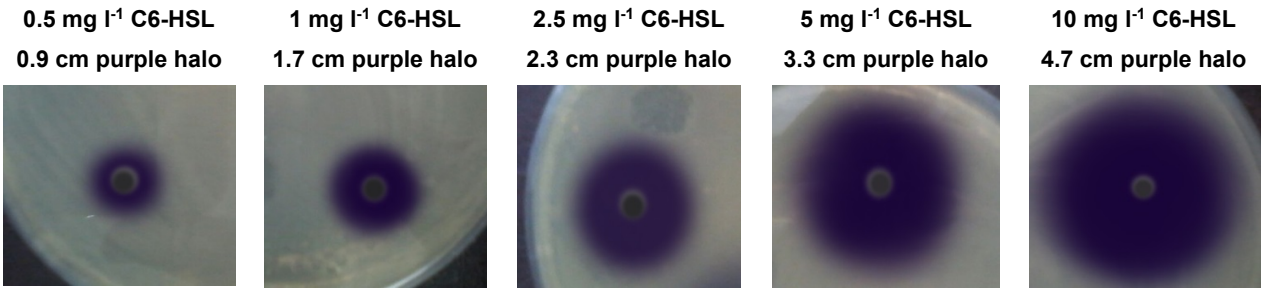
A dose-response curve between the hexanoyl L-homoserine lactone [C6-HSL] and the violacein diameter

One mg of C6-HSL was dissolved in 1 ml of dimethyl sulfoxide (DMSO) to prepare a stock solution of C6-HSL. CV026 was grown in TSB containing 20 mg l^{-1} kanamycin until 0.1 OD at 600 nm. CV026 was then spread on a buffered (pH 6.5) TSA plate. Ten µl C6-HSL solution was dropped into a well in the plate agar. Plates were put in an incubator at 28°C for 24 h. This assay was performed with five different concentrations of C6-HSL including 10, 5, 2.5, 1, and 0.5 mg l^{-1}. The diameter of purple-pigmented halos and the C6-HSL concentration displayed a linear regression line. A standard curve was obtained with the following equation: diameter of purple-pigmented halo = 0.386 [C6-HSL] + 1.0734 and regression coefficient $R^2 = 0.953$ (**Figures 8a, 8b**).

Kinetic of AHL degradation by agar well diffusion assay

Each QQ isolate was grown in a 10 ml buffered TSB (pH 6.5) supplemented with 5 mg l^{-1} C6-HSL. P3/pME6863 and P3/pME6000 strains were cultured under the same conditions as positive and negative controls, respectively. The mixture solutions were incubated at 28 °C with constant agitation (120 × *g*). One ml of each culture medium was taken and filtered (0.22-µm) at 12 h and 24 h. Ten µl of the filtrate was spotted into a well in a TSA plate spread with 100 µl bacterial suspension of CV026. After 24 h incubation, the diameter of purple halos was measured and converted into the residual concentration of C6-HSL in the filtrates.

a)



b)

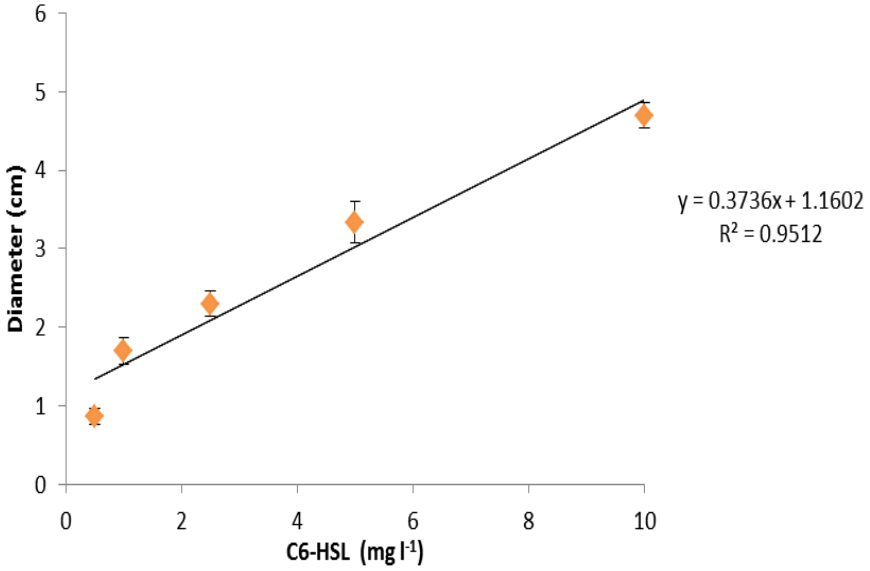


Figure 8. a) Production of the pigment by *Chromobacterium violaceum* CV026 in the presence of different concentrations of *N*-hexanoyl homoserine lactone (C6-HSL); **b)** Standard curve resulted from the relationship between concentrations of C6-HSL and diameter of zones induced by *C. violaceum* CV026. The error bars indicate the standard deviation of 3 replicates.

Quorum quenching characterization

The positive isolates for AHL degradation were examined for enzyme- or non-enzyme-bound AHL degradation by heat and proteinase K treatments. One hundred µl of a

proteinase K solution (500 µg ml^{-1}) (Sigma Aldrich, Germany) was supplemented to 850 µl of TSB overnight isolate cultures, followed by incubation at 28 °C for 24 h. 228 µl (1 mM) of Pefabloc SC plus (AEBSF) [4-(2-Aminoethyl)-benzenesulfonylfluoride hydrochloride] solution (1 mg ml^{-1}) (Sigma Aldrich, Germany) was added into the reaction mixture to terminate proteinase K activity for 1 hour at room temperature. Alternatively, the isolates were subjected to autoclaving at 121 °C, 20 min. The proteinase K and heat treated samples were mixed with C6-HSL and QQ activity was evaluated by the diffusion assay as described above.

Determination of extracellular/intracellular AHL degradation in QQ isolates

AHL degrading isolates cultured in TSB were centrifuged at 6000 × *g* for 15 min and cell-free supernatants, as an extracellular fraction, were obtained by filter sterilization (0.2 µm). C6-HSL was added to reach 5 mg l^{-1} and incubated at 28 °C for 24 h. Ten µl of mixture reactions was spotted into a well in a TSA plate seeded with 100 µl overnight CV026 culture. The plates were incubated for 24 h to allow the purple pigmented halo to develop.

Identification of QQ isolates

Biochemical characterization

Morphological properties such as shape, size, and pigmentation of QQ colonies were investigated on TSA plates after purification. Furthermore, Gram staining was done to verify Gram reaction, size, shape and type arrangement of cells, microscopically. AHL degrading bacteria were biochemically characterized using the following tests: oxidase and catalase activity, motility test, citrate utilization test, gas production from glucose, indole production, nitrate reduction, gelatin hydrolysis, oxidative fermentation, production of hydrogen sulfide, urease and hemolytic activity, aesculin hydrolysis,

voges–proskaüer test (acetoin production), l-arabinose fermentation, glucose and mannitol fermentation.

PCR assay and 16S rDNA sequencing

Total DNA was extracted from AHL degrading bacteria by the boiling method. Amplification of partial 16S rDNA gene of bacteria was performed using FUP-5'-ACGGCTACCTTGTTACGACTT-3' and RUP-5'-AGAGTTTGATCCTGGCTCAG-3' as a universal set of forward and reverse primers, thermocycler apparatus (MJ mini, BioRad, USA). The PCR reaction was set up with an initial denaturation at 95 °C for 5 min, 37 cycles at 94 °C for 1 min, annealing at 59 °C for 1 min, extension at 72 °C for 1 min and an eventual extension at 72 °C for 5 min. PCR products were analyzed by 1.2 % agarose gel containing Red Safe (Intron Biotechnology, Korea) staining through electrophoresis. All PCR products were purified and sequenced bi-directly using a capillary DNA analyzer (ABI3730; Applied Biosystems, Foster City, CA, USA). Continuous sequences were assembled with the BioEdit software version 7.0.5 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Results of DNA sequencing were compared to the GenBank database using Basic Local Alignment Search Tool Nucleotide *(*BLAST-N) server at NCBI (National Center for Biotechnology Information databases) (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to find the closest sequences and establish a tentative identification (acknowledging that 16s rDNA information might not always be sufficient to allow unambiguous identification at the species level). Sequences of the identified isolates were submitted to NCBI with an accession code.

PCR amplification and homology analysis of *aiiA* gene

The presence of the AHL-lactonase gene (*aiiA*) in AHL degrading bacteria was evaluated by amplification of the *aiiA* gene as previously described by Dong et al. (2002) and Nusrat et al. (2011) with some modifications. The *aiiA* gene was amplified using forward and reverse primers, *aiiA* F-5'-ATGACAGTAAAGAAGCTTTA-3' and *aiiA*

R1-5'-TATATATTCAGGGAACACTT-3', respectively. The PCR conditions were as follows: initial denaturation (at 95 °C for 6 min), 40 cycles for denaturation (at 94 °C for 1 min), annealing (at 58 °C for 45 sec), extension (at 72 °C for 50 sec) and a final extension (at 72 °C for 15 min). The amplified samples were resolved by 1.2% agarose gel electrophoresis. Appropriate PCR products for several samples (n=10) were directly sequenced as described above. Sequencing data were processed with the BioEdit software and then the BLAST program was used for comparison with the information in GenBank. Phylogenetic analyses were conducted using the obtained AHL lactonases sequences together with known AHL lactonases on NCBI using maximum likelihood (ML) method by MEGA 6. To assess the robustness of the branches, bootstrap test with 1,000 pseudoreplicates was performed, following the rule of branch consistency (Tamura et al., 2013). In the end, the nucleotide sequences of the *aiiA* genes were registered in the GenBank database. In addition, genetic similarity and multiple amino acids alignment were performed using the CLC Main Workbench 5 software for different AHL lactonase.

Data analysis

In the present study, SPSS 16.0 software (SPSS Inc, Chicago, USA) was applied for statistical analysis. Independent samples t-test was used for the comparison of test samples in determination of extracellular/intracellular mechanism assay and quorum quenching characterization, and one way ANOVA was chosen for Kinetic of AHL degradation test with a *p*-value of 0.05.

RESULTS

Isolation and screening of bacteria for QQ activity

In total 132 isolates were obtained from skin, gill, and intestine of rainbow trout. They were screened for AHL degradation (QQ) activity. QQ activity was verified by a plate

agar assay using the CV026 strain as a biosensor for AHLs and *Y. ruckeri* as a natural AHL producer. The negative control, P3/pME6000, was completely purple (**Figure 9A**). The presence of a non-purple zone in the CV026 strip adjacent to the QQ isolate was used as an initial criterium for QQ ability **(Figure 9B)**. In total twenty four QQ isolates were selected for further examination.

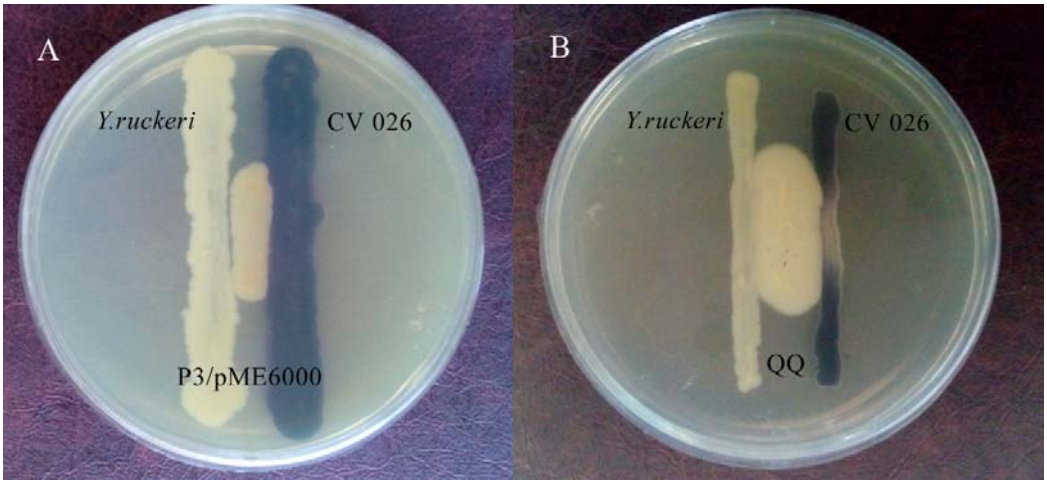


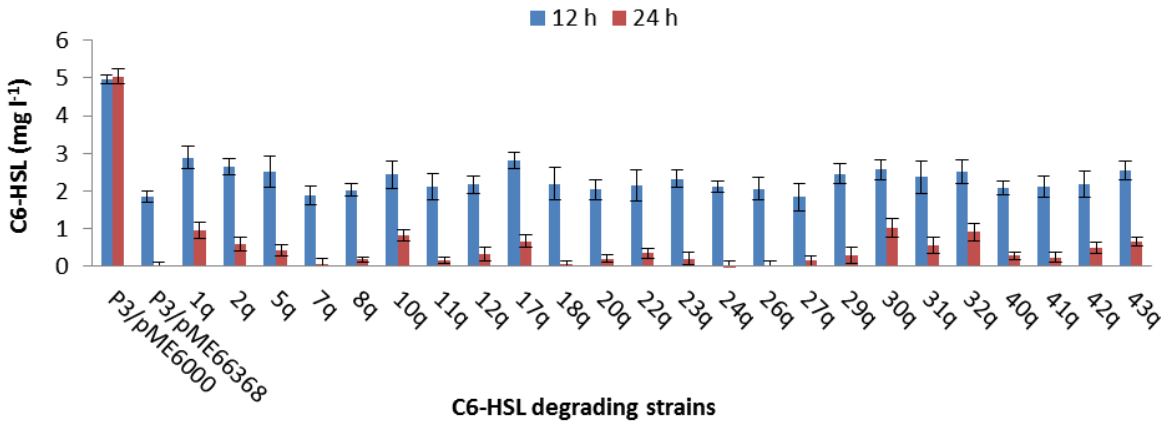
Figure 9. (**A**) Quorum quenching (QQ) activity assay on TSA plate by *Pseudomonas* sp. P3/pME6000 an AHL-degrading negative strain; (**B**) QQ activity by one of the QQ isolates in the present study. *Yersinia ruckeri* and CV026 were used as a natural AHL producer and AHL biosensor strains, respectively. The part adjacent to QQ isolate (middle streak) of CV026 shows no pigment, indicating AHL degradation. P3/pME6000, as a negative control, was not able to inhibit QS.

Determination of the AHL degradation kinetics

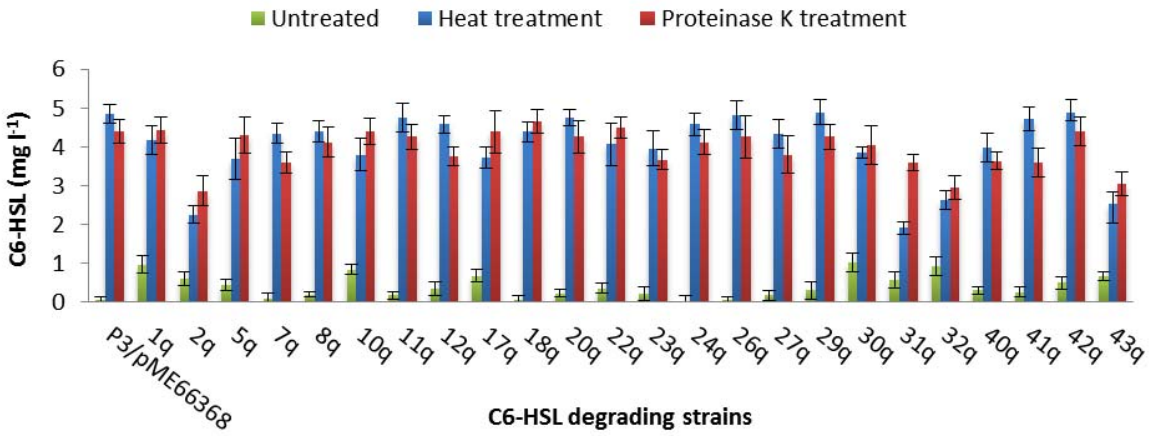
Kinetic of AHL degradation by each QQ isolate was investigated by adding a C6-HSL solution to the isolate cultures. Remaining AHL concentration was verified in samples taken after 12 h and 24 h incubation. All selected isolates degraded C6-HSL in comparison with negative control and almost all QQ bacteria were able to lower the C6-HSL concentration below 1 mg l^{-1} after 24 h incubation. Four QQ isolates 7q, 18q, 26q (*Bacillus thuringiensis*) and 24q (*B. cereus*) along with the positive control completely degraded AHL during 24 h incubation. A comparison of QQ activity in different genera

revealed that *Bacillus* strains degraded C6-HSL faster ($p < 0.05$) than non-*Bacillus* strains during the first 24 h such as for strains belonging to *Stenotrophomonas*, *Enterobacter*, *Citrobacter* and *Pseudomonas* genera. No chemical degradation was observed in the negative control (P3/pME6000 treatment, buffered at 6.5). The results are shown in **Figure 10a**.

a)



b)



c)

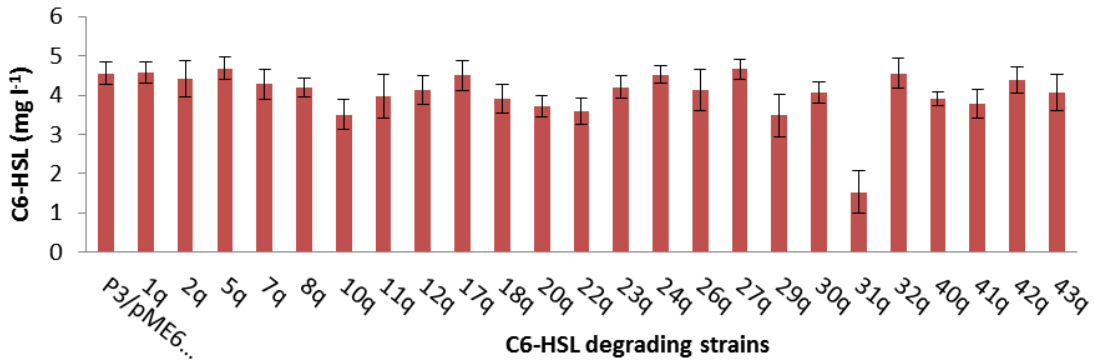


Figure 10. a) Kinetic of the degradation of *N*-hexanoyl homoserine lactone (C6-HSL) by the QQ isolates in reaction mixtures containing 5 mg l^{-1} of C6-HSL at 12 h and 24 h incubation (averages ± S.D.); **b)** AHL degradation in heat, proteinase K and untreated cell cultures (averages ± S.D.). All isolates lost degradation ability after both treatments. Exceptions include *Stenotrophomonas maltophilia* strains (exposure to heat and proteinase K) and *Pseudomonas syringae* (exposure to heat); **c)** Residual C6-HSL level after treatment with the extracellular matrix of QQ isolates (average ± S.D.). The supernatant of isolate 31q, *Pseudomonas syringae,* contained extracellular C6-HSL degradation activity. All other isolates did not display extracellular C6-HSL degradation activity. *Pseudomonas* sp. P3/pME6000 and P3/pME6863 were applied as negative and positive controls, respectively. The error bars indicate the standard deviation of 3 replicates (Independent samples t-test, ANOVA, $p< 0.05$).

Determination of mechanism of action in QQ isolates

To verify whether AHL degradation in the isolates was enzymatic, heat and proteinase K treatments were applied. Both treatments significantly reduced AHL degradation activity in most QQ isolates compared with the untreated controls ($p < 0.05$; **Figure 10b**). However, isolates 2q, 32q, 43q belonging to *Stenotrophomonas maltophilia*, and isolate 31q, *Pseudomonas syringae,* still displayed some AHL degradation activity after heat treatment, indicating that they might produce heat stabile enzymes. Besides, *S. maltophilia* strains retained their QQ ability although they were partly sensitive to proteinase K.

Determination of extracellular/intracellular QQ activity

The supernatants of isolates were used to evaluate whether AHL degradation activity was intracellularly or extracellularly localized. None of the supernatants of QQ isolates showed AHL degradation activity (**Figure 10c**), indicating intracellular QQ activity except for isolate 31q identified as *Pseudomonas syrignae.*

Identification of QQ isolates

Biochemical characterization

The isolates were subjected to some biochemical tests and the results are tabulated in **Table S1**.

16S rDNA gene sequencing

The 16S rDNA gene in QQ positive strains was amplified and sequenced. BLAST analysis of 16S rDNA sequences demonstrated AHL degrading isolates most closely belonged to *Bacillus* (13 isolates), *Stentrophomonas* (3 isolates), *Enterobacter* (4 isolates), *Acinetobacter*, *Citrobacter*, *Agrobacterium* and *Pseudomonas* (each one 1 isolate) genera**.** AHL degradation activity *in E. hormachea* (similarity 100%), *A. radioresistens* (similarity 98%) and *C. gillenii* (similarity 100%) have not been described before. Identified 16S rDNA sequences were submitted to the GenBank database with an accession number **(Table 4).**

Analysis of the *aiiA* homologous genes

The observations in the agar well diffusion assay and inactivation of degradation caused by heat and proteinase K treatments suggest the presence of QQ genes in the isolates. QQ bacteria were investigated for *aiiA* homologous genes coding for AHL lactonase

enzyme by PCR amplification using specific primers for *aiiA* gene. An amplicon of 750 bp was obtained in *B. thuringiensis*, *B. cereus*, *Bacillus* sp., *S. maltophilia*, *E. hormachea*, *A. radioresistens*, *Ag. tumefaciens* and *C. gillenii* as can be expected for an AHL lactonase gene **(Figure 11)**. Using the same primer set, an *aiiA* homologous gene seems to be absent in *P. syringae* and *E*. *cloacae* (data not shown).

DNA sequencing and alignment of lactonase gene

Molecular analysis of *aiiA* sequences in the QQ isolates demonstrated that at least two different AHL lactonase genotypes (genotypes A and B) are present among the isolates. Comparisons of the *aiiA* sequences with other known AHL lactonase sequences available in the GenBank database (*aiiA*, *attM*, *ahlD* and *ahlK*) revealed high similarity (up to 99%). Although the sequences of the genotypes A and B are not completely similar to the previously described sequences in the GenBank, both of them phylogenetically belong to the known *aiiA* type lactonases major group (**Figure 12**). The degree of similarity among the isolated *aiiA* genes and the accession numbers are listed in **Table 5**.

In accordance with the phylogenetic results, the comparison of deduced amino acids sequence showed a high degree of similarity (up to 99%) to the protein data available in the GenBank database for AiiA group. Also, multiple alignments of AHL-lactonase amino acids sequence indicated the presence of the motif “HXHXDH” and zinc-binding sites in both *aiiA* genotypes. The conserved Histidine (H) at the positions 104, 106 and 169 in metal site 1 and Aspirate acid (D) 108, His 109 and His 235 in metal site 2 were present. Moreover, Tyrosine (Y) residue was found at position 194 in all sequences (**Figure 13**).

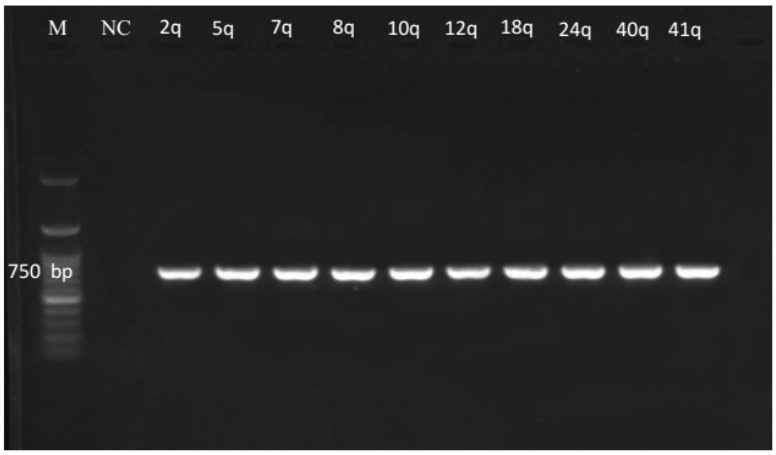


Figure 11. PCR amplification of *aiiA* gene coding lactonase enzyme. Bands with 750 bp on electrophoresis gel agar showing the existence of *aiiA* gene in QQ isolates. The first and second lanes indicate 100kb DNA ladder (M) and negative control (NC), respectively. The numbers in the top holes show the AHL degrading strains: 2q: *S. maltophilia*; 5q: *E. hormaechei*; 7q: *B. thuringiensis*; 8q: *Bacillus* sp.; 10q: *C. gillenii*; 12q: *B. cereus*; 18q: *B. thuringiensis*; 24q: *B. cereus*; 40q: *A. radioresistens* and 41q: *Ag. tumefaciens.*

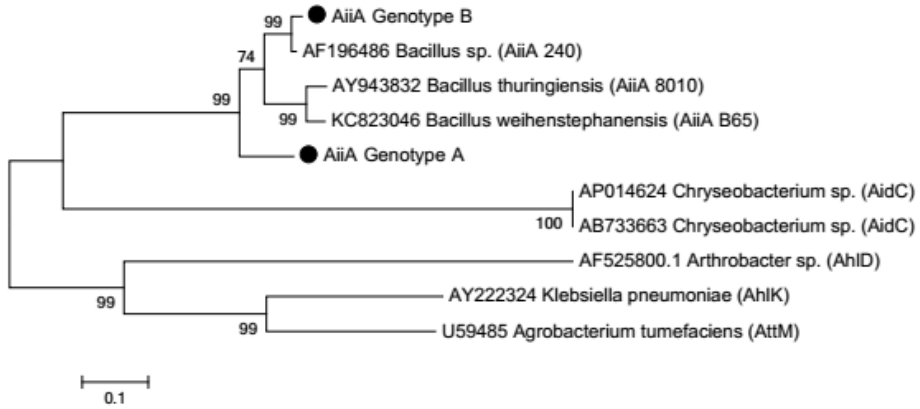


Figure 11. Phylogenetic tree resulting from likelihood presenting the relationship among *aiiA* genotype A and *aiiA* genotype B enzymes and known AHL degrading enzymes: *aiiA* 240 (*Bacillus* sp., accession number: AF196486), *aiiA* 8010 (*B. thuringiensis*, accession number: AY943832); *aiiA* B65 (*B. weihenstephanensis*, accession number: KC823046); *aidC* (*Chrysemobacterium* sp., accession number: AP014624); *aidC* (*Chrysemobacterium* sp., accession number: AB73663); *ahlD* (*Arthrobacter* sp., accession number: AF525800); *ahlk* (*Klebsiella pneumoniea*, accession number: AY222324); *attM* (*Agrobacterium tumefaceins*, accession number: U59485).

Table 4 Identified AHL degrading isolates following BLAST-N search in GenBank based on 16S rDNA sequences

Isolate code	Source	Location	Closest species	Similarity (%)	Accession no.
1q	Skin of healthy rainbow trout	Fars, Iran	*Enterobacter hormaechei*	100%	MF687147
2q	Gill of healthy rainbow trout	Fars, Iran	*Stenotrophomonas maltophilia*	99%	MF687148
5q	Skin of healthy rainbow trout	Fars, Iran	*Enterobacter hormaechei* subsp. *hormaechei*	100%	MF687149
7q	Gill of healthy rainbow trout	Fars, Iran	*Bacillus thuringiensis*	100%	MF687150
8q	Intestine of healthy rainbow trout	Fars, Iran	*Bacillus sp.*	100%	MF687151
10q	Intestine of healthy rainbow trout	Fars, Iran	*Citrobacter gillenii*	100%	MF687152
11q	Intestine of healthy rainbow trout	Fars, Iran	*Bacillus thuringiensis*	100%	MF687153
12q	Intestine of healthy rainbow trout	Fars, Iran	*Bacillus cereus*	99%	MF687154
17q	Skin of healthy rainbow trout	Fars, Iran	*Enterobacter cloacae*	99%	MF687155
18q	Skin of healthy rainbow trout	Fars, Iran	*Bacillus thuringiensis*	99%	MF687156
20q	Intestine of healthy rainbow trout	Fars, Iran	*Bacillus cereus*	99%	MF687157
22q	Gill of healthy rainbow tout	Fars, Iran	*Bacillus thuringiensis*	100%	MF687158
23q	Intestine of healthy rainbow trout	Fars, Iran	*Bacillus thuringiensis*	100%	MF687159
24q	Intestine of healthy rainbow trout	Fars, Iran	*Bacillus cereus*	99%	MF687160
26q	Intestine of healthy rainbow trout	Fars, Iran	*Bacillus thuringiensis*	100%	MF687161
27q	Intestine of healthy rainbow trout	Fars, Iran	*Bacillus cereus*	100%	MF687162
29q	Intestine of healthy rainbow trout	Fars, Iran	*Bacillus cereus*	100%	MF687163
30q	Intestine of healthy rainbow trout	Fars, Iran	*Enterobacter sp.*	99%	MF687164
31q	Gill of healthy rainbow trout	Fars, Iran	*Pseudomonas syringae pv. tomato*	100%	MF687165
32q	Intestine of healthy rainbow trout	Fars, Iran	*Stenotrophomonas maltophilia*	99%	MF687166
40q	Gill of healthy rainbow trout	Fars, Iran	*Acinetobacter radioresistens*	98%	MF687167
41q	Gill of healthy rainbow trout	Fars, Iran	*Agrobacterium tumefaciens*	99%	MF687168
42q	Intestine of healthy rainbow trout	Fars, Iran	*Bacillus thuringiensis* strain	100%	MF687169
43q	Skin of healthy rainbow trout	Fars, Iran	*Stenotrophomonas maltophilia*	100%	MF687170

Table 5 Sequences homology of *aiiA* gene from QQ isolates using BLAST-N analysis.

Isolate Code	Closest Species Based on 16S rDNA	References Strains (Database GenBank)	Similarity (%)	Accession no
2q	*Stenotrophomonas maltophilia*	*Bacillus* sp. 240B1 putative metallohydrolase (*aiiA*) gene (Genotype B)	98%	MF687171
5q	*Enterobacter hormaechei*	*Bacillus mycoides* strain ATCC 6462 plasmid pBMX_1 (Genotype A)	99%	MF687172
7q	*Bacillus thuringiensis*	*Bacillus mycoides* strain ATCC 6462 plasmid pBMX_1 (Genotype A)	99%	MF687173
8q	*Bacillus sp*	*Bacillus* sp. 240B1 putative metallohydrolase (*aiiA*) gene (Genotype B)	98%	MF687174
10q	*Citrobacter gillenii*	*Bacillus* sp. 240B1 putative metallohydrolase (*aiiA*) gene (Genotype B)	98%	MF687175
12q	*Bacillus cereus*	*Bacillus* sp. 240B1 putative metallohydrolase (*aiiA*) gene (Genotype B)	98%	MF687176
18q	*Bacillus thuringiensis*	*Bacillus* sp. 240B1 putative metallohydrolase (*aiiA*) gene (Genotype B)	98%	MF687177
24q	*Bacillus cereus*	*Bacillus mycoides* strain ATCC 6462 plasmid pBMX_1 (Genotype A)	99%	MF687178
40q	*Acinetobacter radioresistens*	*Bacillus mycoides* strain ATCC 6462 plasmid pBMX_1 (Genotype A)	99%	MF687179
41q	*Agrobacterium tumefaciens*	*Bacillus mycoides* strain ATCC 6462 plasmid pBMX_1 (Genotype A)	99%	MF687180

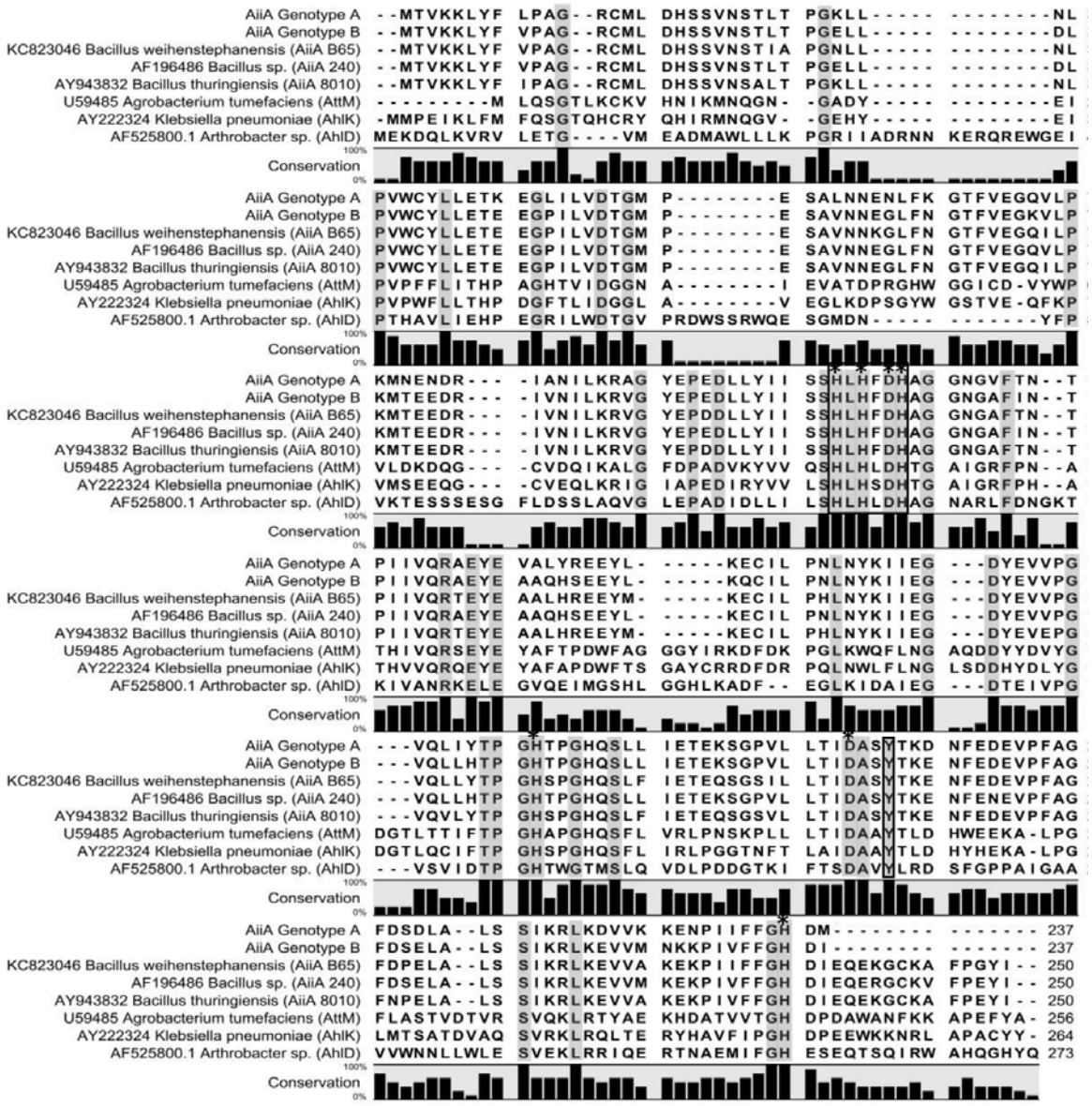


Figure 13. Alignment of amino acid sequences of AHL lactonases of A and B genotypes with amino acid sequences of known sequences: AHL lactonase of AiiA 240 (*Bacillus* sp., accession number: AF196486), AiiA 8010 (*B. thuringiensis*, accession number: AY943832); AiiA B65 (*B. weihenstephanensis*, accession number: KC823046); AidC (*Chrysemobacterium* sp., accession number: AP014624); AidC (*Chrysemobacterium* sp., accession number: AB73663); AhlD (*Arthrobacter* sp., accession number: AF525800); Ahlk (*Klebsiella pneumoniea*, accession number: AY222324); AttM (*Agrobacterium tumefaceins*, accession number: U59485). The motif of HXHXDH and Tyrosine (194) were put into boxes; Conserved sequences were marked by gray shadow; Metal ligands for the dinuclear zinc form proposed by Thomas et al. (2005) were represented with an asterisk.

DISCUSSION

Various bacteria utilize QS systems to estimate their cell density and regulate genes (e.g. virulence factors) in response to that. There are also many bacteria which are able to degrade signal molecules, such as acyl homoserine lactones (AHL). In this study, AHL degrading bacteria were isolated from various trout tissues (gills, skin, and intestines). Many of the isolated species have already been described for having AHL degrading capacity, while for some species this capacity is novel (Carlier et al., 2003; Shepherd & Lindow 2009; Ma et al., 2013; Chu et al., 2014; Noorashikin et al., 2016; Lopez et al., 2017).

There are few publications reporting AHL degradation by *Stenotrophomonas maltophilia* (here the putative identification of strains 2q, 32q and 43q). Singh et al. (2013) reported for the first time the QQ capacity in *S. maltophilia* isolated from the roots of a grass species. We identified three different putative species of *Enterobacter* with QQ activity including *E. hormachea*, *E. cloacae,* and *Enterobacter* sp. Although the QQ ability in *E. hormachea* is reported here for the first time, some research previously reported AHL degradation in other species of *Enterobacter* (Rajesh & Rai 2014a; Noorashikin et al., 2015; Gopu et al., 2016). AHL degradation activity has already been observed in *Acinetobacter* spp. (Kang et al., 2004; Kim et al., 2014; Lopez et al., 2017) and our current finding revealed that *A. radioresistens* shows AHL degradation capacity. However, we could not find any publication referring QQ activity in this species. There is no published evidence for AHL degradation by *Citrobacter,* while the *C. gillenii* strain isolated here, could degrade AHL. Finally, the capacity of *Bacillus* species to degrade AHL has been described by Dong et al. (2002), Momb et al. (2008), Chu et al. (2010), Defoirdt et al. (2011b), an observation that was also made in this study. AHL degrading *Bacillus* species might have the technological advantage of having the capacity to form spores, which make them more suitable for inclusion in for instance aquaculture feed. The *Bacillus* strains isolated here tended to degrade AHL faster than some non-*Bacillus* strains, which make them additionally attractive in QQ applications.

Except for *P. syringae* (as in other reports on the *Pseudomonas* genus; Christiaen et al., 2011; Cheong et al., 2013), no AHL degrading capacity was found in the supernatants. Additionally, heat treatment resulted in the loss of QQ ability in most isolates (*S. maltophilia* and *P. syringae* displayed less sensitivity to heat treatment than other isolates; Singh et al. (2013) and Christiaen et al. (2011) observed the same phenomenon in *S. maltophilia* and *Pseudomonas* strains). Taken together this illustrates that in most isolates AHL degradation is done by cell-bound enzymes as it is the case for AHL lactonases (Czajkowski & Jafra 2009; Christiaen et al., 2011; Rajesh & Rai 2014b). Heat-resistant compound(s) have also been described by Mandrich et al. (2010) in thermophilic archaea. The extracellular QQ activity of *P. syringae* might have some technological advantages in a QQ strategy. For instance, fast degrading *Bacillus* strains (producing cell bound lactonases) could be combined with *P. syringae* strains producing extracellular AHL degrading enzymes.

Additionally, treatment with proteinase K led to the loss of AHL degrading ability in the QQ isolates, suggesting degradation of AHLs by lactonase or acylase enzymes. The limited effect of proteinase K on *S. maltophilia* AHL degrading activity might be caused by different phenomena. E.g. proteinase K might be degraded or proteinase K might not enter the cells, or the AHL degrading enzyme is resistant to proteinase K activity. Further investigations are required to understand the exact mechanisms of QQ in this strain.

In the lactonase family, AiiA lactonase enzyme is the first well-studied group (Dong et al., 2001; Riaz et al., 2008). In fact, AiiA lactonase homologs have been characterized in numerous bacteria. In the present study, the PCR products of aiiA gene, responsible of encoding AHL lactonase, for ten bacterial species showed high genetic similarity (up to 99%) with other *aiiA* homologous gene existing in NCBI database. Conversely, PCR products from *P. syringae* and *E. cloacae* were free of *aiiA* gene. Previously, it has been indicated *Pseudomonas* spp often utilizes AHL acylase to degrade signal molecules (Huang et al., 2003; Huang et al., 2006; Shepherd & Lindow 2009). Based on these observations, it could be suggested that acylase enzyme is probably the main enzyme to degrade AHLs in *P. syringae* pathovar *syringae.* PCR amplification failed to reveal

aiiA gene in *E*. *cloacae* in our research. To our knowledge, there is no report for the existence of lactonase or even acylase enzyme in this species and it seems further studies are required to unravel the AHL degradation mechanism in *E*. *cloacae.* Nevertheless, it has been demonstrated for some other species of *Enterobacter*, such as *E. ludwigii*, *E. aerogenes,* and *E. asburiae* PT39 that they are able to interfere with QS using AiiA homologs lactonase (Rajesh & Rai 2014a; Rajesh & Rai 2014b; Gopu et al., 2016). Interestingly, we could also indicate for the first time that *E*. *hormaechei* encodes *aiiA* homologous gene. The current study is also the first note of QQ ability by an *aiiA* homologous gene in *S. maltophilia*. Previous studies have demonstrated that the QQ activity in *Ag*. *tumefaciens* is lactonase based as encoded by *hqiA*, *attM* and *aiiB* genes (Carlier et al., 2003; Carlier et al., 2004; Torres et al., 2017). Here the existence of an *aiiA* gene homologue was demonstrated in *Ag*. *tumefaciens*. Furthermore, *C. gillenii* also displayed the presence of *aiiA* gene which is the first report for this strain.

The AHL lactonases are divided into three main types including the Metallo-lactamase superfamily, the α/β-hydrolase-fold family (Mei et al., 2010; Wang et al., 2010) and the phosphotriesterase (PTE) family (Uroz et al., 2008). It seems that zinc-binding motifs are conserved in almost all of the Metallo-lactamase superfamily enzymes (Momb et al., 2008; Liao et al., 2009). In our study, the motif “HXHXDH” was observed in both types of identified genotypes, confirming AHL lactonase activity, as the conserved motif “HXHXDH” is essential for zinc-binding and AHL degradation (Thomas et al., 2005). Besides, we observed Tyrosine residue (Y) at the position 194 in the sequences. Elias et al. (2008) reported the Tyrosine residue is conserved in all lactonase enzymes and has a key role in the positioning of lactone ring of the substrate.

In conclusion, 24 known and novel AHL degrading strains were isolated from rainbow trout. These bacteria were able to deactivate natural and synthetic AHL *in vitro* tests and most of them showed cell-bound QQ activity. Heat and proteinase K treatments inhibited QQ capacity in nearly all isolates, indicating enzymatic activity. Various studies have demonstrated that QQ bacteria can inhibit QS *in vivo* experiments and improve the resistance of hosts against pathogens (Tinh et al., 2007; Chu et al., 2014). Hence, the

current isolates could be considered and evaluated for the treatment and prevention of diseases in aquaculture.

CHAPTER 4

Effect of Quorum Quenching Bacteria on Growth, Virulence Factors and Biofilm formation of *Yersinia ruckeri in vitro* and an *in vivo* Evaluation of Their Probiotic Effect in Rainbow Trout

Adapted from:

Torabi Delshad, S., Soltanian, S., Sharifiyazdi, H. & Bossier, P. (2018). Effect of Quorum Quenching Bacteria on Growth, Virulence Factors and Biofilm formation of *Yersinia ruckeri in vitro* and an *in vivo* Evaluation of Their Probiotic Effect in Rainbow Trout. *Journal of Fish Diseases.1-10.*

ABSTRACT

Five N-acyl homoserine lactone degrading bacteria (quorum quenching strains, QQ) were selected to evaluate their impacts on growth, virulence factors and biofilm formation in *Yersinia ruckeri in vitro*. No difference was observed among the growth pattern of *Y. ruckeri* in monoculture and co-culture with the QQ strains. In order to investigate regulation of virulence factors by quorum sensing in *Y. ruckeri*, cultures were supplemented with 3oxo-C8-HSL. The results indicated that swimming motility and biofilm formation are positively regulated by QS ($p < 0.05$) whereas caseinase, phospholipase, hemolysin productions are not influenced by 3oxo-C8-HSL ($p > 0.05$). The QQs were able to decrease swimming motility and biofilm formation in *Y. ruckeri*. QQ bacteria were supplemented to trout feed at 10^8 CFU gram^{-1} (for 40 days). Their probiotic effect was verified by *Y. ruckeri* challenge either by immersion or injection in trout. All strains could significantly increase fish survival with *Bacillus thuringiensis* and *Citrobacter gillenii* showing the highest and lowest relative percentage survival (RPS) values (respectively 85 and 38%). Besides, there was no difference between the RPS values by either immersion or injection challenge expect for *B. thuringiensis.* The putative involvement of the QQ capacity in the protection against *Yersinia* is discussed.

Keywords:
Biofilm; Enteric redmouth disease; Probiotic bacteria; *Yersinia ruckeri*

INTRODUCTION

Enteric redmouth disease (ERMD) or yersiniosis, which is caused by *Yersinia ruckeri,* is a major disease in farmed salmonids. The pathogen is a Gram-negative enterobacterium which has been reported from various fish species worldwide. *Y. ruckeri* uses a wide range of virulence factors such as extracellular enzymes or products (ECPs) with haemolysin, protease, phospholipase and amylase activities (Romalde & Toranzo, 1993), siderophores (Fernandez et al,. 2004), iron uptake system named ruckerbactin (Fernández et al., 2004), a type three secretion system (Gunasena et al., 2003), and a type four secretion system (Méndez et al., 2009) to establish infection. In spite of the availability of vaccins, antibiotics are still used to control ERMD. As an alternative to antibiotics, treatment methods interfering with virulence factor production could be considered in a so-called antivirulence therapy. The expression of virulence factors in many bacteria depends on their cell density, so-called quorum sensing (QS). Sensing population density by bacteria occurs by signal molecules involved in QS systems allowing for negative of positive regulation of virulence factors production (Eberl, 1999; Fuqua et al*.,* 1994). Inhibition of QS, called quorum quenching (QQ), could be applied for control of virulence in bacteria (Defoirdt et al., 2004; Natrah et al., 2011; Chen et al., 2013). Acylated homoserine lactones (AHLs) are produced by many Gram-negative fish pathogenic bacteria including *Aeromonas salmonicida*, *Aeromonas hydrophila*, *Vibrio anguillarum*, *V. harveyi*, *V. salmonicida,* and *Y. ruckeri* and play an important role in QS system as signal molecules (Cao & Meighen 1989; Milton et al., 1997; Swift et al., 1997; Bruhn et al., 2005; Kastbjerg et al., 2007). AHL degradation is being evaluated as a way to inhibit QS for which there are different strategies (Kalia & Purohit, 2011). Various bacteria have been identified with enzymatic AHL degradation ability. Some of them have also been studied for their QQ ability *in vitro* and *in vivo*, with the aim to control disease (Chu et al., 2010; Defoirdt et al., 2011b; Christiaen et al., 2011). As *Y. ruckeri* produces several AHLs, the control of ERMD through QQ could potentially be an effective alternative for conventional treatments such as antibiotic therapy. To date few studies, if any, have been conducted regarding

QS in *Y. ruckeri* and QQ has not been considered (Kastbjerg et al., 2007). Therefore, in the current work, the role of quorum sensing in the regulation of some virulence factors of *Y. ruckeri* was studied. It was also tried to use QQ bacteria, *in vitro* and as well as *in vivo*, for quorum sensing interference and virulence factors production modulation.

MATERIAL AND METHODS

Strains and chemicals used in the current study

Five isolates with positive QQ activity including *Bacillus cereus* strain 24QT (accession number: MF687160), *B. thuringiensis* strain 7QT (accession number: MF687150), *Stenotrophomonas moltophilia* strain 2QT (accession number: MF687148), *Enterobacter hormaechei* subsp. *hormaechei* strain 5QT (accession number: MF687149) and *Citrobacter gillenii* strain 10QT (accession number: MF687152) were selected from our previous study (Torabi Delshad et al., 2018) to evaluate their inhibitory effect on QS-regulated processes in *Y. ruckeri*.

Chromobacterium violaceum strain CV026, a mini-Tn5 mutant derived from the *C. violaceum* strain ATCC31532 (McClean et al., 1997) was used to detect QS signal molecules. This reporter is able to synthesize the purple pigment violacein in the presence of exogenous acyl homoserine lactone (AHL) molecules with acyl side chain of four to eight carbons. P3/pME6863 and P3/pME6000 strains were used as positive and negative controls in AHL degradation assays, respectively. P3/pME686 is a recombinant of *Pseudomonas fluorescence* that carries the *aiiA* gene from *Bacillus* sp. A24 encoding lactonase enzyme (Molina et al., 2003). *Yersinia ruckeri* strain CCUG14190 a pathogenic bacterium in aquaculture was provided from Aquaculture and Artemia Reference Center (ARC), Belgium. The authenticity of the strain was confirmed by a PCR assay using a pair specific primer.

Standard OOHL, N-3-oxo-octanoyl-homoserine lactone (3-oxo-C8-HSL), used in the present study was purchased from Sigma (Sigma-Aldrich, Germany). Tryptone soy (TS) agar or broth was used as a medium for growth of all strains at incubation temperature

28 °C. CV026 strain was grown in TS medium supplemented with 20 mg l^{-1} of kanamycin to preserve the plasmid harboring the gene coding for violacein production. 3-[N-morpholino] propane sulfonic acid (MOPS) was added to all media used for AHL assays to buffer the medium to pH 6.8 avoiding chemical degradation of AHLs (Yates et al., 2002).
All animal experiments were approved by the State Committee on Animal Ethics, Shiraz University, Shiraz, Iran (IACUC no: 4687/63).

Determination of the effect of QQ strains on growth of *Y. ruckeri*

Selection of natural rifampicin resistant (RR) mutant of *Y. ruckeri*

Natural rifampicin resistant mutant of *Y. ruckeri* was used in this experiment to be able to distinguish it from the QQ strain present in the medium culture. In order to make a rifampicin resistant *Y. ruckeri*, first, a stock solution of rifampicin (Sigma Aldrich, Germany) was prepared by adding 50 mg rifampicin in 1 ml 99.9% methanol, rapped with aluminum foil and kept at -20 °C. *Y. ruckeri* was inoculated into 5 ml TSB + 1 µg ml^{-1} rifampicin and incubated at 28 °C overnight. The strain was cultured for 4 days and everyday 10 µl of the previous culture was inoculated into fresh 5 ml medium culture with increasing concentrations of rifampicin (namely 2, 4 and 10 µg ml^{-1}). Finally, bacteria grown in the highest concentration of rifampicin was tested for resistance to rifampicin by an antibiogram test and then stored with 30% glycerol (v/v) at -80 °C.

A standard curve for relationship between diameter of pigment area and concentrations of 3-oxo-C8-HSL

One mg of 3-oxo-C8-HSL was dissolved in 1 ml of dimethyl sulfoxide (DMSO) to prepare a stock solution of 3-oxo-C8-HSL. CV026 was grown in TSB containing 20 mg l^{-1} kanamycin until 0.1 OD at 600 nm. CV026 was then spread on a buffered (pH 6.5) TSA plate. Ten µl of 3-oxo-C8-HSL solution was dropped into a well in the plate agar.

Plates were put in an incubator at 28°C for 24 h. This assay was performed with five different concentrations of 3-oxo-C8-HSL including 10, 5, 2.5, 1, and 0.5 mg l^{-1}. The diameter of purple-pigmented halos and the 3-oxo-C8-HSL concentration displayed a linear regression line.

Coculture of rifampicin-resistant *Y. ruckeri* with QQ strains

An experiment was carried out to determine the impact of QQ isolates on the growth of *Y. ruckeri*. For this assay, RR *Y. ruckeri* and an AHL degrading isolate were cultured together in TSB, both with an initial concentration of 10^6 CFU ml^{-1} (OD_{600} = 1). Moreover, a monoculture of RR-*Y. ruckeri* was prepared and incubated under the same condition (at 28 °C with shaking at 150 × *g*). At regular time intervals, samples were plated on normal TSA plates and rif+TSA plates (50 mg l^{-1}), respectively in triplicates. After incubation at 28 °C, RR-*Y. ruckeri* was enumerated by counting colonies on rif+TSA plates. QQ strains were enumerated as the difference between CFUs on TSA and rif+TSA plates. Monoculture and co-cultures as described above were used in another assay to study the impact of QQ strains on the degradation of AHLs produced by *Y. ruckeri*. One ml of each culture was sampled and filtered (0.22 µm) at different times. The filtrates were applied on the center of the TSA plates seeded by 100 µl CV026 and the plates were incubated at 28 °C for 24h. The degradation of natural AHLs from RR-*Y. ruckeri* was screened by measuring the diameter of purple zone produced by CV026 on the plates, followed by a comparison between co-culture and monoculture. Remaining AHLs concentrations in cultures was determined using the standard curve.

Effect of 3-oxo-C8-HSL on virulence factors of *Y. ruckeri*

To understand the role of QS molecules in the regulation of virulence factors in *Y. ruckeri*, virulence factor production was verified in the presence of synthetic 3-oxo-C8-HSL. Experiments were carried out using fully grown *Y. ruckeri* (OD_{600} =1) in TSB in

triplicates and all plates were incubated at 28 °C. The same amount of solvent (DMSO) used for 3-oxo-C8-HSL, was mixed with TS agar or broth as a negative control.

Swimming motility assay

For swimming motility assay, 1 mg l^{-1} 3-oxo-C8-HSL was added into semi-solidified sterilized (by autoclave) soft TSA (0.3% agar). Ten µl of overnight cultured *Y. ruckeri* was spotted on the center of the plates and plates were incubated for 24 h. The motility (in mm) was measured.

Hemolytic activity assay

Hemolytic activity test was performed by adding 5% defibrinated sheep blood into autoclaved TSA followed by addition of 1 mg l^{-1} 3-oxo-C8-HSL. *Y. ruckeri* was pipetted on the center of the plates. The halo surrounding the strain was measured after 48 h incubation.

Phospholipase activity assay

Autoclaved TSA was mixed 1% egg yolk emulsion (Sigma Aldrich, Germany) and 1 mg l^{-1} 3-oxo-C8-HSL. The mixture was poured onto plates and *Y. ruckeri* was inoculated on the center of solidified plates. The clear zone was measured after 48 h.

Protease (caseinase) activity assay

To prepare caseinase assay plates, double strength TSA was mixed with 4% (w/v) skimmed milk powder (Sigma Aldrich, Germany) suspension in equal volume after autoclaving at 121^{o}C for 20 minutes, separately. The mixture was supplemented with 1 mg l^{-1} 3-oxo-C8-HSL and poured on plates. Then, 10 µl of *Y. ruckeri* was spotted on plates followed by incubation for 2 days to measure the clear zone.

Biofilm formation assay

For biofilm formation assay, 200 µl diluted overnight cultured bacteria in TSB were inoculated into a 96-well microtiter plate containing 1 mg l^{-1} 3-oxo-C8-HSL. The bacteria were allowed to adhere without agitation for 4 days. Non-adherent cells were removed by rinsing the wells with 300 µl sterile saline (0.9%) three times. The attached bacteria were fixed in 99% methanol (200µl/well) for 2 hours. Crystal violet 0.1% was used for staining and washed with running tap water after 20 minutes. The plate was then air dried and attached cells were solubilized with 200 µl 95% ethanol. Absorbance was gauged by the wave lambda (OD_{570}) using a microplate reader (Infinite M200 microplate reader, Tecan).

Determination of the effect of QQ bacteria on virulence factors production in *Y. ruckeri*

Results of the previous step revealed that swimming motility and biofilm formation functioned under control of QS in *Y. ruckeri*. The effect of AHL degrading strains on swimming motility and biofilm formation was investigated to find out whether QQ bacteria could reduce the intensity of these activities. These assays were performed as described earlier with minor modification. As QQ activity of the tested AHL degrading bacteria was found to be cell-bound (based on the results of our previous study: Torabi Delshad et al., 2018), it was required to prepare cell-free lysate of them. The fully grown QQ strains (OD_{600} 1.0) in TSB were centrifuged (at 12000 × *g* for 10 min), the supernatant was discarded and cell pellets were re-suspended in 10 ml of potassium phosphate buffer (PPB, 100 mM; pH 7.0). The cell suspension (equalized to an OD of 1.0 at 600 nm) was sonicated on ice and the cell-free lysate was obtained after centrifugation and filtration through a 0.2 µm pore size filter. All tests were conducted with negative control (PPB) in triplicates.

Swimming motility assay

Motility assay was performed by mixing autoclaved double strength soft agar (0.3% agar) and cell-free lysates in equal ratio (v/v). Ten µl of *Y. ruckeri* culture (OD_{600} of 1.0) was placed in the center of the plates. Motility activity of *Y. ruckeri* was measured after 48 h incubation.

Biofilm formation assay

A 96 well plate was filled with 100 µl *Y. ruckeri* (OD_{600} of 1.0) and then cell-free lysate of each QQ strain with an OD of 1.0 at 600 nm (100 µl). Biofilm formation assay was performed according to the protocol as explained previously.

***In vivo* assay**

Fish

Healthy rainbow trout (*O. mykiss*) (average weight 20 ± 3 g for feeding trial, 76 ± 10 g for other experiments) were provided from a commercial farm (Fars province, Iran) and transferred to Aquatic Animal Health and Diseases Department, School of Veterinary Medicine, Shiraz University, Iran). Fish were kept in fiberglass tanks (1 m^3) continuously aerated by an aeration system to be acclimatized for two weeks. Feeding was performed with a commercial diet (45% crude protein; Beyza Feed Mill Co, Iran) based on 3% body weight twice daily. In order to eliminate feces and feed remains, the tanks were siphoned and 10% of the water was exchanged every day.

Ten fish were randomly inspected by bacteriological, parasites and fungal examinations to ensure they were healthy. Within the adaptation and experimental periods, parameters of water quality were: 14 °C, pH at 7, dissolved oxygen (DO) at 7.8 ± 0.8. The photoperiod regime was 16L: 8D. Unionized ammonia and nitrite were determined

using an ammonia checker and a nitrite checker, respectively and were below the permissible limits (HI-715 and HI-708-11, HANNA, Milan, Italy).

Determination of the pathogenicity of selected QQ strains

Five QQ strains including *S. moltophilia*, *E. hormaechei*, *B. thuringiensis*, *C. gillenii* and *B. cereus* isolated from rainbow trout in our previous study (Torabi Delshad et al., 2018) were tested to verify any possible pathogenicity to fish and the 50% lethal dose (LD_{50}) was calculated before the beginning of the feeding trial. To carry out this experiment, rainbow trout (average weight: 76 ± 10 g) were randomly taken and stocked in glass aquaria (10 fish per aquarium). This assay was done in 5 experimental groups and one control group each with two replicates using six dosages ranging 1.3×10^4 - 1.3×10^9 CFU g $fish^{-1}$ for each strain (the cell numbers obtained by a hemocytometer slide). The cells were washed, re-suspended in sterile saline 0.9% (w/v) (0.1 ml) and finally injected intraperitoneally in MS222 sedated fish (90 mg l^{-1}). Fish from the negative control group was exposed to sterile saline instead of bacterial suspensions. Mortality and/or clinical signs were monitored over 2 weeks.

Feeding with QQ strains

The experiments were conducted in 5 different treatments and a control group (20 fish per tank) in triplicates. To evaluate the ability of QQ strains to mitigate ERMD in rainbow trout, each QQ strain was inoculated into TSB and incubated at 28 °C for 24 h, followed by centrifugation at 3000 × *g* for 10 min. The harvested cells were resuspended in sterile saline to final concentration 10^8 CFU ml^{-1} as verified by TSA plating. The QQ strains were then sprayed on commercial fish feed to obtain 10^8 CFU per 1 gram of feed and then air dried. Saline solution without bacteria was sprayed on the feed and regarded as a negative control group. The food preparation was performed daily and the feeding trial was carried based on 3% body weight twice daily for 40 days.

Determination of the pathogenicity and LD_{50} in *Y. ruckeri*

Before beginning an *in vivo* challenge, seven different doses of *Y. ruckeri* (10^2 – 10^8 CFU ml^{-1} for immersion and 0.13 × 10 – 1.3 × 10^6 CFU g $fish^{-1}$ for intraperitoneal (I.P.) injection were prepared to estimate pathogenicity and LD_{50} value for fish with 76 ± 10 g. Briefly, the immersion baths were prepared by adding the full-grown *Y. ruckeri* in TSB (OD 1.0 at 600 nm) into aerated tanks containing 5 l freshwater and the mentioned doses were obtained by a hemocytometer slide. For injection, the cells were washed, re-suspended in sterile saline 0.9% (w/v) and adjusted to the desired doses using a hemocytometer slide. The assay was performed in sixteen groups of 10 fish (seven treatments and one negative control for each exposure routes) in triplicates. Considering the fact that *Y. ruckeri* growth is temperature-dependent (15-18 °C) (Roberts, 1983; Danley et al., 1999), the water temperature was adjusted to 18.0 ± 0.5 °C during all *in vivo* tests. Fish were inoculated with *Y. ruckeri* by either immersion or injection (I.P.) routes and monitored for 4 days to record mortalities. The moribund fish were used to recover the pathogen and the isolates from their internal organs were subjected to biochemical and molecular (PCR) characterization. Control fish received sterile solution without the bacterium. Fish were anesthetized with MS222 (90 mg l^{-1}) before injection. The LD_{50} value was calculated using the equation defined by Reed & Muench (1938).

Challenge

After feeding fish with QQ strains for 40 days, a challenge test was conducted to evaluate the influence of QQ strains on the resistance of rainbow trout to ERMD. First, fish were clinically inspected and no signs of disease were recorded.

Each treatment and control group was divided into two sub-groups with 10 fish in triplicates. The bacterium preparations were made as explained above. The first sub-group (41 ± 21 g) was IP injected with 0.1 ml of *Y. ruckeri* 2.4 × 10^2 CFU g $fish^{-1}$ (based on the calculated LD_{50} for injection). The second sub-group was challenged by

immersion bath containing 10^6 CFU ml^{-1} (in accordance with the calculated LD_{50} for immersion) for 1 min. 0.9 % sterile saline was applied for the fish from control groups in both challenge methods. Upon the challenge, the exposed fish were transferred to fresh water and observed for clinical signs of ERMD and mortalities for 2 weeks. Relative percent of survival (RPS) was calculated according to the formula proposed by Amend (1981). RPS= (1- % mortality in treatment/ % mortality in control) × 100.

Re-isolation of *Y. ruckeri*

Bacterial re-isolation and biochemical characterization were carried out from the internal organs of moribund fish as illustrated above.

The genomic DNA of re-isolated *Y. ruckeri* was extracted and the bacterium 16S rDNA (a 409 bp fragment) was amplified by PCR, using specific primers ruck1, 5´-CAG CGG AAA GTA GCT TG-3´ and ruck2 5´-TGT TCA GTG CTA TTA ACA CTT AA-3´, respectively (LeJeune & Rurangirwa, 2000). The amplification was performed using an Eppendorf thermocycler (Eppendorf, Germany) with a thermocycling program: an initial denaturation at 94 ˚C for 6 min, 35 cycles of denaturation at 94 ˚C for 1 min, annealing at 54.5 ± 1.0 ˚C for 45 sec, extension at 72 ˚C for 50 sec) and a final extension phase at 72 ˚C for 5 min. The amplicons with 409 base pairs were resolved in 1.2 % (w/v) agarose gel (Gibco BRL, Karlsruhe, Germany). A pure culture of *Y. ruckeri* was utilized as a positive control.

Data analysis

Statistical analysis was performed using the SPSS software version no. 16 (SPSS Inc, Chicago, USA). The numeric data are displayed as mean ± SD. Independent sample t-test was used to determine any significant difference between the growth of *Y. ruckeri* in monoculture and in each co-culture and also to compare each AHL treatment with controls in the virulence factors experiment. Additionally, one-way analysis of variance (ANOVA) followed by Tukey's test was applied to compare the effects of QQ strains on

virulence factors and also survival rate of fish. *P* values < 0.05 were considered significant. The normality of the data was investigated by Kolmogorov-Smirnov test. LD_{50} of the QQ strains and *Y. ruckeri* was obtained using the equation defined by Reed & Muench (1938).

RESULTS

The effect of QQ strains on the growth of RR *Y. ruckeri*

The growth of RR *Y. ruckeri* in the presence/absence of QQ strains was investigated through a coculture assay. The growth of RR *Y. ruckeri* was apparently not influenced by the QQ strains. ($p > 0.05$) **(Figure 14)**.

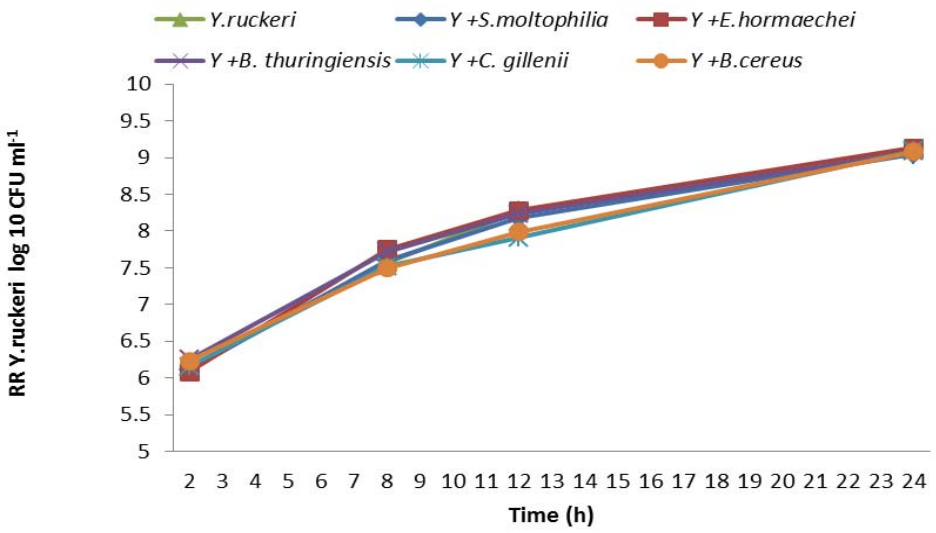


Figure 14. Growth curve of *Y. ruckeri* with and without AHL degrading strains (three replicates). The absence of error bars indicates that the error bars are too small to be visible (Independent sample t-test, *p<0.05*).

Inhibition of AHL production in *Y. ruckeri* by QQ strains

A standard curve was obtained with the following equation: diameter of purple-pigmented halo = 0.386 [3-oxo-C8-HSL] + 1.020 and regression coefficient R^2 = 0.932 **(Figure S1)**. Using the obtained equation we evaluated the effect of the QQ strains on AHL production of *Y. ruckeri.* All AHL degrading strains were able to completely quench AHL produced by *Y. ruckeri* as the violet pigmentation in agar plates was absent in contrast to the existence of purple halo in the plates seeded with monoculture supernatant **(Figure 15, 16)**.

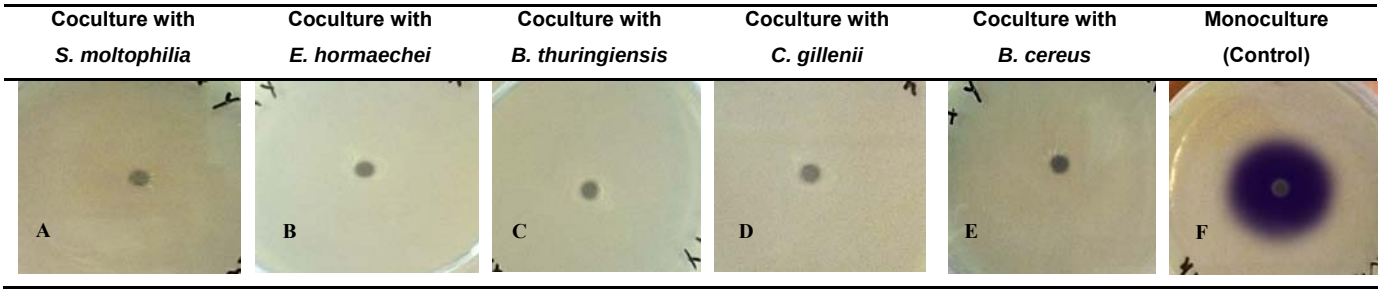


Figure 15. Degradation of AHL produced by *Y. ruckeri* in cocultures with QQ strains after 24 hours incubation **(A, B, C, D, E)**. Pigmentation induced by *Chromobacterium violaceum* strain CV026 in TSA plate, indicating the presence of AHLs in *Y. ruckeri* monoculture as a control **(F)**.

Effect of 3-oxo-C8-AHL on virulence factors of *Y. ruckeri*

The *Y. ruckeri* strain used in this study has been shown to produce some tentative virulence factors including swimming motility, biofilm formation, phospholipase, caseinase and hemolytic activities (data not shown). 3-oxo-C8-AHL, as a dominant signal molecule produced by *Y. ruckeri*, was used to find out whether the above virulence factors are regulated by quorum sensing. The results indicated a significant ($p < 0.05$) increase in swimming motility and biofilm formation upon the addition of AHL

into the culture media of *Y. ruckeri*. In addition, apparently phospholipase, caseinase and hemolysin productions are not regulated by quorum sensing in *Y. ruckeri* **(Figure 17, Table 6)**.

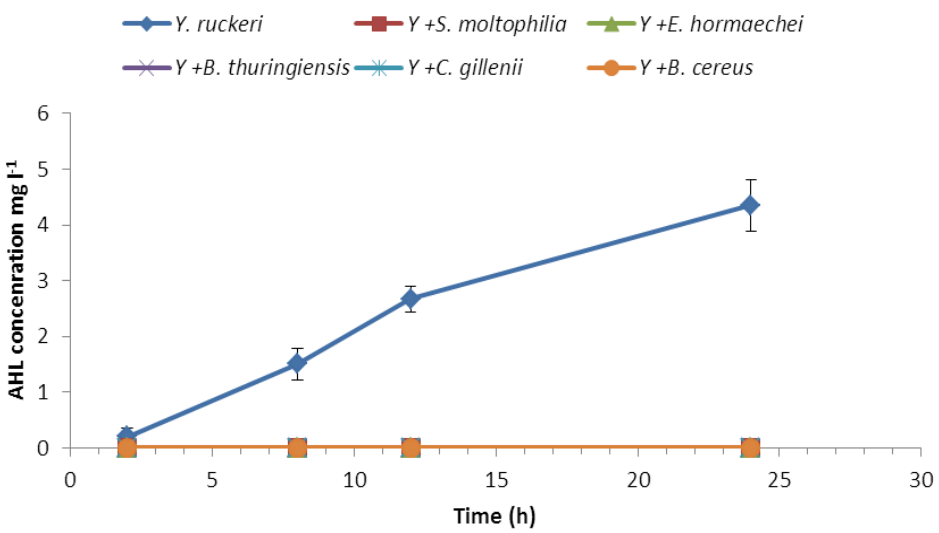


Figure 16. Concentrations of AHLs produced by *Y. ruckeri* cultured with and without QQ strains over time. The error bars indicate the standard deviation of 3 replicates.

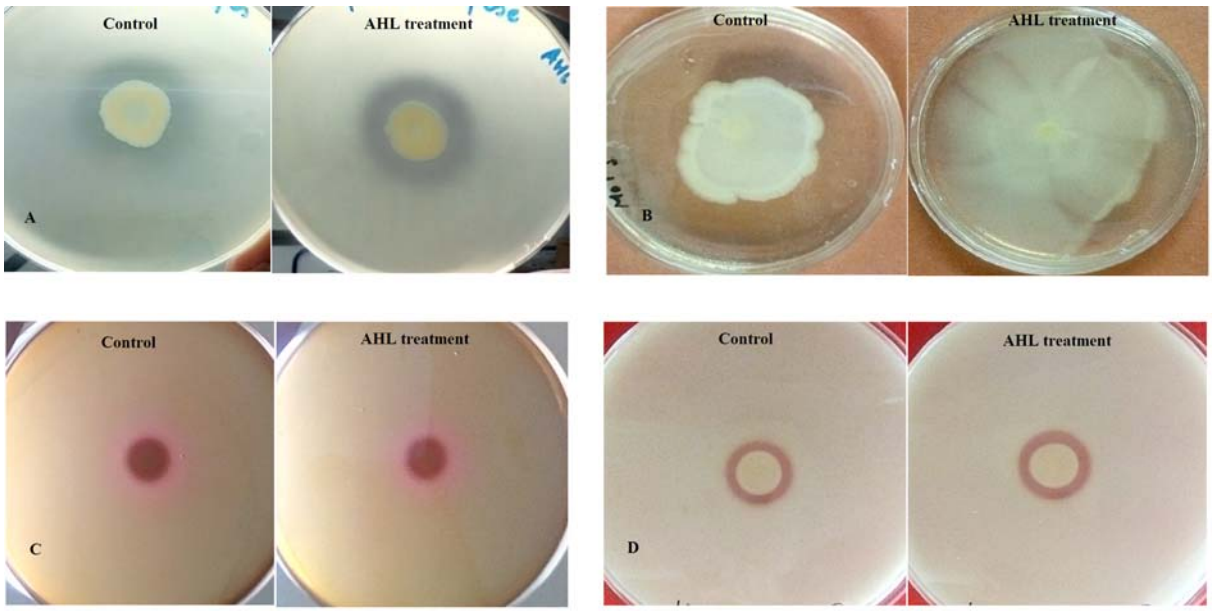


Figure 17. Phospholipase activity **(A)**, Swimming motility **(B)**, Hemolytic activity **(C)** and Caseinase activity **(D)** in *Y. ruckeri* after 48 hours with and without adding 3-oxo-C8-HSL.

Table 6 Effects of 3-oxo-C8-HSL on virulence factors and biofilm formation of *Y. ruckeri* (average ± SD) after 48 h incubation

Virulence factor	AHL treatment	Control	Regulation by QS
Swimming motility (mm)	75.7 ± 5.1 [a]	48.0 ± 3.6 [b]	+
Hemolytic activity*	2.2 ± 0.2 [a]	1.8 ± 0.1 [a]	-
Phospholipase activity*	1.8 ± 0.2 [a]	1.8 ± 0.2 [a]	-
Caseinase activity*	2.0 ± 0.2 [a]	1.8 ± 0.8 [a]	-
Biofilm formation **	0.207 ± 0.009 [a]	0.165 ± 0.016 [b]	+

**Ratio of the diameter of clear halo (mm) to the diameter of colony (mm)*

***Blank-corrected OD_{570} of Crystal violet stained biofilms*

Different letters between columns indicate significant differences (Independent sample t-test, p<0.05)

Average ± standard deviation of four and three replicates for biofilm formation and other tests, respectively

Table 7 Swimming motility and biofilm formation in *Y. ruckeri* (average ± SD) with and without QQ strains extract after 48 h incubation.

Treatment with QQ strains / Virulence factor	*S. moltophilia*	*E. hormaechei*	*B. thuringiensis*	*C. gillenii*	*B. cereus*	*Control*
Swimming motility (mm)	19.0 ± 1 [ac]	19.0 ± 2 [ac]	15.3 ± 0.6 [a]	21.0 ± 2 [c]	16.6 ± 0.6 [ac]	48.0 ± 3.6 [b]
Biofilm formation [*]	0.139 ± 0.006 [a]	0.134 ± 0.009 [a]	0.129 ± 0.008 [a]	0.137 ± 0.010 [a]	0.129 ± 0.007 [a]	0.165 ± 0.016 [b]

**Blank-corrected OD_{570} of Crystal violet stained biofilms*

Different letters among columns indicate significant differences (ANOVA with Tukey's post-hoc test; $p<0.05$)

Average ± standard deviation of four and three replicates for biofilm formation and other two tests, respectively.

Determination of the effect of QQ bacteria on swimming motility and biofilm formation in *Y. ruckeri*

All strains were able to decrease motility of *Y. ruckeri* compared to control group ($p < 0.05$). *B. thuringiensis* and *C. gillenii* displayed the highest and lowest impacts on swimming motility, respectively **(Table 7)**.

Our findings revealed that biofilm formation in *Y. ruckeri* was significantly diminished in the presence of QQ strains in comparison with control group ($p < 0.05$), although no significant difference was seen among QQ treatments ($p > 0.05$). Data are shown in **table 7**.

Safety of the QQ strains

The LD_{50} of the AHL degrading strains were established to verify for their pathogenicity to fish by IP injection. No mortality occurred, during 4 days, with all experimental dosages, indicating that the LD_{50} of the QQ strains are above 1.3×10^9 CFU g $fish^{-1}$. No

clinical signs nor abnormal activities were noticed in the trouts during two weeks monitoring.

Evaluation of the pathogenicity and LD_{50} of *Y. ruckeri*

The LD_{50} of this pathogen was determined either through immersion or by IP injection. The obtained amounts were 10^6 CFU ml^{-1} and 1.3×10^2 CFU g $fish^{-1}$ for fish with 76 ± 10 g for immersion and IP injection, respectively (**Table 8**). No mortality was observed in rainbow trout exposed to sterile saline, and all fish were clinically healthy. The isolates from moribund fish were biochemically characterized and identified by PCR as *Y. ruckeri* (data not shown).

Table 8 Accumulated mortality (%) after challenge with *Y. ruckeri* (CCGU14190) for calculating 96 h-LD_{50}

Dosage of *Y. ruckeri* (CFU ml^{-1})	**10^2**	**10^3**	**10^4**	**10^5**	**10^6**	**10^7**	**10^8**
Accumulated mortality (%)							
Immersion	0	10	26	30	36	60	83
Injection	23	36	43	56	80	96	100

In vivo test

After 40 days feeding with the diet supplemented with QQ strains, experimental trouts were exposed to *Y. ruckeri* through bath exposure and IP injection. Exposed fish were monitored and moribund or dead fish were collected for further examinations. Mortality started 24h post-challenge and increased up to day7 post exposure with clinical signs of Enteric redmouth disease such as typical hemorrhagic septicemia in mouth, lips, fins,

annus and internal organs. The treated groups displayed significantly reduced mortality relative to the control groups ($p < 0.05$). The treatment with *B. thuringiensis* with 10% and 20% mortality and the treatment of *C. gillenii* with 43% and 50% mortality showed the highest and lowest resistance to *Y. ruckeri*, respectively in both immersion and IP injection routes. Likewise, all of the treatments presented a significantly higher growth performance than the control group ($p < 0.05$) (data not shown). In addition, the fish fed by *S. moltophilia* displayed a better growth rate compared with other treatments. No significant difference ($p > 0.05$) was found between the averages the relative percentage survivals (RPS) by immersion and by injection for each QQ bacterium except for *B. thuringiensis* **(Table 9)**.

Table 9 Relative percentage survivals (RPS)* (%) (average ± SD) of rainbow trouts (n = 10, with triplicates), 2 weeks after exposure to *Y. ruckeri* (10^6 CFU ml^{-1} and 2.4 × 10^2 CFU g $fish^{-1}$for immersion and IP injection, respectively).

Treatment with QQ strains / **Relative percentage survivals (RPS) (%)**	***C. gillenii***	***E. hormaechei***	***S. moltophilia***	***B. cereus***	***B. thuringiensis***	**Control**
Immersion	38 ± 5[a]	47 ± 12[a]	61 ± 11[a]	80 ± 11[a]	85 ± 2[a]	-
IP injection	42 ± 12[a]	50 ± 6[a]	62 ± 6[a]	73 ± 6[a]	77 ± 2[b]	-

Different letters in each column indicate significant differences (ANOVA with Tukey's post-hoc test; p<0.05)

**RPS = 1-(mortality treatment/mortality control) × 100*

Re-isolation *Y. ruckeri* from moribund fish

The isolates from challenged fish were biochemically investigated and identified as *Y. ruckeri*. The result was confirmed by a specific PCR amplification, showing a 409 bp band on agarose gel (**Figure 18**).

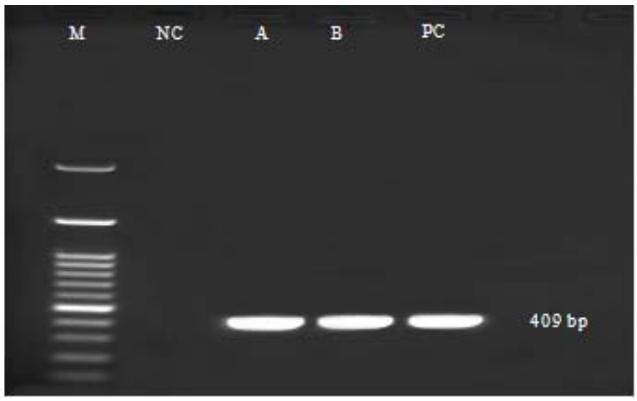


Figure 18. PCR amplification of strains isolated from infected fish by *Y. ruckeri*, using specific primers. The first and second lanes indicate 100kb DNA ladder **(M)** and negative control **(NC)**, respectively. 409 bp-bands on agarose gel in lanes: sample isolated from fish challenged through immersion **(A)**; sample isolated from fish challenged through IP injection **(B)**; Positive control was obtained from pure *Y. ruckeri* culture, confirming the presence of *Y. ruckeri* in experimental fish **(PC)**.

DISCUSSION

AHL degrading bacteria are able to inhibit quorum sensing system in pathogens as well as decrease the virulence factors towards the hosts without negative impact on growth rate of pathogens (Defoirdt et al., 2004). To date, only a few numbers of studies have been performed on QS in *Y. ruckeri.* Temprano et al. (2001) could identify genes involved in *Y. ruckeri* quorum sensing including *yruR* and *yruI* which possess homology to genes encoding the LuxR/LuxI protein family. It has been demonstrated that *Y. ruckeri* can produce a wide range of AHLs, specifically an N-3-oxo-octanoyl homoserine lactone (OOHL) (Kastbjerg et al., 2007).

In the present study, the effects of five QQ strains isolated from rainbow trout on growth and AHL production of rifampicin-resistant (RR) *Y. ruckeri* in a co-cultivation system was investigated. The findings revealed that none of QS strains inhibited the growth of RR *Y. ruckeri* and that this pathogen in all co-culture systems could grow with the same dynamics as in the monocultures. In addition, we studied AHL degradation activity of each QQ strain in a co-culture with RR *Y. ruckeri*. All strains showed a complete

quenching of AHL. We evaluated for the first time the influence of the exogenous 3-oxo-C8-HSL on swimming motility in *Y. ruckeri* showing increased swimming motility. This result was in line with the observations by Nievas et al., (2012) who indicated that motility is affected by exogenous addition of increasing concentrations of synthetic AHLs in symbiotic bradyrhizobial strains of peanut. Besides, it has been reported that bacterial motility is controlled by signaling communication in *S. marcescens*, *V. cholerae, E. coli*, and *Y. pseudotuberculosis* (Ang et al., 2001; Zhu et al., 2002; Sperandio et al., 2002; Atkinson et al., 2008). The present study is the first to investigate the connection between QS and biofilm formation by *Y. ruckeri*. 3-oxo-C8-HSL supplemented to the culture medium of *Y. ruckeri* significantly increased the biofilm formation. There are several studies showing that QS is affecting biofilm formation in other bacteria such as *Aeromonas hydrophila* and *Pseudomonas putida* (Lynch et al., 2002; Arevalo-Ferro et al., 2005). The production of virulence factors was increased by the addition of 3-oxo-C8-HSL but these changes were not significant for hemolysin, phospholipase and caseinase productions. Our findings are in agreement with the results of the only published study until now on the correlation between QS and virulence phenotypes of *Y. ruckeri* indicating that the addition of different pure exogenous AHLs failed to induce protease production in this bacterium (Kastbjerg et al., 2007) despite the fact that proteases are often QS-regulated (Passador et al., 1993; Eberl et al., 1996; Swift et al., 1997; Rasch et al., 2005, Chu et al., 2014).

The tested QQ bacteria could decrease the swimming motility and biofilm formation of *Y. ruckeri* (with the highest and lowest effect belonging to *B. thuringiensis* and *C. gilleni*, respectively). These results indicate that these QQ bacteria have the potential to interfere with the production of some virulence factors.

Two *in vivo* tests were performed with these QQ bacteria, showing that the RPS values are more or less equal when *Yersinia* infection is caused by immersion or injection.

The information about the routes of entry in pathogenic bacteria is very important to understand the early stage of a disease, as it might assist in the development of efficient therapeutic strategies. The gills, the gut and the skin have been identified as important routes of infection for fish pathogens (Tobback et al., 2009). The gut has been

suggested to be a portal of entry for *Y. ruckeri* (Busch & Lingg 1975; Valtonen et al*.,* 1992) and *Y. ruckeri* was recently shown to adhere to different glycoproteins in carp intestinal mucus (Schroers et al., 2008). The gills are in constant contact with the aquatic environment and therefore, directly accessible to pathogen attack. The presence of QQ bacteria at the portal of entry of *Y. ruckeri* might be considered as a defense barrier against the pathogen. Our previous findings (Torabi Delshad et al., 2018) indicate that the used QQ bacteria can be found in the gill, skin, and intestine tissues of rainbow trout. Hence if infection is established by immersion there is the potential that supplemented QQ bacteria can interfere with *Yersinia* and probably the QQ capacity of the tested strains downregulate virulence factor important during the early stage of infection by *Yersinia*. Such mechanism could be substantiated if QQ mutants of the isolated strains would be tested.

It is more difficult to imagine how the QQ capacity of the supplemented strains could protect trout upon challenge by *Yersinia* injection, as in this case there is no opportunity for physical contact between the QQ strains and *Yersinia*. 3-oxo-C8-HSL has been detected in organs of infected fish by Bruhn et al. (2005), suggesting that these molecules can circulate in the host. Moreover, AHLs have been proven to act as inhibitors of the host immune response. There is some evidence showing that *N*-(3-oxododecanoyl)-L-homoserine lactone [OdDHL] can inhibit lymphocyte proliferation and tumour necrosis factor-α production, and down regulate IL-12 production in lipopolysaccharide-stimulated macrophages (Telford et al., 1998), cytokine production *in vitro* (Ritchie et al., 2003), and the differentiation and proliferation of both T_H1 and T_H2 cells (Ritchie et al., 2005; Pritchard et al., 2005). Hence there is the potential that QQ strains influence the concentration of AHL molecules in the host upon *Yersinia* infection by injection and that in this way the clearance of *Yersinia* by the immune system is facilitated. Obviously, this interpretation is speculative and would need further experimentation, including the determination of AHL concentration in serum. Alternatively, it could be that the QQ strains directly stimulate the immune system, facilitating the clearance of injected *Yersinia* cells. In view of the different RPS values of

the tested QQ strains, further research should under that hypothesis focus on the difference in dynamics of the immune response upon *Yersinia* injection.

In conclusion, the QQ strains in this study degraded OOHL (3-oxo-C8-HSL) and *Y. ruckeri* signaling molecules. Our AHL degrading strains could effectively reduce swimming motility and biofilm formation of *Y. ruckeri*. In addition, the results of *in vivo* experiments suggest these QQ strains act as probiotic candidates and are able to control ERMD in trout. Yet it is not clear if the QQ capacity is directly involved in the probiotic effect.

CHAPTER 5

The catecholamine (Norepinephrine and Dopamine) and the Glucocorticoid Stress Hormones Increase Growth and Virulence Factors of *Yersinia ruckeri in Vitro* and *in Vivo*

ABSTRACT

In this study, we evaluated the impact of the catecholamines and glucocorticoids on growth, swimming motility, biofilm formation and some virulence factors activities of pathogenic *Yersinia ruckeri*. Norepinephrine and dopamine (at 100 μM) significantly increased the growth of *Y. ruckeri* in culture media containing serum. An increase in swimming motility of the pathogen was found following the exposure to the hormones however, no effect was seen on caseinase, phospholipase and hemolysin productions. Further, antagonists for the catecholamine receptors were observed to block some the influences of the catecholamines. Indeed, the effects of catecholamines were inhibited by chlorpromazine (the dopaminergic receptor antagonist) for dopamine, labalatol (α- and β-adrenergic receptor antagonist) and phenoxybenzamine (the α-adrenergic receptor antagonist) for norepinephrine, but propranolol (the β-adrenergic receptor antagonist) showed no effect. Additionally, the glucocorticoid treatments in different concentrations led to an increase in the growth, swimming motility and biofilm formation in *Y. ruckeri* and showed no effect on caseinase, phospholipase and hemolysin activities. Pretreatment of *Y. ruckeri* with the catecholamines resulted in a significant enhancement of its virulence towards rainbow trout and the antagonists could neutralize the effect of the stress hormones *in vivo*. In summary, our results show that the catecholamines and glucocorticoids increase the virulence of *Y. ruckeri* which is pathogenic to trout through increasing the motility, biofilm formation and growth.

Keywords:

Yersinia ruckeri; catecholamines; glucocorticoids; virulence factors; growth

INTRODUCTION

Enteric redmouth disease (ERMD) causes remarkable economic losses in the salmonid culture. The infection occurs by *Yersinia ruckeri*, a Gram-negative bacterium, in salmonids, especially rainbow trout (Furones et al., 1993). The misuse of antibiotics in order to control and prevent bacterial diseases has led to the development of antibiotic resistance and subsequently, new methods for controlling bacterial infections in aquaculture are urgently required (Karunasagar et al., 1994; Moriarty, 1998; Defoirdt et al., 2011a). Several virulence factors, e.g. biofilm formation and swimming motility are produced by *Y. ruckeri* that enable it to enter and overcome the immune system of host. Antivirulence therapy, through inhibiting the production of virulence factors, is an attractive alternative strategy to antibiotics for controlling infections caused by bacteria (Defoirdt, 2013). Therefore, a better understanding of mechanisms involved in bacterial infection is very important as this knowledge can lead to the exploration and development of novel agents to treat and control diseases. Host stress has been considered to affect the outcome of interactions between host-pathogen, resulting in decreased activity of the host immune system (Verbrugghe et al., 2012). Upon exposure to stress, the catecholamines and glucocorticoids release into the circulation and influence the immune responses as a conserved process in vertebrates and invertebrates (Ottaviani & Franceschi, 1996). There is some evidence indicating that specific sensing systems have been developed in bacteria for detecting stress hormones secreted by their host, and recognizing these stress hormones leads to an increase in virulence of the pathogens (Lyte, 2004). It has been found that the catecholamines (norepinephrine and dopamine) as an important part of the stress response in animals (Reiche et al., 2004), are capable of enhancing the growth in various bacteria including (*Yersinia enterocolitica*, *Listeria monocytogenes*, *Escherichia coli, Pseudomonas aeruginosa, Aeromonas hydrophila*, *Salmonella spp.* and *Vibrio parahaemolyticus*) in serum-based media (Lyte & Ernst, 1992; Coulanges et al., 1997; Kinney et al., 1999; Belay et al., 2003; Nakano et al., 2007a; 2007b; Verbrugghe et al., 2012; Gao et al., 2018). Due to the presence of iron-binding proteins with high-affinity

such as transferrin and lactoferrin in the host body, such media are iron-limited. Furthermore, the effects induced by the catecholamines in bacteria can be blocked by antagonists of adrenergic and dopaminergic receptors (Freestone et al., 2007; Sharaff & Freestone, 2011). Therefore, interfering with binding the catecholamines to their receptors by the different antagonists of the receptors and subsequently, reducing the virulence of bacteria can be regarded as an alternative method to antibiotic therapy. With respect to enteric redmouth disease in aquaculture, detecting and responding to catecholamines by *Y. ruckeri* might also be important, as aquatic animals (including fish) also release these hormones (Ottaviani & Franceschi, 1996).

Besides, an endocrine stress response is triggered in fish through activation of the hypothalamic-pituitary-interrenal (HPI-) axis to secrete glucocorticoids, particularly cortisol, into the blood upon exposure to a stressful condition (Barton, 2002; Sharaff & Freestone, 2011). Cortisol causes a series of physiological and behavioral changes in the host (Sapolsky et al., 2000; Blas et al., 2007; Cockrem, 2013), leading to an adaptation of fish with altered states (McEwen & Wingfield, 2003; Korte et al., 2005; Øverli et al., 2007). To the best of our knowledge, there are few studies on the effects of the glucocorticoids on the growth and virulence factors of bacteria and according to these reports cortisol is able to influence the growth in bacteria such as *Porphyromonas gingivalis*, *Fusobacterium nucleatum* and *Flavobacterium columnare* (Jentsch et al., 2013; Akcal et al., 2014; Declercq et al., 2016).

In the current study, for the first time, we investigated the effect of the catecholamine (dopamine and norepinephrine), the antagonists of the catecholamine receptors and some the glucocorticoids on the growth and virulence factors in pathogenic *Y. ruckeri* (including swimming motility and biofilm formation), and determined the impact of these compounds (excluding the glucocorticoids) on the virulence of *Y. ruckeri* towards rainbow trout *in vivo*.

MATERIAL AND METHODS

Bacterial strains, cultural conditions and chemicals

Yersinia ruckeri strain CCUG14190 was used and cultured in tryptone soy (TS) agar or broth at 28 °C. Norepinephrine was dissolved in hydrochloride acid (HCl 0.1N) at 10 mM, while dopamine was dissolved in distilled water at 10 mM. The antagonists used in the present study (Sigma-Aldrich, Germany) are shown in **table 10**. The compounds were sterilized using a 0.22 µm filter and stored at -20°C.

For all tests, filter sterilized stress hormone stock solutions were added into the autoclaved agar (cooled down to 50°C) to reach a final concentration of 100 µM. The inhibitor agents were used at 50 µM and 100 µM. In case organic solvents were employed to prepare stock solutions, control treatments were prepared using the same volume of the solvent to verify that the obtained effects were not caused by the solvent. All animal experiments were approved by the State Committee on Animal Ethics, Shiraz University, Shiraz, Iran (IACUC no: 4687/63).

Regarding glucocorticoids hormones, the stock solutions were prepared in 1 mg l^{-1} with ethanol and the hormones were used at 30 ng ml^{-1}, 300 ng ml^{-1} and 3000 ng ml^{-1}. Ethanol was added into the controls at the same volume to ensure an appropriate control. Glucocorticoids hormones tested in our experiments are represented in **table 11**.

Table 10 Bacterial strain, catecholamines and inhibitors used in this study

Strain/ Compound	**Specificity of inhibitor**	**Solvent**
Yersinia ruckeri CCUG14190		
Dopamine (Dopa)		Water
Norepinephrine (NE)		HCl 0.1 N
Chlorpromazine hydrochloride	Dopaminergic	Distilled Water
Labetalol hydrochloride	α- and β-adrenergic	Ethanol
S-propanolol hydrochloride	β-adrenergic (irreversible)	Ethanol
Phenoxybenzamine hydrochloride	α-adrenergic (irreversible)	DMSO

Table 11 Glucocorticoids used in the current study

Code	Compound	Category
Control	Control + Ethanol	
C1	20β-dihyrocortisone	Phase I metabolites
C2	11-deoxycortisol	Precursors
C3	tetrahydrocortisol	Phase I metabolites
C4	11-deoxycorticosterone	Precursors
C5	cortisone	Phase I metabolites
C6	cortisol	Active hormones
C7	tetrahydrocortisone	Phase I metabolites
C8	17α-hydroxyprogesterone	Precursors
C9	corticosterone	Active hormones

Bacterial growth assays

Catecholamines

For the bacterial growth assays, *Y. ruckeri* was cultured overnight in TSB at 28°C. After that, the bacterium was re-inoculated at a concentration of 10^2 CFU ml^{-1} into fresh TSB containing 30% (v/v) adult bovine serum and without serum (Sigma-Aldrich, Germany) as well as with and without 100 µM norepinephrine or dopamine. Additionally, the catecholamine receptor antagonists in different concentrations (50 µM and 100 µM) were added along with the catecholamines to evaluate their inhibitory effects on catecholamine-induced growth. The cultures were incubated in 200 µl volumes in 96-well plates at 28°C for 48 h, and the turbidity at 600 nm was recorded every eight hours using a Multireader machine (Infinite M200, TECAN, Austria). Growth curves were obtained for triplicate cultures.

Glucocorticoids

Growth assay for the glucocorticoid hormones was performed as described above. The glucocorticoid hormones were added in different concentrations (30, 300 and 3000 ng ml^{-1}) into TSB inoculated with *Y. ruckeri* in 96-well plates and the culture media were incubated at 28°C for 48 h. The experiments were carried out in three independent

cultures and *Y. ruckeri* culture treated with ethanol (the solvent of the used hormones) was considered as a negative control.

Motility assay

Catecholamines

In order to assess the efficacy of the catecholamines on motility, TS soft agar plates (containing 0.3% agar) were used. The strain (OD_{600} = 1.0) was spotted on the center of the plates (10 µl) and the diameter of the motility zones was measured after 24 h of incubation at 28 ^{o}C.

Glucocorticoids

Swimming motility assay was repeated for glucocorticoid hormones similar to the way used for catecholamines. Three different amounts of the glucocorticoids were supplemented to the autoclaved soft TSA and upon inoculation with *Y. ruckeri* the culture media were incubated at 28 ^{o}C for 24 h.

Biofilm formation

Catecholamines

Biofilm formation was quantified by crystal violet staining. In brief, 200 µl of an overnight culture of *Y. ruckeri* (OD_{600} of 1.0) was inoculated in TS broth with or without the catecholamines and antagonists into the wells of a 96 well plate. The bacteria were allowed to adhere without agitation for 24 h at 28°C. The cultures were removed and washed three times with 300 µl of sterile saline for removing the non-adherent bacteria. The remaining attached cells were fixed with 150 µl of 99% methanol per well for 20 min, after removing the methanol plates were air-dried. Then, biofilms were stained for

15 min using 150 µl of a 0.1% crystal violet solution per well. The wells were rinsed off with running tap water. The plates were air dried and the adherent cells were solubilized with 150 µl of 95% ethanol. Finally, absorbance was recorded at 570 nm by a Multi-reader (Infinite M200, Tecan, Austria). A sterile medium was regarded as a negative control.

Glucocorticoids

Biofilm formation test was performed as explained above using the glucocorticoid hormones.

Caseinase, hemolysin and phospholipase assays

All these assays were performed by adding different concentrations of stress hormones into autoclaved TSA. The caseinase assay plates were prepared by mixing equal volumes of autoclaved double strength TS agar and a 4% skim milk powder suspension (Sigma Aldrich, Germany), sterilized separately at 121ºC for 20 and 5 minutes, respectively. Then, 10 µl of *Y. ruckeri* (OD_{600} =1.0) was spotted on plates followed by incubation for 48 h. Clearing zones surrounding the bacterial colonies and colony diameters were measured. In order to prepare hemolytic assay plates, autoclaved TS agar was supplemented with 5% defibrinated sheep blood (Sigma Aldrich, Germany). Colony diameters and clearing zones were measured after 48 h incubation at 28°C. Finally, phospholipase assay was carried out by mixing 1% egg yolk emulsion (Sigma Aldrich, Germany) with autoclaved TSA. *Y. ruckeri* was inoculated on the center of solidified plates and the clear zone and colony diameter were measured after 48 h.

***In vivo* assay**

Fish

Healthy rainbow trout (*Oncorhynchus mykiss*) (average weight 63 ± 10 g) were provided from a commercial farm (Fars province, Iran) and transferred to Aquatic Animal Health and Diseases Department, School of Veterinary Medicine, Shiraz University, Iran. Fish were kept in fiberglass tanks (1 m^3) continuously aerated by an aeration system to be acclimatized for two weeks. Feeding was performed with a commercial diet (45% crude protein; Beyza Feed Mill Co, Iran) based on 3% body weight twice daily. In order to eliminate feces and feed remains, the tanks were siphoned and 10% of the water was exchanged every day. Ten fish were randomly inspected by bacteriological, parasites and fungal examinations to ensure they are healthy. Within the adaptation and experimental periods, parameters of water quality were: 14 °C, pH at 7, dissolved oxygen (DO) at 7.8 ± 0.8. The photoperiod regime was 16L: 8D. Unionized ammonia and nitrite were determined using an ammonia checker and a nitrite checker, respectively, and were below the permissible limits (HI-715 and HI-708-11, HANNA, Milan, Italy).

Pre-treatment of *Y. ruckeri* and *in vivo* assay for Rainbow trout challenge tests

The efficacy of the catecholamines and the antagonists on the virulence of *Y. ruckeri* were investigated through an *in vivo* challenge test with rainbow trout (10 fish per treatment). *Y. ruckeri* was pretreated with the catecholamines and the inhibitors to avoid any direct effect on rainbow trout. Shortly, *Y. ruckeri* was cultured an overnight in TS broth in the presence of norepinephrine (with and without the inhibitors) or dopamine (with and without chlorpromazine) to reach OD 1.0 at 600 nm. The compounds were washed through centrifugation at 3000 × *g* for 10 min followed by re-suspending in sterile saline solution 0.9% (w/v) prior to addition to the water. The immersion baths were prepared by adding the pretreated *Y. ruckeri* into aerated tanks containing 5 l freshwater and the dose 10^6 CFU ml^{-1} was adjusted by a hemocytometer slide. Fish were exposed to the pretreated *Y. ruckeri* for 1 min. Untreated *Y. ruckeri* cultures were prepared in the same way and used as negative controls. Besides, sterile saline solution 0.9% (w/v) was added into water bath at the same volume as the bacterial

preparation and regarded as control group. Upon the challenge, the exposed fish were transferred to fresh water and the survival rate was counted for one week after the addition of the pathogen. The moribund fish were used to recover the pathogen and the isolates from kidney were subjected to biochemical and molecular (PCR) characterization as explained in **chapter 4** in detailes. Each treatment was carried out in four duplicates.

Statistical analyses

Data analysis was carried out using the SPSS statistical software (version 16, SPSS Inc, Chicago, USA). All data were compared with one-way analysis of variance (ANOVA), followed by Tukey's post hoc test with a *p*-value of 0.05.

RESULTS

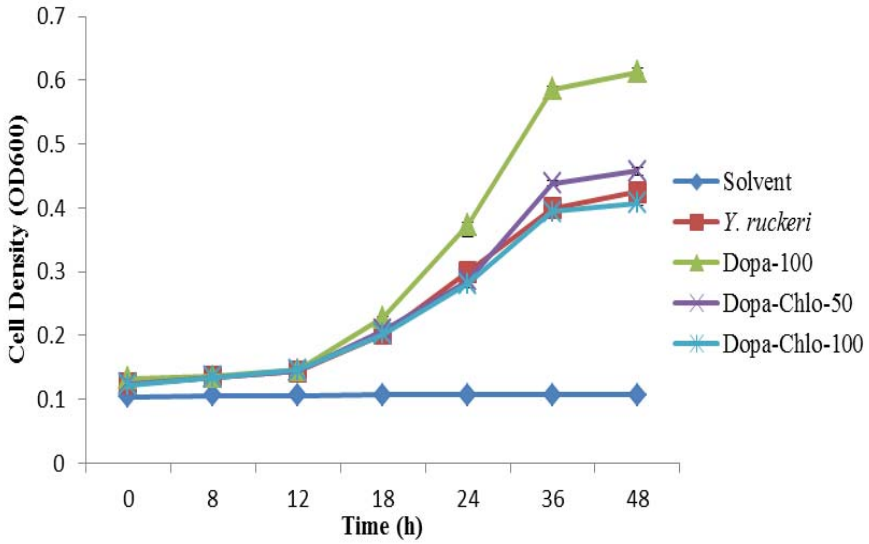
Effects of the catecholamines and antagonists on the growth of *Y. ruckeri* in serum-supplemented medium

The addition of norepinephrine or dopamine led to an enhancement in the growth of *Y. ruckeri* in TSB containing 30% (v/v) serum compared to untreated cultures **(Figure 19)**. The growth of *Y. ruckeri* in medium without serum showed no change in the presence of the catecholamines (data not shown).

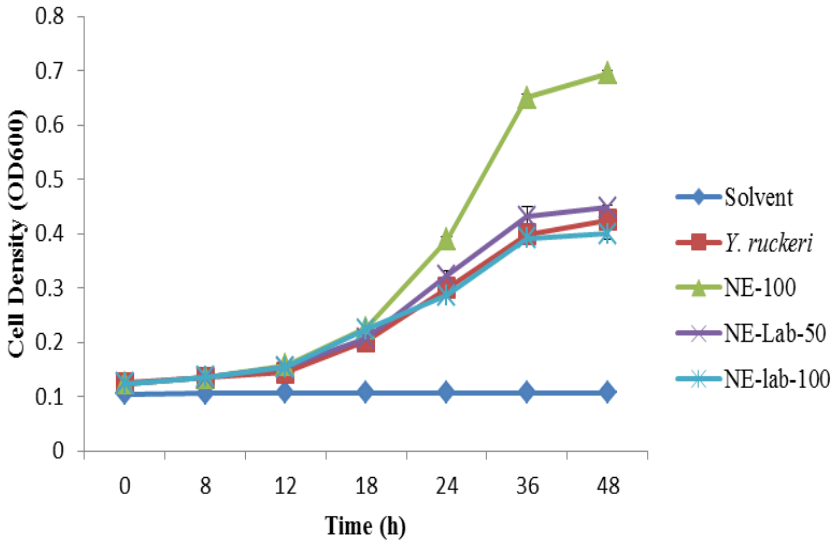
Further, we investigated the inhibitory effect of the antagonists of catecholamine receptors on the growth-inducing impact of catecholamines in serum-supplemented TSB (with 100 µM catecholamines). Chlorpromazine (the dopaminergic antagonist) inhibited the growth conferred by dopamine but displayed no effect on norepinephrine-induced growth **(Figure 19A)**. Additionally, the antagonists with α-adrenergic activity (not antagonists with only β-adrenergic activity) could neutralize growth induced by norepinephrine **(Figures 19B, 19C, 19D)**, and the adrenergic antagonists were not capable of neutralizing the growth caused by dopamine (data not shown). The growth of

Y. ruckeri was not influenced by the antagonists and solvents used for dissolving the antagonists when tested alone (data not shown), indicating that the growth inhibition by the antagonists was not resulting from toxicity.

(A)



(B)



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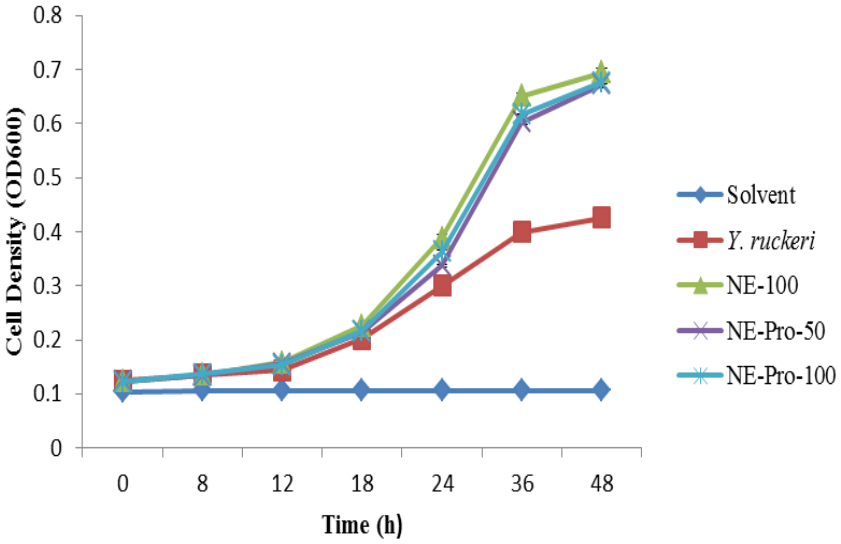

Cell Density (OD600)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
8
12
18
24
36
48
Time (h)
Solvent
Y. ruckeri
NE-100
NE-Pro-50
NE-Pro-100

(D)

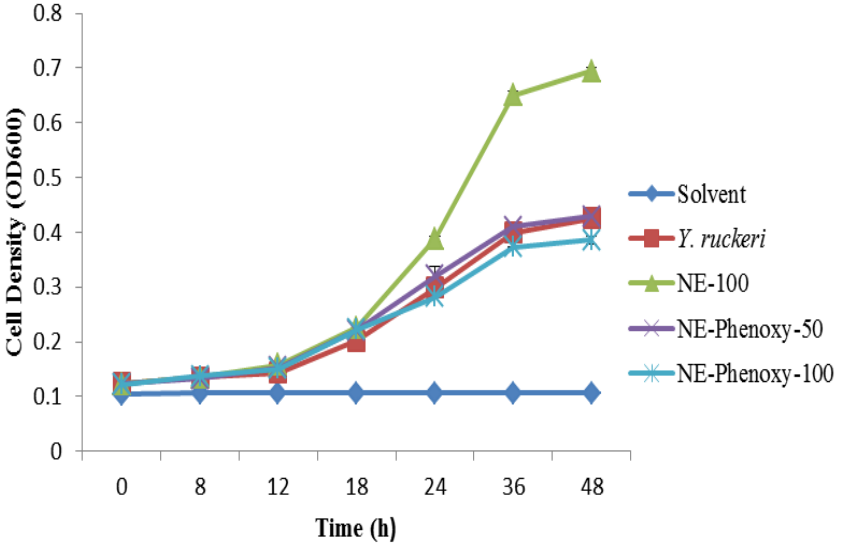

Cell Density (OD600)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
8
12
18
24
36
48
Time (h)
Solvent
Y. ruckeri
NE-100
NE-Phenoxy-50
NE-Phenoxy-100

Figure 19. The impact of the catecholamines and the antagonists on growth in *Y. rucheri* during 48 h incubation **(A)** Dopamine (Dopa; 100 µM) with or without chlorpromazine (chlo) (the dopaminergic antagonist) at 50 µM or 100 µM, **(B)** Norepinephrine (NE; 100 µM) with or without labetalol (Lab) (the α- and β-adrenergic antagonist) at 50 µM or 100 µM, **(C)** Norepinephrine (NE; 100 µM) with or without propranolol (Pro) (the β-adrenergic antagonist) at 50 µM or 100 µM, **(D)** Norepinephrine (NE; 100 µM) with or without phenoxybenzamine (Phenoxy) (the α-adrenergic antagonist) at 50 µM or 100 µM. Error bars representing the standard deviation of three replicates are too small to be visible. *Y.ruckeri* refers to the untreated pathogen (supplemented with only the same volume of solvent used for dissolving the catecholamines and antagonists). Solvent in the graphs alludes to sterile medium culture, which was used as a control.

Effects of glucocorticoid on the growth of *Y. ruckeri*

Supplementation of culture media with glucocorticoid hormones resulted in an increase in the cell density of *Y. ruckeri.* All the compounds were significantly able to induce the growth in *Y. ruckeri* ($p < 0.05$), with some exceptions at 30 and 300 ng ml^{-1}. Moreover, the comparison of three concentrations in each treatment indicated that the growth of *Y. ruckeri* induced by some glucocorticoid hormones is significantly dose-dependent and enhanced with the increase in the concentration. The results of the growth assay are illustrated in **table 12** and **figure 20**.

Table 12 The effect of different concentrations of glucocorticoid hormones on the growth of *Y. ruckeri* after 48 h incubation.

Code	Compound	Cell density (OD_{600})		
		30 ng/ml	**300 ng/ml**	**3000 ng/ml**
Control	Control + Ethanol	0.44 ± 0.02^{a}	0.45 ± 0.03^{a}	0.44 ± 0.02^{a}
C1	20β-dihyrocortisone	0.51 ± 0.02^{bc}	0.56 ± 0.07^{bcd}	0.59 ± 0.01^{bc}
C2	11-deoxycortisol	0.70 ± 0.04^{e}	0.75 ± 0.01^{f}	0.79 ± 0.01^{d}
C3	tetrahydrocortisol	0.56 ± 0.02^{cd}	0.57 ± 0.01^{cd}	0.58 ± 0.01^{bc}
C4	11-deoxycorticosterone	0.47 ± 0.01^{ab}	0.56 ± 0.01^{bcd}	0.64 ± 0.02^{c}
C5	cortisone	0.51 ± 0.02^{bc}	0.52 ± 0.03^{abc}	0.53 ± 0.05^{b}
C6	cortisol	0.61 ± 0.02^{d}	0.67 ± 0.04^{e}	0.75 ± 0.05^{d}
C7	tetrahydrocortisone	0.48 ± 0.02^{ab}	0.49 ± 0.04^{ab}	0.53 ± 0.03^{b}
C8	17α-hydroxyprogesterone	0.57 ± 0.03^{cd}	0.62 ± 0.03^{de}	0.63 ± 0.03^{c}
C9	corticosterone	0.58 ± 0.02^{e}	0.78 ± 0.01^{f}	0.81 ± 0.03^{d}

Results are expressed as mean ± standard deviation of three replicates.

Values in the same column with different superscript letters are significantly different (ANOVA with Tukey's post-hoc test; p < 0.05)

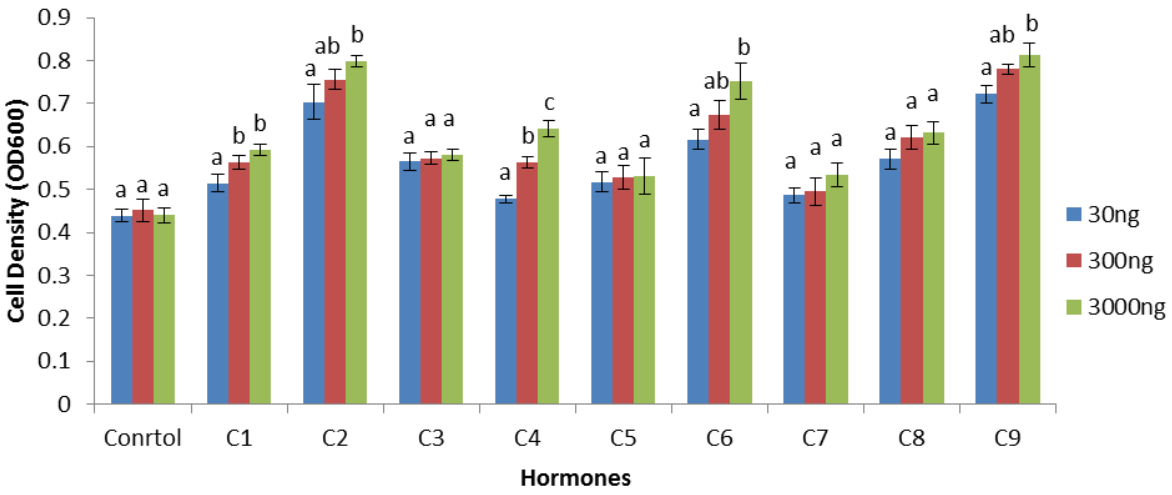


Figure 20. Growth of *Y. ruckeri* with different concentrations of the glucocorticoid hormones. Error bars represent the standard deviation of three replicates. Different letters above columns show significant difference among three concentrations in each treatment (ANOVA with Tukey's post-hoc test; $p < 0.05$)

Effects of the catecholamines and antagonists on the swimming motility

We evaluated the effect of the catecholamines on the swimming motility of *Y. ruckeri* in soft agar. Both norepinephrine and dopamine could significantly increase the swimming motility of *Y. ruckeri* ($p < 0.05$) **(Figure 21)**.

We also investigated the effect of the catecholamine receptor antagonists on the swimming motility induced by the catecholamines. The results demonstrated that chlorpromazine (the dopaminergic antagonist) inhibited the motility caused by dopamine **(Figure 21A)**, but adrenergic receptor antagonists did not affect (data not shown). Furthermore, phenoxybenzamine (the α-adrenergic antagonists), labetalol (the α- and β-adrenergic antagonist) were able to neutralize swimming motility induced by norepinephrine **(Figure 21B, 21C, 21D)**, no change was seen by applying propranolol (the β-adrenergic receptor antagonist) and chlorpromazine (the dopaminergic antagonist) (data not shown). Finally, we also tested the swimming motility of *Y. ruckeri* using the antagonists as well as equivalent volumes of the solvents in the absence of the catecholamines and no influence was observed (data not shown).

Effects of the glucocorticoids on the swimming motility

The glucocorticoid hormones could increase the swimming motility in comparison with control group. The swimming motility indicated a significant increase in all the hormones and at all the concentrations except 11-deoxycorticosterone and tetrahydrocortisone at 30 ng ml^{-1}. Increasing the concentration of some of the glucocorticoid hormones caused a significant increase in swimming motility of *Y. ruckeri* ($p < 0.05$). Data related to the swimming motility affected by the glucocorticoids are illustrated in **table 13** and **figure 22**.

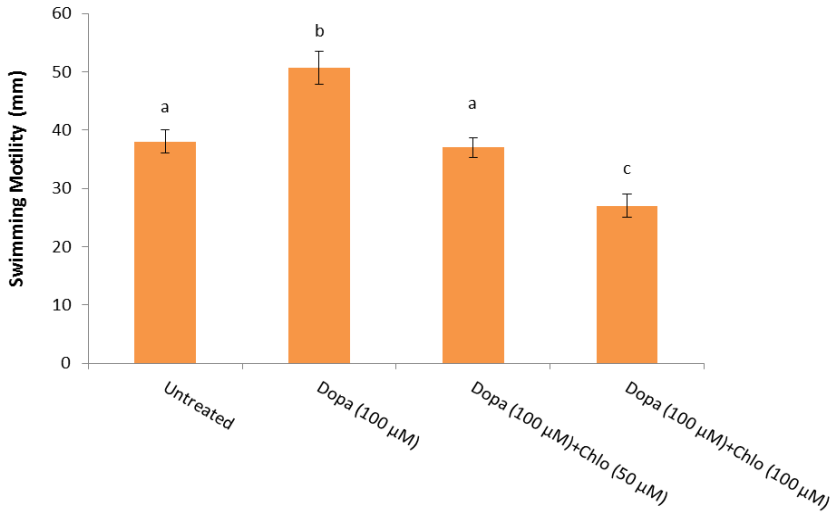
Effects of the catecholamines and antagonists on biofilm formation

A significant increase was found in biofilm formation by using the catecholamines, and the influence was blocked by the antagonists **(Figure 23)**. However, propranolol (the β-adrenergic receptor antagonist) was not able to inhibit the effect of norepinephrine on biofilm formation. Besides, the use of only the antagonists and solvents had no effect on biofilm formation (data not shown).

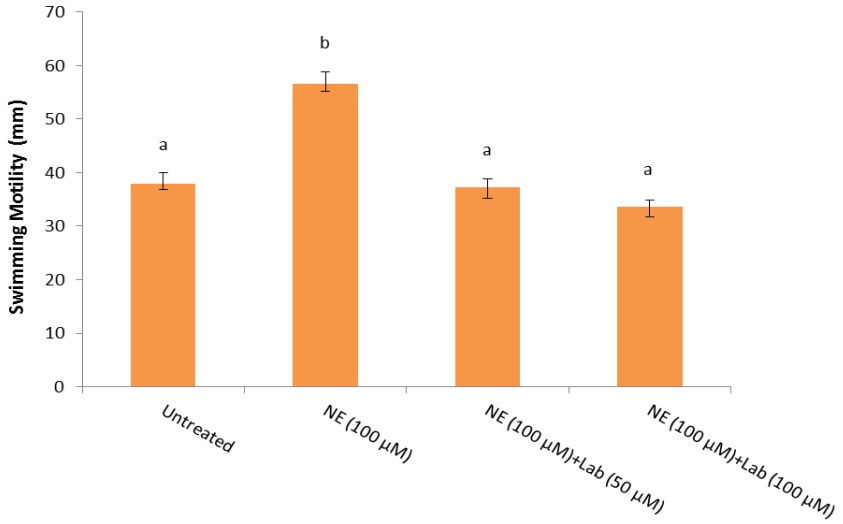
Effects of the glucocorticoids on biofilm formation

The results of the biofilm assay demonstrated that the glucocorticoid hormones affected biofilm formation through a significant increase in biofilm formation ($p < 0.05$) at every three concentrations except 20β-dihydrocortisone and tetrahydrocortisone at 30 ng ml^{-1}. Biofilm formation in *Y. ruckeri* significantly enhanced with increasing the concentration of some the glucocorticoid hormones ($p < 0.05$) **(Table 14, Figure 24)**.

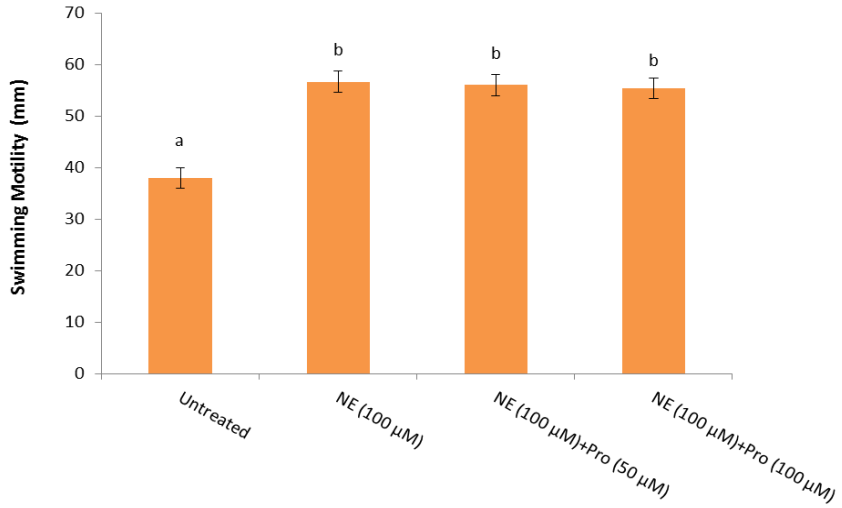
(A)


Swimming Motility (mm)
60
50
40
30
20
10
0
a
b
a
c
Untreated
Dopa (100 µM)
Dopa (100 µM)+Chlo (50 µM)
Dopa (100 µM)+Chlo (100 µM)

(B)


Swimming Motility (mm)
70
60
50
40
30
20
10
0
a
b
a
a
Untreated
NE (100 µM)
NE (100 µM)+Lab (50 µM)
NE (100 µM)+Lab (100 µM)

(C)



(D)

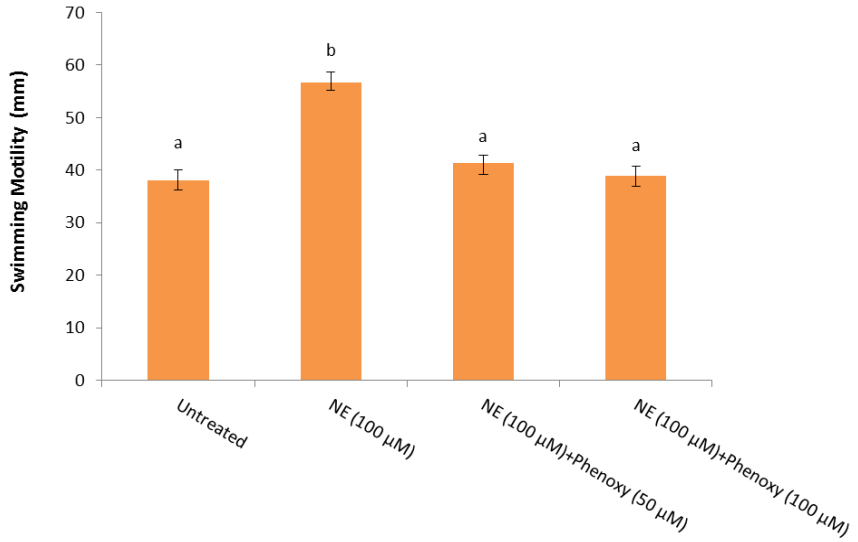


Figure 21. The impact of the catecholamines and the antagonists on swimming motility in *Y. rucheri* after 24 h incubation on soft agar **(A)** Dopamine (Dopa; 100 µM) with or without chlorpromazine (Chlo) (the dopaminergic antagonist) at 50 µM or 100 µM, **(B)** Norepinephrine (NE; 100 µM) with or without labetalol (Lab) (the α and β-adrenergic antagonist) at 50 µM or 100 µM, **(C)** Norepinephrine (NE; 100 µM) with or without propranolol (Pro) (the β-adrenergic antagonist) at 50 µM or 100 µM, **(D)** Norepinephrine (NE; 100 µM) with or without phenoxybenzamine (Phenoxy) (the α-adrenergic antagonist) at 50 µM or 100 µM. Error bars indicate the standard deviation of three replicates (ANOVA with Tukey's post-hoc test).

Table 13 The effect of different concentrations of the glucocorticoid hormones on swimming motility of *Y. ruckeri* after 24 h incubation

Code	Compound	Swimming motility (mm)		
		30 ng/ml	**300 ng/ml**	**3000 ng/ml**
Control	Control + Ethanol	41.7 ± 3.5^{a}	42.3 ± 3.0^{a}	42.7 ± 1.5^{a}
C1	20β-dihyrocortisone	52.0 ± 3.0^{b}	65.0 ± 2.6^{cd}	66.0 ± 5.3^{b}
C2	11-deoxycortisol	67.0 ± 2.0^{c}	72.3 ± 3.7^{de}	74.7± 2.5^{cd}
C3	tetrahydrocortisol	63.7 ± 3.5^{c}	65.3 ± 3.7^{d}	66.3 ± 3.8^{b}
C4	11-deoxycorticosterone	50.3 ± 2.5^{ab}	56.0 ± 2.0^{bc}	67.0 ± 3.0^{bc}
C5	cortisone	53.7 ± 1.1^{b}	56.0 ± 1.0^{bc}	60.7 ± 4.0^{b}
C6	cortisol	67.3 ± 4.6^{c}	70.0 ± 5.0^{de}	76.0.0 ± 2.0^{cd}
C7	tetrahydrocortisone	50.3 ± 3.0^{ab}	53.7 ± 4.0^{b}	68.0 ± 2.6^{bcd}
C8	17α-hydroxyprogesterone	63.7 ± 4.5^{c}	65.7 ± 2.5^{d}	66.3 ± 3.2^{b}
C9	corticosterone	72.7 ± 3.0^{c}	76.7 ± 1.5^{e}	77.3 ± 3.0^{d}

Results are expressed as mean ± standard deviation of three replicates.

Values in the same column with different superscript letters are significantly different (ANOVA with Tukey's post-hoc test; $p < 0.05$)

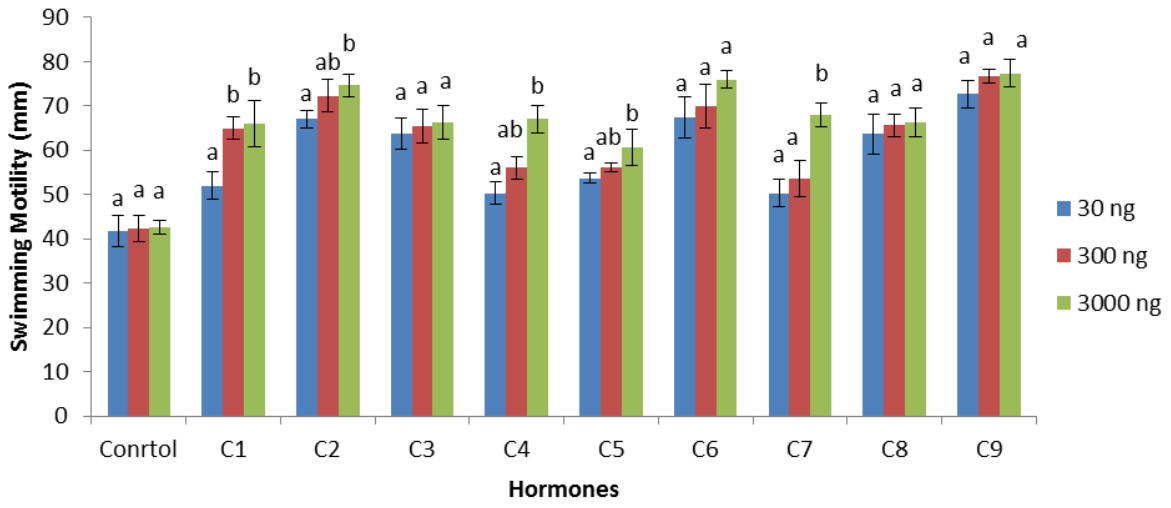
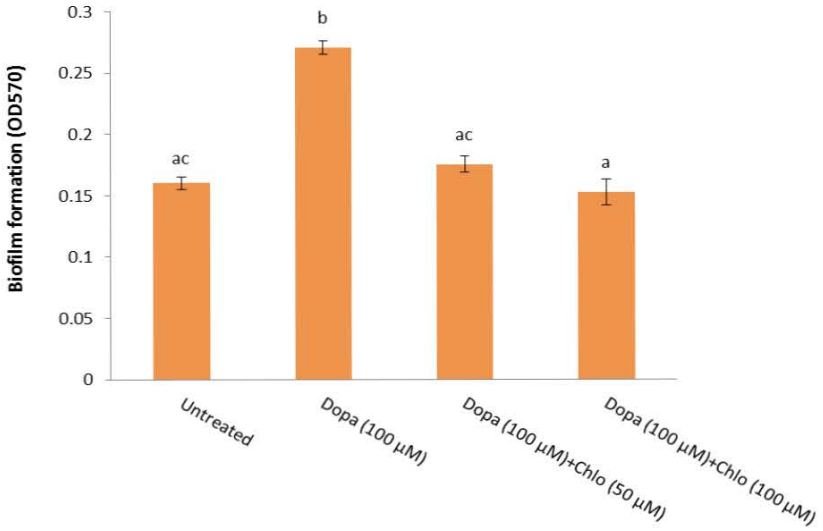
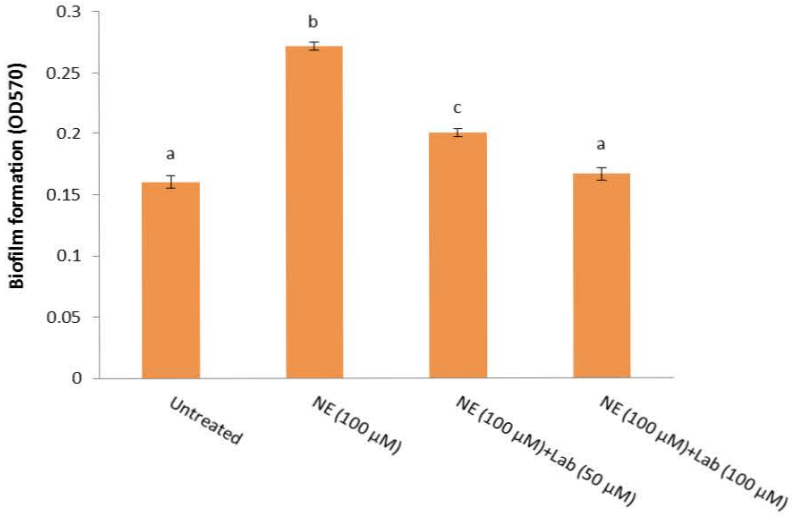


Figure 22. Swimming motility of *Y. ruckeri* with different concentrations of glucocorticoid hormones. Error bars represent the standard deviation of three replicates. Different letters above the columns show significant difference among three concentrations in each treatment (ANOVA with Tukey's post-hoc test; *$p < 0.05$*)

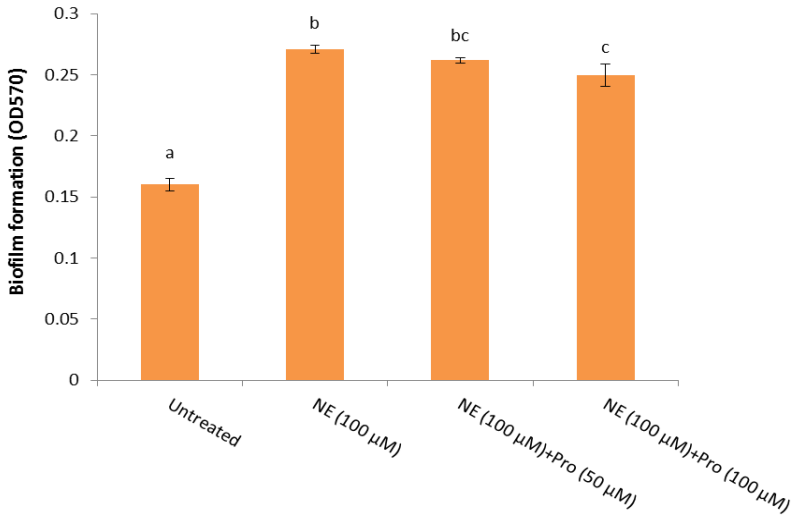
(A)


Biofilm formation (OD570)
0.3
0.25
0.2
0.15
0.1
0.05
0
ac
b
ac
a
Untreated
Dopa (100 µM)
Dopa (100 µM)+Chlo (50 µM)
Dopa (100 µM)+Chlo (100 µM)

(B)


Biofilm formation (OD570)
0.3
0.25
0.2
0.15
0.1
0.05
0
a
b
c
a
Untreated
NE (100 µM)
NE (100 µM)+Lab (50 µM)
NE (100 µM)+Lab (100 µM)

(C)



(D)

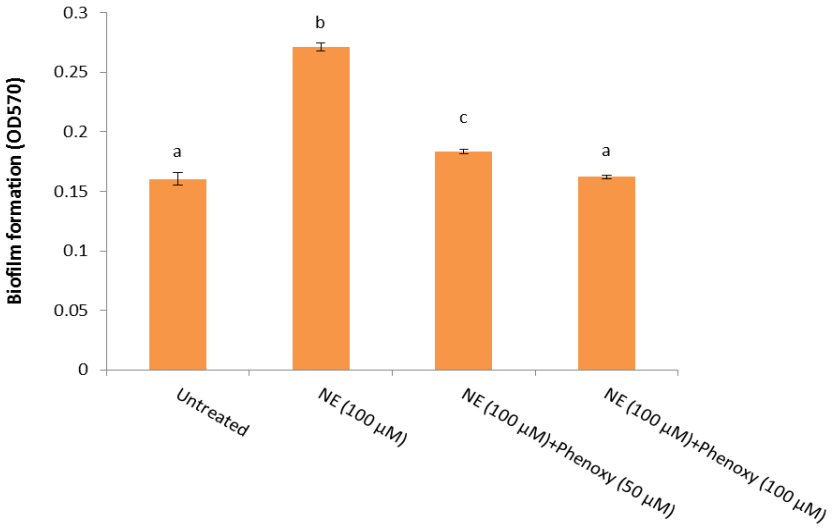


Figure 23. The impact of the catecholamines and antagonists on biofilm formation by *Y. rucheri* **(A)** Dopamine (Dopa; 100 µM) with or without chlorpromazine (Chlo) (the dopaminergic antagonist) at 50 µM or 100 µM, **(B)** Norepinephrine (NE; 100 µM) with or without labetalol (Lab) (the α- and β-adrenergic antagonist) at 50 µM or 100 µM, **(C)** Norepinephrine (NE; 100 µM) with or without propranolol (Pro) (the β-adrenergic antagonist) at 50 µM or 100 µM, **(D)** Norepinephrine (NE; 100 µM) with or without

phenoxybenzamine (Phenoxy) (the α-adrenergic antagonist) at 50 μM or 100 μM. Error bars represent the standard deviation of three replicates (ANOVA with Tukey's post-hoc test; $p < 0.05$).

Table 14 The effect of different concentrations of the glucocorticoid hormones on biofilm formation by *Y. ruckeri*

Code	Compound	Biofilm formation (OD_{570})		
		30 ng/ml	**300 ng/ml**	**3000 ng/ml**
Control	Control + Ethanol	0.15 ± 0.00^{a}	0.15 ± 0.00^{a}	0.15 ± 0.00^{a}
C1	20β-dihyrocortisone	0.17 ± 0.00^{ab}	0.17 ± 0.01^{b}	0.18 ± 0.01^{b}
C2	11-deoxycortisol	0.25 ± 0.01^{e}	0.26 ± 0.01^{e}	0.27 ± 0.01^{ef}
C3	tetrahydrocortisol	0.19 ± 0.02^{bc}	0.20 ± 0.00^{cd}	0.20 ± 0.01^{bc}
C4	11-deoxycorticosterone	0.18 ± 0.01^{b}	0.22 ± 0.01^{d}	0.24 ± 0.02^{de}
C5	cortisone	0.18 ± 0.01^{b}	0.19 ± 0.01^{bc}	0.19 ± 0.01^{b}
C6	cortisol	0.21 ± 0.01^{cd}	0.22 ± 0.01^{d}	0.25 ± 0.01^{de}
C7	tetrahydrocortisone	0.17 ± 0.00^{ab}	0.20 ± 0.01^{cd}	0.20 ± 0.01^{bc}
C8	17α-hydroxyprogesterone	0.18 ± 0.01^{b}	0.21 ± 0.01^{cd}	0.23 ± 0.01^{cd}
C9	corticosterone	0.23 ± 0.01^{de}	0.23 ± 0.01^{e}	0.28 ± 0.01^{f}

Results are expressed as mean ± standard deviation of three replicates.

Values in the same column with different superscript letters are significantly different (ANOVA with Tukey's post-hoc test; $p < 0.05$)

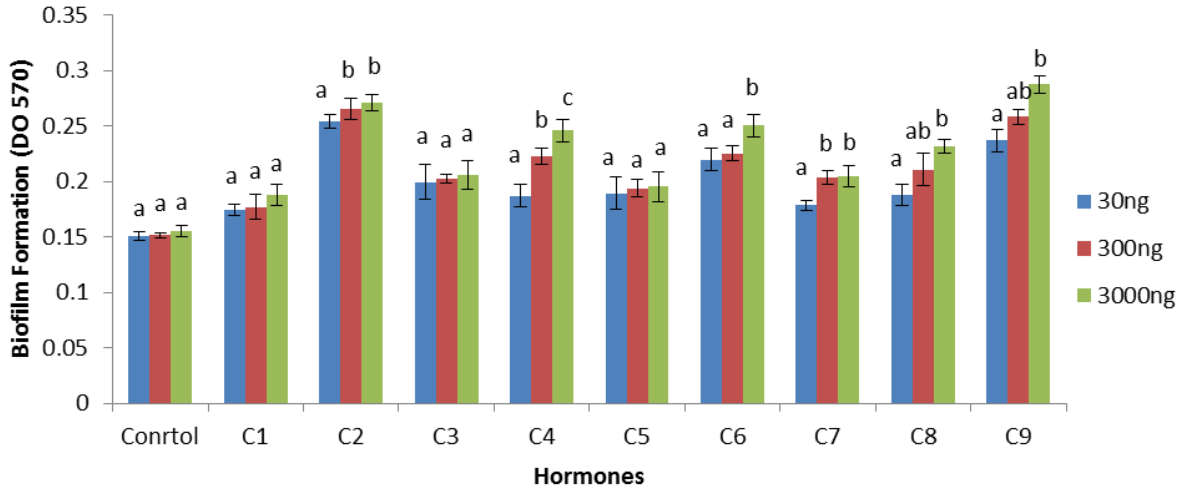


Figure 24. Biofilm formation by *Y. ruckeri* with different concentrations of the glucocorticoid hormones. Error bars represent the standard deviation of three replicates. Different letters above columns show significant difference among three concentrations in each treatment (ANOVA with Tukey's post-hoc test; $p < 0.05$).

Effects of the catecholamines and the glucocorticoids on hemolysin, protease and phospholipase activities

Finally, none of dopamine, norepinephrine and the glucocorticoids affected hemolysin, protease and phospholipase activities in *Y. ruckeri* (data not shown).

Effects of the catecholamines and the antagonists of their receptors on the virulence of *Y. ruckeri* towards rainbow trout

The *in vivo* effects of the catecholamines and the antagonists were evaluated by pretreating *Y. ruckeri* with both the catecholamines and antagonists. Phenoxybenzamine (the α-adrenergic antagonist) and labetalol (the α- and β-adrenergic antagonist) were all able to block the increased virulence induced by norepinephrine, and chlorpromazine (the dopaminergic antagonist) could inhibit the effect of dopamine. Propranolol did not show any antagonist ability to neutralize the effect of norepinephrine **(Table 15)**. Pretreatment of *Y. ruckeri* with only the antagonists resulted in no change in virulence of *Y. ruckeri* (data not shown). *Y. ruckeri* was re-isolated from the kidney of the moribund fish and identified as the cause of the mortalities.

Table 15 Impact of pretreatment of *Y. ruckeri* with the catecholamines and the antagonists on the virulence of the bacterium towards rainbow trout (average ± standard deviation of four replicates, one week after exposure to *Y. ruckeri* (10^6 CFU ml^{-1}))

Treatment	Survival (%)
Control	100^{a}
Y. ruckeri	50 ± 8^{b}
Y. ruckeri + Dopa	35 ± 6^{c}
Y. ruckeri + Dopa + Chlo100	45 ± 6^{bc}
Y. ruckeri + NE	27.5 ± 5^{c}
Y. ruckeri + NE + Lab100	47.5 ± 5^{b}
Y. ruckeri + NE + Pro100	30 ± 8^{c}
Y. ruckeri + NE + Pheno100	45 ± 6^{b}

Values with a different superscript letter are significantly different from each other (ANOVA with Tukey's post-hoc test; P < 0.05).

Dopa: dopamine; NE: norepinephrine; Chlo: chlorpromazine; Lab: labetalol; Pro: propranolol; Phenoxy: phenoxybenzamine

DISCUSSION

Yersinia ruckeri is a virulent pathogen in aquaculture worldwide. Stressful conditions are considered one of the important factors involved in the occurrence of enteric redmouth disease (Hunter et al., 1980).

A pathogen encounters a new environment upon entrance into a host, and environmental changes cause triggering the regulatory cascades that in turn influence gene expression (of virulence). Indeed, pathogens are able to detect the molecules produced by the host such as catecholamines, resulting in the production of virulence factors in bacteria (Alksne & Projan, 2000; Burton et al., 2002; Karavolos et al., 2008; Pande et al., 2014; Yang et al., 2014).

Norepinephrine and dopamine caused *Y. ruckeri* to grow in the presence of serum (which is added to mimic the host body condition through limiting the availability of iron by high affinity ferric iron-binding proteins such as transferrin (Freestone et al., 2008)), and the addition of inhibitors of eukaryotic catecholamine receptors with the catecholamines could partially block this effect. Catecholamines have been found to induce the growth of different Gram-negative bacteria (such as *Vibrio parahaemolyticus, V. mimicus, V. harvei, Aeromonas hydrophila, V. campbellii* and *V. anguillarum* in medium containing serum (Kinney et al., 1999; Lyte, 2004; Nakano et al., 2007b; Pande et al., 2014; Yang et al., 2014; Gao et al., 2018). This growth stimulation in serum-supplemented medium may be justified through facilitating the availability of Iron for bacteria by the catecholamines (Lyte, 2004; Nakano et al., 2007b). Iron is essential for some physiological functions of bacterial pathogens such as multiplication and the receiving of iron from the iron-binding protein (e.g. transferrin) is necessary for growth of bacteria when cultured in the presence of serum (Freestone et al., 2003). It has been indicated that catecholamines form complexes with transferrin, reducing the ferric iron to ferrous iron (Fe^{3+} to Fe^{2+}), an iron with a much lower affinity to iron binding proteins. Upon complex formation, the bond between iron and transferrin will be weakened and subsequently iron will be available for the bacteria (Sharaff & Freestone, 2011; Freestone, 2013). It is important to note that serum might have other effects on the pathogens in addition to limiting iron availability. For instance, serum might contain some proteins with antimicrobial activity (Zasloff, 2002).

In fish during various stressful events, the catecholamine hormones are secreted into the blood from chromaffin cells (Nilsson et al., 1976; Mazeaud et al., 1977; Nilsson, 1984; Schreck, 1990). Average of the concentration of the catecholamines in rainbow trout have been reported 2.07 ± 1.26 ϱmol ml^{-1} for norepinephrine and 1.33 ± 0.87 ϱmol ml^{-1} for dopamine (Gingerich & Drottar, 1989). Although the doses of the catecholamines (100 µM) that enhanced bacterial growth in this study were greater than what would be found systemically in the circulation, during stressful conditions at local sites these concentrations could be found and affect bacterial growth (Eaton et al., 1982; Kinney et al., 2000; Belay & Sonnenfeld, 2002; Belay et al., 2002; Burton et al.,

2002; Freestone et al., 2003). As it has been indicated that the concentration of a stress hormone may be raised by 20-40 times the physiological level; and release of 0.17-0.54 mg of stress hormone per minute has been reported as well (Genco et al., 1999).

In the current study, we demonstrated that the catecholamines norepinephrine and dopamine are able to significantly induce the swimming motility and biofilm formation of *Y. ruckeri*. Our results agree well with other studies which previously indicated that the catecholamine stress hormones were shown to increase the motility of *Campylobacter jejuni* (Cogan et al., 2007), *E. coli* (Kendall et al., 2007), *Salmonella typhimurium* (Bearson & Bearson, 2008), and the fish pathogens such as *Edwardsiella tarda* (Wang et al., 2011) *Vibrio campbellii* and *V. anguillarum* (Pande et al., 2014) and *Aeromonas hydrophila* (Gao et al., 2018). Furthermore, our findings are in agreement with previous works reporting the effect of the catecholamines on biofilm formation in *Staphylococcus epidermidis* (Lyte et al., 2003), *Vibrio campbellii* and *V. anguillarum* (Pande et al., 2014) and *V. harvei* (Yang et al., 2014).

Sandrin et al. (2014) with the same results revealed that the catecholamine was able to induce biofilm formation in *Streptococcus pneumoniae* on abiotic surfaces in media containing serum. A recent work has found that addition of a non-therapeutic level of norepinephrine (100 μM) could stimulate a high increase in growth but in contrast to our results, displayed an inhibitory effect on biofilm formation (Gonzales et al., 2013). Hence, further research is required to better understand this contradictory effect of the catecholamine on biofilm formation.

Our results indicated that the catecholamines do not influence the virulence factors of *Y. ruckeri* such as hemolysin, protease and phospholipase productions. There are some studies in good agreement with our findings which demonstrated dopamine and norepinephrine did not show any effect on lipase, phospholipase, caseinase, chitinase and hemolytic activity in *Vibrio* strains (Pande et al., 2014; Yang et al., 2014). Although, Yang et al. (2014) showed that gelatinase could be moderately affected by the catecholamines. Additionally, Gao et al. (2018) investigated the effect of norepinephrine on lipase, hemolysin and protease productions in *A. hydrophila* and found that all the virulence factors were not significantly influenced by NE except lipase activity.

Bacteria have developed systems to detect the alterations in the stress hormone values using receptors, which have been found to be specific and able to distinguish between different stress hormones (Freestone et al., 2007; Freestone et al., 2008; Sharaff & Freestone, 2011).

Moreover, antagonists of the catecholamine receptors have widely been employed to identify and characterize catecholamine receptors in mammals. Considering the roles of the catecholamines in virulence factors production and sensing these stress hormones by respective receptors in bacteria, it has been generated a hypothesis suggesting that blocking these receptors by specific antagonists may inhibit the effect of the catecholamines. Accordingly, the effects of various antagonists on the growth and production of virulence factors in *Y. ruckeri* were investigated in the current study. Our finding demonstrated that the antagonists of catecholamine receptors are able to inhibit the induction effect of the catecholamines on growth and virulence factors, indicating the existence of a regulatory system for catecholamine receptors.The same result has also been reported for some pathogens such as *E. coli* O157:H7, *Salmonella enterica*, *Yersinia enterocolitica, Vibrio harveyi, V. campbellii* and *V. anguillarum* (Freestone et al., 2007; Pande et al., 2014; Yang et al., 2014). The results demonstrated that the responses to norepinephrine could be inhibited by α- but not β-adrenergic receptor antagonists, whereas these antagonists did not show any effect on dopamine receptors. In contrast, the antagonists of dopaminergic receptors could neutralize induction caused by dopamine but did not block stimulation by norepinephrine. In the current study, the presence of putative adrenergic and dopaminergic receptors in *Y. ruckeri* was showed using α and β receptor antagonists. Similarly, it has been revealed that the response mechanisms for the catecholamines in enteric pathogens of terrestrial animals show a degree of specificity similar to mammalian catecholamine receptors (Freestone et al., 2007).

Pretreatment of the pathogen with the norepinephrine, dopamine and the antagonists was performed to prevent the direct impact of the compounds on the host body. Another point was simulating the transmission of the pathogen from a host with increased stress hormones (e.g. because of damage in cell or tissue) to a new host. Considering the

increase in swimming motility and biofilm formation caused by the catecholamines, which are important in initial stages of infection, it may be concluded that sensing the catecholamines by the pathogen enhances the chance of entrance to a new host.

The results from *in vivo* challenge assay demonstrated that pretreatment of *Y. ruckeri* with the catecholamines caused an increase in the virulence of the pathogen towards rainbow trout. Pande et al. (2014) also indicated that *V. campbellii* pretreated with norepinephrine and dopamine showed an increase in the virulence towards giant freshwater prawn larvae. Besides, the similar results obtained in another research revealed that the virulence of *V. harveyi* enhanced towards gnotobiotic brine shrimp larvae when was pretreated with the catecholamines. Gao et al. (2018) found that injection of *A. hydrophila* into crucian carp followed by NE resulted in a significant increase in fish mortality rate compared to other groups. Additionally, in our work, the antagonists were able to decrease the effect induced by the stress hormones.

Upon exposure to a stressful condition, an endocrine stress response is triggered in fish to release the glucocorticoids, particularly cortisol, into the blood (Barton, 2002; Sharaff & Freestone, 2011). In the present study, we investigated the possible effects of the glucocorticoids on the growth, swimming motility, biofilm formation and some virulence factors of *Y. ruckeri*. There are limited studies on the impact of cortisol on bacteria until now (Jentsch et al., 2013; Akcal et al., 2014; Declercq et al., 2016).

The glucocorticoids used in this study belonged to three functional categories including precursors, active hormones and metabolites (Arlt et al., 2005) **(Table 11)**. All these compounds contribute to the synthesis and metabolism processes of the glucocorticoids. Briefly, 17OH-progesterone and 11-deoxycortisol can be considered as the precursor for cortisol (as an active hormonal form). Alternatively, in case of corticosterone, 11-deoxycorticosterone acts as a precursor. Finally, tetrahydro metabolites such as tetrahydrocortisol and tetrahydrocortisone as well as 20β-hydroxycortisone, a final metabolite, are conjugated rapidly with glucuronic acid and eliminated via the exit routes.

The value of cortisol in the circulation is commonly considered an indicator of the level of conferred stress in fish (Barton & Iwama, 1991; Wendelaar, 1997). Moreover, it has

been reported that plasma cortisol amount in trout blood reaches to average values of 140 ± 5 µg l^{-1} following an acute stress situation (Bertotto et al., 2012). While we used the cortisol concentrations of 30 µg l^{-1}, 300 µg l^{-1} and 3000 µg l^{-1} in this study.

Our results revealed that all the used glucocorticoids were able to stimulate the growth in *Y. ruckeri*. This is in agreement with the reports by Akcal et al. (2014) and Jentsch et al. (2013) who indicated that the growth rate of *P. gingivalis* and *Fusobacterium nucleatum* significantly increased after the addition of cortisol in the culture medium. Besides, in relation to the effects of the glucocorticoids on biofilm formation and swimming motility in *Y. ruckeri*, our findings demonstrated that these phenotypes were influenced by these hormones and significantly increased. Franco et al. (2012) obtained the same results and demonstrated that cortisol could enhance the biofilm formation of *S. mutans in vitro*. Our findings revealed that apparently the response of *Y. ruckeri* (growth, swimming motility and biofilm formation) to some of the glucocorticoids is significantly dose-dependent, whereas other tested glucocorticoids did not show any significant effect on *Y. ruckeri* as their concentrations increased.

Further studies investigating the expression of various virulence factors in the presence of the glucocorticoids are required to better clarify the interaction between the glucocorticoids and *Y. ruckeri*.

In conclusion, it can be suggested that stress hormones such as the catecholamines and the glucocorticoids may show a specific effect on growth and virulence factors of *Y. ruckeri*. This specific effect may be one of the mechanisms involved in the interaction between stress and bacterial diseases, suggesting that these hormones can act as an additional risk factor for the development of bacterial infections in aquaculture. Further research is necessary to evaluate the possible effects of different stress hormones on the different bacteria.

CHAPTER 6

GENERAL DISCUSSION

General discussion

Currently, aquaculture is regarded as the fastest growing sector of the agriculture industry in many countries as annual fish production has enhanced from 28 to 73.8 million tons from 1998 to 2014 (FAO, 2016). Unfortunately, as the production of fish increases, so the outbreak of diseases rises. At present, disease is considered the major factor in the loss of fish in aquaculture. In 2009, 90% of all the deaths associated with farmed rainbow trout were found to result from disease (National Agricultural Statistic Service, 2010). This can often lead to notable socio-economic consequences, especially in rural areas and/or developing countries. Given the impact of disease on the industry, controlling and preventing disease is very important. ERMD is an example of serious diseases in aquaculture. Preventing ERMD was initially a successful experience in the application of an inactivated whole-cell vaccine via immersion, which successfully prevented major disease occurrence (Austin & Austin, 2007). Nevertheless, the success of the vaccine led to a decrease in research into other methods of disease control. During the 1980s, most of the studies regarding ERM were based on the fish immune system and its response towards live or inactivated *Y. ruckeri* vaccines (Austin & Austin, 2007), it continued until the early 1990s that research started to focus on the molecular analysis of the pathogenicity of *Y. ruckeri* (Romalde & Toranzo, 1993, Davies, 1991a, Davies, 1991b, Romalde et al., 1991, Furones et al., 1990).

On the other hand, antibiotic-resistant pathogens, a concern for human and animal health, lead to the urgent need for the development of alternative therapeutic strategies such as antivirulence therapy. Since virulence regulation in numerous bacterial pathogens is controlled by quorum sensing (QS), thus, QS might be considered an appropriate target for antivirulence therapy, which inhibits virulence instead of cell growth. Various microorganisms have been shown to be capable of producing small molecular QS inhibitors and/or macromolecular QQ enzymes (pathogen-pathogen signaling), which can be considered as a strategy for bacteria to obtain benefits in competitive conditions. Moreover, it has been proven that bacteria are able to detect the

stress hormones released into the host body and respond to it (pathogen-host signaling) (Freestone et al., 2007). Catecholamine stress hormones were found to induce increasing the virulence factors from the bacteria such as *V. harveyi*, *V. anguillarum and V. campbellii* (Pande et al., 2014; Yang et al., 2014) and on the other hand, antagonists of adrenergic and dopaminergic receptors of mammals can also block catecholamine-induced effects in bacteria (Freestone et al., 2007; Sharaff & Freestone, 2011).
Furthermore, there is limited research into the effects of the glucocorticoids on bacteria reporting that cortisol is able to influence the growth of bacteria such as *Porphyromonas gingivalis*, *Fusobacterium nucleatum* and *Flavobacterium columnare* (Jentsch et al., 2013; Akcal et al., 2014; Declercq et al., 2016). Hence, interference with this connection between the pathogen and the host has been proposed to be as an antivirulence therapy approach in control of diseases in aquaculture.

Chapter 3. Various bacteria belonging to different genera have been found to show AHLs degradation activity. It is not known why AHL degradation systems have been developed in bacteria. Some research has been carried out into the biological role of AHL-degrading enzymes in bacterial communities. There is some evidence indicating that the AHL degradation products may be employed as a source of nitrogen or/and carbon and an energy reservoir (Leadbetter & Greenebrg, 2000; Huang et al., 2003; Park et al., 2003; Sio et al., 2006; Yang et al., 2006; Yoon et al., 2006; Chai et al., 2007). Similarly, *V. paradoxus* is found to utilize the fatty acid released from AHL as an energy source (Leadbetter & Greenberg, 2000). The findings of some studies suggest the hypothesis that AHL-degrading enzymes of Gram-positive bacteria, e.g. AHL lactonases from *Bacillus* play a key role to inhibit the antibacterial activity of AHLs and help bacteria to survive in natural environment (Kaufmann et al., 2005). Some data demonstrated that AiiA lactonase might participate in microbial competitiveness in the rhizosphere and enhance bacterial survival on the plant root. Moreover, it has been suggested that AiiA lactonase can be involved in bacterial metabolism (Park et al., 2008). The AttM AHL lactonase from *Ag. tumefaciens* has been found to be involved in

the control of the conjugation and Ti plasmid transfer between members of its own population (Zhang et al., 2002; Carlier et al., 2003).

It is now generally accepted that the ability to inactivate QS signal molecules might be useful for controlling infection by bacterial pathogens (Defoirdt et al., 2004). In order to identify bacteria that are able to interfere with the QS system and therefore have the potential to be used as biotherapeutic microorganisms, we have isolated QQ bacteria from gill, skin and intestine of rainbow trout. Isolation of bacteria belonging to genera known to AHL degradation ability e.g. *Bacillus*, *Stentrophomonas, Agrobacterium*, *Enterobacter, Acinetobacter, Pseudomonas* (Carlier et al*.,* 2003; Shepherd & Lindow, 2009; Ma et al*.,* 2013; Chu et al*.,* 2014; Noorashikin et al*.,* 2016; Lopez et al*.,* 2017). Moreover, these microorganisms indeed have a considerable biotherapeutic potential. The isolation of strains belonging to bacteria without known QSI activity (e.g. *Citrobacter*) on the other hand, shows the added value of our investigation. It also highlights the need for further screening, since it is not unlikely that the list of QSI bacteria will grow tremendously taking into account bacterial diversity in various ecosystems (Ma et al., 2013). It should be mentioned that in the isolation step, propebly duo to the irregular use of antibiotics by farmers, the bacteria from fish were obtained after many attempts. It might be because the misuse of various antibiotics with precautionary purposes has resulted in clearance of all microflora in intestine of fish.
Apart from the identification of some bacteria inhibiting QS with biotherapeutic potential, we also demonstrated that the mechanisms of action employed in the inhibition of QS vary between isolates. We have found isolates with intracellular QQ activity as well as one isolate (*P. syrignae*) with extracellular activity as other reports on the *Pseudomonas* genus (Christiaen et al., 2011; Cheong et al., 2013). The isolates *S. maltophilia* and *P. syringae* produced and secreted heat-stable inhibitory compounds, whereas QQ activity of other isolates was stopped following heat treatment. The same phenomenon has been previously observed in *S. maltophilia* and *Pseudomonas* strains by Singh et al. (2013) and Christiaen et al. (2011). Furthermore, for *P. syrignae*, K proteinase treatment did not abolish enzymatic activity. This diversity in mechanisms offers a perspective

regarding the use or application of QQ bacteria or of the active compounds they produce.

As it was mentioned in chapter 2 in details, four QQ enzyme groups secreted by bacteria, including AHL acylase, AHL lactonase, and oxidoreductases and paraoxonases (PON), have been identified and characterized. The first defined nature QQ enzyme was AHL-lactonase encoded by the *aiiA* gene, which was detected in *Bacillus* isolate 240B1 (Dong et al., 2000). AHL lactonases inactivate signal molecules by hydrolyzing the linkage in the homoserine lactone ring of AHL molecules (Dong et al., 2000; 2001; Leadbetter, 2001). Various Gram-positive and Gram-negative bacteria have been found to carry the homologs of this enzyme. According to sequence homologies, AHL-lactonases can be divided into two main clusters including the AiiA cluster (e.g. *Bacillus*) (Dong et al., 2002) and the AttM cluster (*Ag. tumefaciens* and *K. pneumoniae* (Carlier et al., 2003; Wang et al., 2004). Moreover, AHL-lactonase targets both short and long acyl chain AHLs (Wang et al., 2004). AHL-acylases are described to irreversibly cleave the amide bond between the acyl chain and homoserine moiety of AHL signals, which results in the release of corresponding fatty acid and homoserine lactone (Leadbetter & Greenberg, 2000). These enzymes are found in several bacteria, including *Variovorax*, *Ralstonia* and *P. aeruginosa, Streptomyces* (Huang et al., 2003; Park et al*.,* 2005). In contrast to lactonases, acylases show substrate specificity in accordance with the length of the acyl chain and degrade long-chain AHLs (Lin et al., 2003). The third group of QQ enzymes, oxidoreductase, is found in a few numbers of bacteria. The oxidoreductases have been indicated to target the acyl side chain by oxidative or reductive activity and thus it acts as a catalyzer for the structural change of AHL signal without degradation (Chan et al., 2011). The structural modification in AHLs results in a change in its recognition and specificity, causing the interference with the activation of QS-related phenotypes (Uroz et al., 2003). The bacteria with oxidoreductase activity have been proposed that are able to target various long-chain AHLs with or without 3-oxo-substitutions (Uroz et al., 2005; Chowdhary et al., 2007). Paraoxonases (PON), the latter class of AHL degrading enzymes, are also known to affect AHLs by hydrolyzing the homoserine lactone ring, identified in mammals, other

vertebrates and invertebrates (Draganov et al., 2005; Elias & Tawfik, 2012). Unlike AHL-lactonases, PONs are defined to be less substrate specific (Billecke et al., 2005), however, the PON enzymes exhibit the most activity with long-chain AHLs, such as C12-oxo- HSL, but less efficient with short-chain AHL molecules (Chun et al., 2004; Yang et al., 2005).

Our QQ isolates were evaluated to detect the *aiiA* gene, indicating the AHL lactonase activity. The *aiiA* was observed in most isolates apart from *P. syringae* and *E*. *cloacae*. Previously, it has been indicated that *Pseudomonas* spp often utilizes AHL acylase to degrade signal molecules (Huang et al. 2003; Huang et al. 2006; Shepherd & Lindow 2009). Accordingly, it could be suggested that acylase enzyme is probably the main enzyme to degrade AHL molecules in *P. syringae.* To our knowledge, there is no evidence for the existence of lactonase or even acylase enzyme in *E*. *cloacae* and it seems further studies are required to reveal the AHL degradation mechanism in this species. Additionally, the presence of the *aiiA* gene is reported in *E*. *hormaechei*, *S. maltophilia* and *C. gillenii.* Previously, it has been demonstrated that the QQ activity in *Ag*. *tumefaciens* is lactonase based, encoded by *hqiA*, *attM* and *aiiB* genes (Carlier et al., 2003; Carlier et al., 2004; Torres et al., 2017). Nevertheless, in the present study, the existence of an *aiiA* gene homologue was detected in *Ag*. *tumefaciens.*

Chapter 4. Quorum sensing is a mechanism of gene regulation in which bacteria coordinate the expression of certain genes in response to the presence of small signal molecules. This regulatory mechanism has been shown to control virulence gene expression in many different pathogens, and a wide range of molecules (both of natural and synthetic origin) able to interfere with quorum sensing systems have been reported. Quorum sensing has been documented to be required for full virulence of *Aeromonas* spp. and *vibrios* towards different aquatic hosts, including fish and crustaceans (Schwenteit et al., 2011; Bjelland et al., 2012; Defoirdt & Sorgeloos, 2012).
According to previous research, in chapter 4, the link between quorum sensing and some virulence factors, biofilm formation and swimming motility in *Y. ruckeri* was investigated for the first time. We found out swimming motility and biofilm formation in *Y.*

ruckeri could be considered to be regulated by QS. Our findings also revealed that virulence factors such as protease, hemolysins and phospholipase productions are not regulated under QS system in *Y. ruckeri*. Further investigations should be performed on gene regulation of these virulence factors by quorum sensing. In the present investigation, we showed five selected QQ strains which were originally isolated from rainbow trout can inhibit the AHLs produced by *Y. ruckeri* as well as swimming motility and biofilm formation. Besides, when QQ strains were cocultured with *Y. ruckeri*, there is no effect on the growth of *Y. ruckeri*, but the production of quorum sensing molecules was decreased compared with the mono-culture of *Y. ruckeri*.

Moreover, we studied the effect of quorum quenching activity of the strains on survival of rainbow trout after a 40 days feeding trail followed by a challenge with *Y. ruckeri*. The QQs could increase survival rates of infected rainbow trout, indicating their potential as a friendly alternative to antibiotics in aquaculture. The presence of QQ bacteria in the skin, gill and the intestine of rainbow trout might be useful to facilitate contact between *Y. ruckeri* and QQ bacteria during the early stage of infection by the pathogen through immersion. Upon encounter, degradation of QS signal molecules by the QQ strains results in a reduction of the virulence of the pathogens (Dong et al., 2007; LaSarre & Federle, 2013).

Although it is more difficult to explain how the supplemented QQ strains could protect rainbow trout following a challenge by injection, as there is no opportunity for physical contact between the QQ strains and *Y. ruckeri*. The cross-kingdom interaction between the pathogen and the host might be used to justify this phenomenon. Interestingly, it has been proven that bacterial QS signal molecules are recognized by eukaryotes, resulting in physiological adaptations such as modifications of the defense system, modulation of the immune response, or alteration in the hormonal condition as well as growth (Hartmann & Schikora, 2012). There is some evidence to indicate that 3-oxo-C12-HSL and C4-HSL can act as immunosuppressive and anti-inflammatory. *In vitro* administration of 3-oxo-C12-HSL caused an inhibition in cytokine IL-12 production, attenuation of innate immunity and consequently modulation of the outcomes of the

adaptive immune response (Telford et al., 1998). These authors also demonstrated that 3-oxo-C12-HSL was able to inhibit mitogen-induced proliferation of lymphocytes, indicating that it directly affected lymphocyte immune responses. The further studies also showed the inhibitory effects of 3-oxo-C12-HSL on the proliferation and function (e.g. cytokine production) of T lymphocytes stimulated by mitogen (Chhabra et al., 2003; Ritchie et al., 2003) and T lymphocytes stimulated by antigen (Ritchie et al., 2005) as well as modulation of antibody production by B lymphocytes (Telford et al., 1998; Ritchie et al., 2003). Moreover, Holm et al. (2015) demonstrated that C4-HSL from RhlI /RhlR system of *P. aeruginosa* is able to affect the macrophage and attenuate its functions.

On the other hand, 3-oxo-C8-HSL has been detected in organs of infected fish, suggesting that these molecules can circulate in the host (Bruhn et al., 2005).Therefore, there is a possibility that QQ strains affect the concentration of AHL molecules in the host following infection by injection, consequently, causing that the clearance of *Y. ruckeri* by the immune system is facilitated. However, this is just a hypothesis and certainly, further investigations are required to understand the mode of action of the QQs as probiotic when a bacterial infection is established through injection.

Chapter 5. Host stress has been found to affect the outcome of many bacterial infections. Following exposure to a stressful condition, stress hormones such as glucocorticoids and catecholamines are produced by many higher organisms and can affect the host immune system (Verbrugghe et al., 2012). The findings of some research have indicated that catecholamines can also influence the growth and virulence of several pathogenic bacteria as well as the pathogen-host interactions. This new concept results in the development of a new research field, entitled microbial endocrinology, which is considered useful for understanding the connection between microflora and their host (Sharaff & Freestone, 2011). In Chapter 5, we evaluated the impact of two types of catecholamines (norepinephrine and dopamine) and nine different glucocorticoids, on the growth and virulence of *Y. ruckeri*. Results indicated that both catecholamines and also glucocorticoids could significantly increase the growth of *Y.*

ruckeri. Serum-supplemented media used for catecholamines is associated with the ability of catecholamines to supply the iron for the pathogen. The enhanced availability of iron, supplied by catecholamines, might be important for the effects of stress on the outcome of an infection (Verbrugghe et al., 2012). Further, we found that both catecholamines and also glucocorticoids can enhance the production of some virulence factors in *Y. ruckeri* that are required for colonization and adhesion to the host, such as swimming motility and biofilm formation. As the catecholamine and glucocorticoids levels systemically increase by stress in a host (Barton, 2002; Freestone et al., 2008; Tort, 2010), indeed, sensing the increase in concentration of stress hormones might act as an alarm to inform the pathogen to leave the host and on the other hand, enhance the success of transmittion of the pathogen to a new host.

In animals, catecholamines exert their effects by binding to specific dopaminergic and adrenergic receptors and consequently, antagonists specific to these receptors can block the binding (Freestone et al., 1999). In the present study, the results demonstrated that α- but not β-adrenergic receptor antagonists could inhibit responses to norepinephrine, but did not show any activity on dopamine-related effects. In contrast, the induction caused by dopamine was neutralized by dopaminergic receptor antagonists but was not neutralized by norepinephrine receptor antagonists, suggesting that there is a degree of specificity in bacterial response system for catecholamine recognition similar to catecholamine receptors present in animals. In respect with a bacterial catecholamine receptor, there are only a few studies reporting the existence of such receptors. Furthermore, these receptors display different activities depending on the type of pathogen, the host and stress hormone (Sharaff & Freestone, 2011). For example, it has been reported that norepinephrine and epinephrine are recognized as QS signal molecules through histidine sensor kinases (QseC and QseE) in *Escherichia coli* O157:H7 and *Salmonella enterica*, whereas norepinephrine has been found to increase the growth and virulence in *Pseudomonas aeruginosa* via the *las* QS system (Hughes et al., 2009; Pullinger et al., 2010; Hegde et al., 2009). Besides, in all the species mentioned above, catecholamines are able to regulate virulence gene

expression. These findings suggest that these receptors with ability of regulation of virulence factors could be regarded as a possible target for antivirulence therapy.

In vivo tests to evaluate the impact of stress hormones on bacterial infections in different hosts can be useful for a better understanding of the interactions between the host immune system and pathogen. In this study, we investigated the impact of norepinephrine, dopamine and the cognate receptor antagonists on the virulence of *Y. ruckeri* in rainbow trout. The pathogen was pretreated with catecholamines prior to inoculation. The pretreatment took place to simulate transmission from a host site (with high levels of catecholamines caused by stress or cell/tissue hurt) to another host. This also prevented any direct effect of the compounds on the host (such as decreased activity of the defense system), ensuring that the mortality of challenged fish was because of enhanced virulence of the pathogen. A significantly higher mortality was observed in fish following treatment of *Y. ruckeri* with norepinephrine or dopamine prior to the challenge and the specific antagonists could neutralize the induced virulence. However, pretreatment of *Y. ruckeri* with the antagonists did not show any effect on the virulence in the absence of catecholamines. There are previous works confirming our finding that indicate that the presence of catecholamines results in the increase of virulence in *V. campbellii* and *V. harveyi* in giant freshwater prawn larvae and brine shrimp larvae, respectively (Pande et al., 2014; Yang et al., 2014).

Interference with pathogen-pathogen and host-pathogen signaling, as an alternative to antibiotics, can be considered a promising approach to make resistant aquatic animals against bacterial diseases. Focusing on the inhibition of quorum sensing, we concluded that QS controls the virulence of *Y. ruckeri* towards rainbow trout. Moreover, the use of AHL-degrading bacteria is an effective way for controlling the disease. In addition to quorum sensing inhibition, interfering with host-pathogen signaling might also be an effective biocontrol strategy for aquaculture.

Future perspectives

1. Further investigations should be performed on QS-dependent gene regulation of the virulence factors in different serotypes of *Y. ruckeri*. They might produce various virulence factors depending on the nature of each serotype.
2. Farm trails would certainly be useful to evaluate the effects of the QQ bacteria on farmed fish against the bacterial infections in natural conditions.
3. From a biocontrol point of view, the efficacy of QQ isolates should be evaluated on different pathogen-pathogen interactions and the mode of action of these strains towards pathogen should be clarified. The combination of QQ approach with other treatments, e.g antibiotics should be investigated for their effects. This could potentially increase the susceptibility of bacteria to antibiotic treatments.
4. The use of live bacteria, the use of genetically modified organisms and the use of purified active compounds (enzymes or small molecules) have led to development of more QS inhibitors. Not only small molecules but also enzymes can be purified and used. For instance, it has recently been indicated that oral administration of a purified AHL lactonase from a *Bacillus* sp. strain could decrease virulence of *Aeromonas hydrophila* towards zebrafish (Cao et al., 2012). Moreover, many enzymes have also been found as drugs and are currently on the market (Vellard, 2003).
5. Previously, it was generally thought that pathogens would not show resistance to quorum sensing disruption. Recently, this assumption is challenged by Defoirdt et al. (2010) who argued that there is also a risk of resistance development using quorum sensing inhibitors. They presented an overview of data indicating that different strains of a certain species can display variation in the expression of QS related-genes. These genes play a role in the production of signal molecule synthases and receptors, as well as in signal molecules transduction. The variation of QS related-genes can be caused by a horizontal gene transfer and this variability may be associated with a difference in fitness under treatment of quorum sensing inhibitors, resulting in an automatically natural selection (Defoirdt

et al., 2010). Thereafter, this hypothesis was confirmed by some reports indicating the ability of resistance development in strains (Maeda et al., 2012; García-Contreras et al., 2013; García-Contreras et al., 2015). Further research is also required to determine the possibility of resistance to more quorum sensing disruption compounds, such as AHL-degrading enzymes.

6. Most research with respect to exploring new antivirulence strategies for preventing and treatment of bacterial diseases have focused on the inhibition of quorum sensing. However, there are some limitations to use this mechanism for instance, there is not yet a technology for practicable applications. Therefore, it is important to evaluate more mechanisms that might be potential targets for antivirulence therapy for aquaculture, including other signaling mechanisms (such as indole signaling) and host factors sensing (e.g. catecholamine or glucocorticoid stress hormones). Additionally, different antivirulence therapies could be applied synergistically to enhance the chance of success.
7. In addition to pathogen-pathogen signaling, interference with host-pathogen signaling has also been suggested as a strategy to control bacterial disease. Indeed, bacteria can detect catecholamine stress hormones by the QseC receptor. Recently, a small molecule, N-phenyl-4-{[(phenylamino) thioxomethyl] amino}-benzenesulfonamide (LED209), was identified using a high-throughput screening, inhibiting the QseC mediated activation of virulence gene expression as well as a virulence (*in vivo*) of several pathogens, such as enterohaemorrhagic *Escherichia coli*, *Salmonella typhimurium* and *Francisella tularensis* (Rasko et al., 2008). Since pathogens such as *Aeromonas*, *Edwardsiella* and *Vibrio* species have been found to respond to catecholamine stress hormones and/or to contain a QseC homologue, this compound might be useful for controlling bacterial diseases in aquaculture. Further investigations are required to find the response of *Y. ruckeri* to LED209 and the existence of QseC homologue.
8. Obligatorily, higher organisms are associated with microbial communities. It has been found that bacterial QS molecules are also sensed by eukaryotes that are

invaded by QS producing-bacteria. Some specific physiological adaptations in the colonized eukaryote can be the consequence of such the cross-kingdom interaction (Hartmann & Schikora, 2012). The eukaryotes respond to the detection of QS signal through modulation of the immune response, modifications of the defense system, or alterations in the hormonal conditions and growth responses (Telford et al. 1998; Chhabra et al., 2003; Ritchie et al., 2003; Khajanchi et al., 2011; Holm et al., 2015). Since there is no much knowledge of the effects of the cross-kingdom interaction on both sides of this interaction, therefore, it is important to more evaluate this signal sensing in different hosts and bacteria in terms of the mode of action and mutual effects caused by the interaction. The findings will be useful for a greater understanding the communications between various kingdoms and more effective application of QQ bacteria for bio-controlling the infectious diseases in eukaryotes, particularly in aquaculture.

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SUMMARY

Summary

Y. ruckeri is one of the most prominent pathogens in the aquaculture industry. It is able to infect a wide range of fish, especially rainbow trout, causing significant losses to the aquaculture industry worldwide. The inhibition of the production of virulence factors that are required to appear disease, e.g. antivirulence therapy, has been proposed as a novel strategy to control bacterial infections. The production of virulence factors in many bacterial pathogens is under strict regulatory control, and one of the regulatory mechanisms is quorum sensing, bacterial cell-to-cell communication. Disruption of quorum sensing is the most intensively studied strategy to inhibit virulence factor production. As *Y. ruckeri* produces a wide range of signal molecules, in this work, we evaluated the impact of pathogen-pathogen signaling as well as sensing of host factors on the virulence of *Y. ruckeri* in rainbow trout. First, a literature research was done on the current knowledge of *Y. ruckeri*, including the virulence, pathogenesis, and control and treatments **(Chapter 2)**. This chapter also discusses quorum sensing, quorum quenching, stress hormones such as catecholamine and glucocorticoids.

In **Chapter 3**, we isolated and characterized bacteria with quorum quenching ability from fish. We further investigated enzymatic and also extracellular/ intracellular activity in the quorum quenching (QQ) isolates. Phylogenic analysis was performed on the QQ bacteria and AHL-lactonase as well. Our results introduced some novel and known QQ bacteria with enzymatic quorum sensing molecule degradation in most isolates as QQ activity could be destroyed by heat and/or proteinase K treatments. All QQ activity proved to be cell-bound except for *Pseudomonas* sp., where it could be detected in the supernatant. The results of *aiiA* gene homology analysis revealed the presence of *aiiA* gene encoding AHL lactonase in all examined isolates except *P. syringae* and *E. cloacae*. The HXHXDH motif, conserved in all AHL lactonases and considered to be essential for AHL degradation, was detected in all AiiAs after sequence alignment.

In **Chapter 4**, we demonstrated the link of QS and virulence regulation in *Y. ruckeri* and the impact of QQ bacteria on virulence factors, motility and biofilm formation (*in vitro*). Our findings indicated that swimming motility and biofilm formation are positively

regulated by QS, whereas caseinase, phospholipase, hemolysin productions are not influenced by signal molecules. The QQs were able to decrease swimming motility and biofilm formation in *Y. ruckeri*. QQ bacteria were supplemented to trout feed for 40 days. Their probiotic effect was verified by *Y. ruckeri* challenge either by immersion or injection in trout. All strains could significantly increase fish survival. Furthermore, the putative involvement of the QQ capacity in the protection against *Yersinia* is discussed. We think these QQ strains act as probiotic candidates and are able to control ERMD in trout. Yet it is not clear if the QQ capacity is directly involved in the probiotic effect.

In **Chapter 5**, we investigated the impact of catecholamines and glucocorticoids on growth, swimming motility, biofilm formation and some virulence factors production of pathogenic *Yersinia ruckeri*. The catecholamines increased growth, swimming motility and biofilm formation of the tested strain, whereas they had no effect on caseinase, phospholipase and hemolysin activities. Further, antagonists for eukaryotic catecholamine receptors were able to neutralize some of the effects of the catecholamines. Indeed, the dopaminergic receptor antagonist chlorpromazine neutralized the effect of dopamine, the α-adrenergic receptor antagonist phenoxybenzamine and α- and β-adrenergic antagonist labetalol neutralized the effect of norepinephrine, whereas the β-adrenergic receptor antagonist propranolol had limited to no effect. Additionally, the cortisols treatments in different concentrations led to increase the growth, swimming motility and biofilm formation in *Y. ruckeri* and showed no effect on caseinase, phospholipase and hemolysin activities. Pretreatment of *Y. ruckeri* with catecholamines significantly increased its virulence towards rainbow trout.

Finally, in **Chapter 6**, the most important results obtained in this thesis are highlighted and discussed. Suggestions for further research are proposed, including perspectives on exploring novel antivirulence strategies.

In conclusion, the work presented in this thesis indicates that quorum sensing and host factors sensing regulate the virulence of *Y. ruckeri*. Interference with these systems can be regarded as novel methods for controlling the disease. Additionally, some quorum quenching bacteria isolated from rainbow trout can be a promising antivirulence agent for infections caused by *Y. ruckeri*.

چکیده

یرسینیا راکری *Yersinia ruckeri* یکی از مهمترین عوامل بیماری زا در آبزی پروری به شمار می‌رود که قادر است طیف وسیعی از ماهیان به خصوص قزل آلای رنگین کمان را آلوده نماید. مهار فاکتورهای حدت باکتری، که جهت بروز بیماری مورد نیاز هستند، با عنوان antivirulence therapy به عنوان یک روش نوین برای کنترل بیماری‌های باکتریایی مطرح شده است. تولید فاکتورهای حدت در بسیاری از باکتری‌های پاتوژن تحت کنترل سیستم‌های تنظیمی می-باشد و یکی از این مکانیسم‌های تنظیمی، کوروم سنسینگ یا ارتباط سلول به سلول است. با توجه به اینکه تعداد زیادی از باکتری‌های پاتوژن از کوروم سنسینگ جهت کنترل بیان ژن برخی از فاکتورهای حدت خود استفاده می‌کنند، مهار کوروم سنسینگ می‌تواند به عنوان یک روش جایگزین برای آنتی‌بیوتیک تراپی درنظر گرفته شود. هدف اصلی این تحقیق، یافتن باکتری‌های مهارکننده کوروم سنسینگ و بررسی اثر کاربرد آنها در کنترل و پیشگیری از بیماری‌های باکتریایی بود. از آنجا که یرسینیا راکری دامنه گسترده ای از سیگنال مولکول‌های حاضر در کوروم سنسینگ را تولید می‌کند در این پژوهش ما اثرات pathogen-pathogen sensing و نیز اثرات host factors sensing را بر تولید فاکتورهای حدت این باکتری به صورت *in vitro* و *in vivo* بررسی نمودیم.

ابتدا یک مطالعه در رابطه با اطلاعات موجود درباره یرسینیا راکری شامل فاکتورهای حدت، بیماری‌زایی، کنترل و درمان و ... انجام شد **(فصل دوم)**. این فصل همچنین حاوی مطالبی درباره کوروم سنسینگ، مهار کوروم سنسینگ (کوروم کوئنچینگ) و نیز هورمون‌های استرس (کاتکولامین‌ها و گلوکوکورتیکوئیدها) بعنوان فاکتورهای میزبان می‌باشد.

در **فصل سوم**، ما تعدادی باکتری با خاصیت کوروم کوئنچینگ از ماهی قزل آلا جداسازی و از طریق آزمایش‌های بیوشیمیایی و توالی یابی 16S rDNA شناسایی نمودیم. همچنین بررسی‌های فیلوژنیک روی این باکتری‌ها و توالی آنزیمی آنها صورت گرفت. نتایج ما منجر به معرفی تعدادی باکتری کوروم کوئنچینگ جدید و یا شناخته شده گردید. از آنجایی که خاصیت کوروم کوئنچینگ اکثر این باکتری‌ها پس از تیمار با حرارت و پروتئیناز کی از بین رفت ما نتیجه گرفتیم که اکثر این باکتری‌ها از طریق مکانیسم آنزیمی قادر هستند که سیگنال مولکول‌ها را تجزیه نمایند. به علاوه بررسی‌ها نشان داد که فعالیت کوروم کوئنچینگ در اکثر باکتری‌های جداسازی شده به استثنای *Pseudomonas* sp. ، به صورت متصل به سلول انجام می‌شود. یافته‌های بررسی‌های *aiiA* gene homolog بیانگر وجود *aiiA* gene، کدکننده آنزیم AHL لاکتوناز، در همه ایزوله‌های مورد آزمایش به استثنای *P. syringae* و *E. cloacae* است. پس از الاینمنت توالی‌های اسید آمینه، موتیف HXHXDH که در همه آنزیم‌های AHL لاکتوناز حفظ شده و مورد نیاز برای تجزیه AHL است در همه AiiA ها مشاهده گردید.

در **فصل چهارم**، ما ارتباط میان کوروم سنسینگ و تولید برخی از فاکتورهای حدت در یرسینیا راکری و همچنین اثر چند باکتری کوروم کوئنچینگ بر روی فاکتورهای حدت، حرکت و تشکیل بیوفیلم این باکتری پاتوژن بررسی نمودیم. نتایج ما نشان داد که در این باکتری، swimming motility و تشکیل بیوفیلم به طور مثبت بوسیله کوروم سنسینگ کنترل می-گردد درحالیکه فعالیت‌های کازئیناز، فسفولیپاز و همولایزین تحت تاثیر وجود سیگنال مولکول‌ها قرار نگرفت. باکتری‌های کوروم کوئنچینگ قادر بودند که میزان swimming motility و تشکیل بیوفیلم را در یرسینیا راکری بطور معنی داری کاهش دهند. ماهی‌ها به مدت ۴۰ روز با غذای حاوی چند باکتری کوروم کوئنچینگ تغذیه شدند و اثر پروبیوتیکی این باکتری‌ها با انجام چلنج با یرسینا راکری به دو روش غوطه وری و تزریق بررسی گردید. همه باکتری‌ها بطور معنی داری منجر به افزایش بقای ماهی‌ها شدند. همچنین نقش این باکتری‌ها در حفاظت ماهی‌ها در مقابل بیماری ناشی از یرسینا راکری مورد بحث قرار گرفت. اثبات اثر پروبیوتیکی مستقیم این باکتری‌ها علیه یرسینا راکری نیاز به آزمایش‌های بیشتر دارد.

در **فصل پنجم**، اثر هورمون‌های استرس کاتکولامین‌ها و گلوکوکورتیکوئیدها بر روی رشد، حرکت و تشکیل بیوفیلم و چند فاکتور حدت در یرسینا راکری بررسی گردید. کاتکوالامین‌ها بر روی رشد، swimming motility، و تشکیل بیوفیلم اثر مثبت داشتند اما تغییری در فعالیت کازئیناز، فسفولیپاز و همولایزین ایجاد ننمودند. همچنین آنتاگونیست‌های رسپتورهای کاتکولامین‌ها قادر بودند برخی اثرات این هورمون‌ها را خنثی نمایند. در واقع، chlorpromazine بعنوان یک آنتاگونیست رسپتور دوپامینرژیک اثرات دوپامین را خنثی کرد و phenoxybenzamine، یک آنتاگونیست رسپتورِ α-adrenergic، و labetalol ، آنتاگونیست رسپتورِ α-and β-adrenergic، اثرات نوراپی‌نفرین را کاهش دادند. در حالیکه، propranolol، آنتاگونیست رسپتورِ β-adrenergic، اثر محدودی داشت. همچنین، انواع گلوکوکورتیکوئیدها در سه غلظت مختلف توانستند رشد، حرکت و تشکیل بیوفیلم را در یرسینا راکری افزایش دهند اگرچه تغییری در عملکرد کازئیناز، فسولیپاز و همولازین بوجود نیامد. پیش تیمار یرسینا راکری با کاتکولامین‌ها موجب افزایش حدت باکتری در چلنج ماهی‌ها گردید. پیش تیمار یرسینا راکری با آنتاگونیست‌های رسپتور آنها (پس از تیمار با کاتکولامین‌ها) اثرات بوجود آمده را خنثی نمود.

در **فصل ششم**، مهمترین نتایج بدست آمده طی این تحقیق مورد بررسی قرار گرفت. همچنین پیشنهادهایی جهت تحقیقات آتی بخصوص در زمینه یافتن راهکارهای جدید و جایگزین برای آنتی بیوتیک تراپی مطرح گردید.

بطور خلاصه نتایج بدست آمده در این پایان نامه، نشان داد که کوروم سنیسنگ و host factors sensing قادر هستند بیماری‌زایی را در یرسنیا راکری تنظیم نمایند و ایجاد اختلال در این سیستم‌ها ممکن است بعنوان روش‌های نوین برای کنترل این بیماری درنظر گرفته شود. همچنین برخی از باکتری‌های کوروم کوئنچینگ جداشده از قزل آلا در این مطالعه می‌توانند به عنوان عوامل antivirulence در آلودگی با یرسینا راکری پیشنهاد گردند.

Samenvatting

Y. ruckeri is één van de meest prominente pathogenen in de aquacultuur industrie. Het is in staat om een brede RANGE vissen te infecteren, in bijzonder regenboogforel, waarbij wereldwijd significante verliezen worden veroorzaakt in de aquacultuur industrie. De inhibitie van de productie van virulentiefactoren die nodig zijn om ziekte te veroorzaken, dit is antivirulence therapy, werd voorgesteld als een nieuwe strategie om

bacteriële infecties te controleren. De productie van virulentiefactoren in veel bacteriële pathogenen staat onder strikte controlee en één van de regulatiemechanismen is quorum sensing, bacteriële cel-tot-cel communicatie. Onderbreking van quorum sensing is de strategie die het meest intensief wordt bestudeerd om de productie van virulentiefactoren te inhiberen. Daar *Y. ruckeri* een wijde RANGE signaalmolecules produceert, hebben we in dit werk de impakt van pathogeen-pathogeen seinen en voelen van gastheerfactoren op de virulentie van *Y. ruckeri* in regenboogforel geëvalueerd. Eerst werd een literatuurstudie uitgevoerd over de huidige kennis over *Y. ruckeri*, met inbegrip van de virulentie, pathogeniciteit en de controle en behandelingen **(Hoofdstuk 2)**. Dit hoofdstuk behandelt ook quorum sensing, quorum quenching, stress hormonen zoals catecholamine en glucocorticoiden.

In **Hoofdstuk 3** hebben we bacterën geïsoleerd en gekarakteriseerd met quorum quenching vermogen uit vissen. We hebben eveneens de enzymatische en ook de extracellulaire/ intracellulaire activiteit in dhe quorum quenching (QQ) isolaten onderzocht. Een fylogenische analysis werd uitgevoerd op de QQ bacteriën evenals AHL-lactonase. Onze resultaten leidden tot enkele nieuwe en reeds gekende QQ bacteriën met enzymatische quorum sensing molecule degradatie in de meeste isolaten daar QQ activiteit kon vernietigd worden door hitte en/of proteinase K behandelingen. Alle QQ activiteit bleek celgebonden behalve voor *Pseudomonas* sp., waar het kon gedetecteerd worden in het supernatans. De resultaten van de *aiiA* gen homologie analyse toonden de aanwezigheid van het *aiiA* gen coderend voor AHL lactonase aan in alle onderzochte isolaten behalve in *P. syringae* en *E. cloacae*. Het HXHXDH patroon, geconserveerd in alle AHL lactonases en als essentieel geschouwd voor AHL degradatie, werd gedetecteerd in alle AiiAs na sequentie alignment.

In **Hoofdstuk 4** hebben we het verband tussen QS en virulentie regulatie in *Y. ruckeri* en de impact van QQ bacteria op virulentie factoren, beweeglijkheid en biofilmformatie (*in vitro*) aangetoond. Onze bevindingen toonden aan dat zwembeweging en biofilmformatie positief gereguleerd zijn door QS, terwijl caseinase-, fosfolipase- en hemolysine-producties niet beïnvloed zijn door signaalmoleculen. De QQs waren in staat om zwembeweging en biofilmformatie in *Y. ruckeri* te verminderen. QQ bacteriën

werden toegevoegd aan forelvoedsel gedurende 40 dagen. Hun probiotisch effect werd geverifieerd aan de hand van een *Y. ruckeri* blootstelling ofwel door immersie of door injectie in forellen. Alle stammen konden de visoverleving significant verhogen. Darenboven, de mogelijke betrokkenheid van de QQ capaciteit in de bescherming tegen *Yersinia* wordt bediscussieerd. We denken dat deze QQ stammen werken als probiotische kandidaten en in staat zijn om ERMD in forel te controleren. Het is echter nog niet duidelijk of de QQ capaciteit direct betrokken is in het probiotisch effect.

In **Hoofdstuk 5** onderzochten we de impakt van catecholaminen en glucocorticoïden op de groei, zwembeweging, biofilmformatie en de productie van enkele virulentie factoren van pathogene *Yersinia ruckeri*. De catecholaminen verhoogden de groei, zwembeweging en biofilmformatie van de geteste stammen, terwijl ze geen effect hadden op caseinase, fosfolipase en hemolysine activiteiten. Verder, antagonisten voor eukaryotische catecholamine receptoren waren in staat om sommige effecten van de catecholaminen te neutraliseren. Inderdaad, de dopaminergische receptor antagonist chlorpromazine neutralizeerde het effect van dopamine, de α-adrenergische receptor antagonist phenoxybenzamine en de α- en β-adrenergic antagonist labetalol neutralizeerde het effect van norepinephrine, terwijl de β-adrenergische receptor antagonist propranolol weing tot geen effect had. Darenboven leidden de cortisolbehandelingen in verschillende concentrations tot een verhoging van de groei, zwembeweging en biofilmformatie in *Y. ruckeri* en toonden geen effect op caseinase, fosfolipase en hemolysine activiteiten. Voorbehandeling van *Y. ruckeri* met catecholaminen verhoogde significant zijn virulentie tegenover regenboogforel.

Finaal, in **Hoofdstuk 6** worden de belangrijkste resultaten, die in deze thesis werden bekomen, benadrukt en besproken. Suggesties voor verder onderzoek worden voorgesteld, onder andere perspectieven voor het verkennen van nieuwe antivirulentie strategieën.

Ter besluit, het voorgestelde werk in deze thesis toon aan dat quorum sensing en gastheerfactoren sensing de virulentie van *Y. ruckeri* beïnvloeden. Interferentie met deze systemen kan worden beschouwd als nieuwe methoden voor het beheersen van de ziekte. Darenboven zijn sommige quorum quenching bacteriën, geïsoleerd uit

regenboogforellen blijken veelbelovende antivirulentie agenten voor infecties veroorzaakt door by *Y. ruckeri*.

SUPPLEMENTARY MATERIALS

Supplementary materials

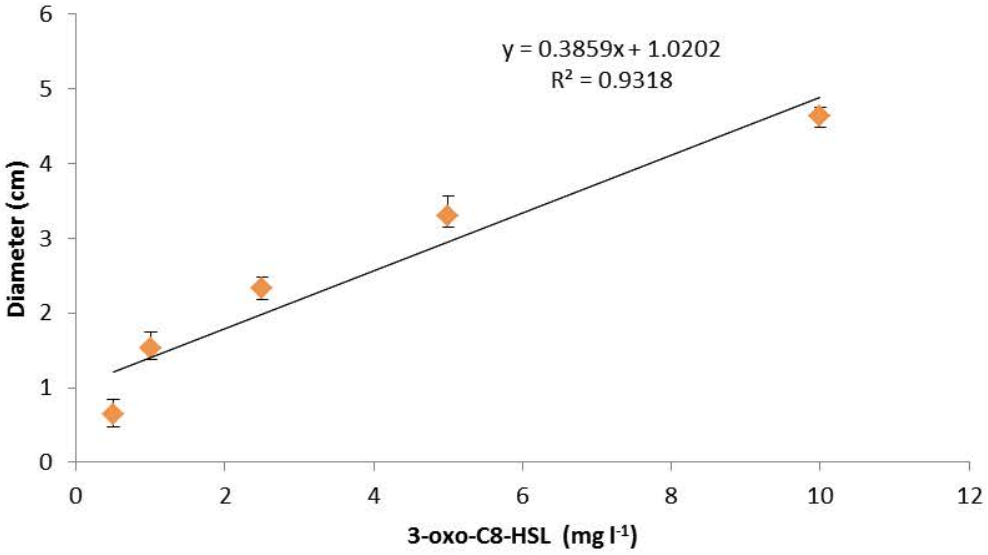
Table S1 Biochemical characteristics of quorum quenching strains

Isolate Code	Shape	Gm	Ox	Cat	bH	Mot	Nit	Ind	Cit	Urea	MR	VP	Aes	G	OF	Arab	Glu	Man	H2S	Closest species
1q	Rod shaped	-	-	+	-	+	+	-	+	+	-	+	N	+	N	-	+	+	-	*Enterobacter hormaechei*
2q	Rod shaped	-	-	+	+	+	-	-	+	+	-	-	+	+	O	-	+	-	-	*Stenotrophomonas maltophilia*
5q	Rod shaped	-	-	+	N	+	+	-	+	+	-	+	+	+	N	-	+	+	-	*Enterobacter hormaechei*
7q	Rod shaped	+	-	+	+	+	+	-	+	-	+	+	-	+	O	-	-	-	-	*B. thuringiensis*
8q	Rod shaped	+	-	+	N	+	+	N	-	-	-	-	-	+	O	-	-	-	N	*B. sp*
10q	Rod shaped	-	-	+	+	+	+	-	-	-	+	-	-	-	F	+	+	+	+	*Citrobacter gillenii*
11q	Rod shaped	+	-	+	+	+	+	-	+	-	+	+	-	+	O	-	-	-	-	*B. thuringiensis*
12q	Rod shaped	+	-	+	+	+	+	-	+	+	-	+	-	+	O	-	+	-	-	*B. cereus*
17q	Rod shaped	-	-	+	-	+	+	-	+	-	-	+	+	+	F	+	+	+	-	*Enterobacter cloacae.*
18q	Rod shaped	+	-	+	+	+	+	-	+	-	+	+	-	+	O	-	-	-	-	*B. thuringiensis*
20q	Rod shaped	+	-	+	+	+	+	-	+	+	-	+	-	+	O	-	+	-	-	*B. cereus*
22q	Rod shaped	+	-	+	+	+	+	-	+	-	+	+	-	+	O	-	-	-	-	*B. thuringiensis*
23q	Rod shaped	+	-	+	+	+	+	-	+	-	+	+	-	+	O	-	-	-	-	*B. thuringiensis*
24q	Rod shaped	+	-	+	+	+	+	-	+	+	-	+	-	+	O	-	+	-	-	*B. cereus*
26q	Rod shaped	+	-	+	+	+	+	-	+	-	+	+	-	+	O	-	-	-	-	*B. thuringiensis*
27q	Rod shaped	+	-	+	+	+	+	-	+	+	-	+	-	+	O	-	+	-	-	*B. cereus*
29q	Rod shaped	+	-	+	+	+	+	-	+	+	-	+	-	+	O	-	+	-	-	*B. cereus*
30q	Rod shaped	-	-	+	-	+	+	-	+	-	-	+	+	+	F	+	+	+	-	*Enterobacter sp.*
31q	Rod shaped	-	-	+	+	+	-	-	+	N	-	-	-	+	O	-	+	-	-	*Pseudomaonac syringae.*
32q	Rod shaped	-	+	+	+	+	-	-	+	+	-	-	+	+	O	-	+	-	-	*Stenotrophomonas maltophilia*

40q	Coccobacilli	-	-	+	-	-	-	-	-	-	-	-	-	-	O	-	-	-	-	*Acinetobacter radioresistens*
41q	Rod shaped	-	+	+	N	+	-	+	-	+	N	N	N	+	N	+	+	+	-	*Agrobacterium tumafeceins*
42q	Rod shaped	+	-	+	+	+	+	-	+	-	+	+	-	+	O	-	-	-	-	*B. thuringiensis*
43q	Rod shaped	-	+	+	+	+	-	-	+	+	-	-	+	+	O	-	+	-	-	*Stenotrophomonas maltophilia*

List of abbreviations mentioned in the above table: Cat=Catalase, Gm=Gram reaction, H_2S=Production of Hydrogen Sulfide, Mot=Motility, Nit=Nitrate reduction, Ox=Oxidase, Urea=Urease activity (Christensen's agar), bH=beta Haemolysis, VP=Voges–Proskaüer, Aes=Aesculin Hydrolysis, G=Gelatin hydrolysis, OF=Oxidative Fermentative, Arab=L-Arabinose fermentation, Glu=Glucose fermentation, Man=Manitol fermentation, MR=Methyl Red, F= Fermentation of glucose under anaerobic conditions, O= The absence of fermentation of glucose under anaerobic conditions; N= No result, (+)= Positive reaction, (-)= Negative reaction

Figure S1. Standard curve resulted from the relationship between concentrations of 3-oxo-C8-HSL and diameter of zones induced by *Chromobacterium violaceum* CV026. The error bars indicate the standard deviation of 3 replicates.



CURRICULUM VITAE

Curriculum vitae

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Sincerely yours

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