FACULTY OF BIOSCIENCE ENGINEERING

THE IMPACT OF INDOLE-3-ACETIC ACID ON VIRULENCE OF PATHOGENIC VIBRIOS

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TABLE OF CONTENTS

SUMMARY

As a consequence of the world's historically high human population showing a dietary revolution towards more fish consumption, there's an increased interest in exploiting our waters to ensure adequate food supplies for everyone (Office of Aquaculture, 2017). With capture fisheries not being able to follow this demand, aquaculture has to take over aquatic production and has become the world's fastest growing food producing sector, with an increasing contribution to global food supply and economic growth (Gutiérrez *et al.* 2020). Such great sector growth could not have been achieved without the extensive use of antibiotic compounds to control infections. However, as for terrestrial animal production, we are currently facing the adverse consequences of this rescue system.

With antibiotic use strictly selecting for resistant bacteria that spread by natural selection while removing their drug-sensitive competitors, increasing antibiotic resistance in pathogenic microorganisms forms a huge challenge for animal medicine (Defoirdt, 2013; Read & Woods, 2014). Moreover, considering the overlap between antimicrobial agents commonly used in aquaculture and in human medicine, occurrence of this (multiple) resistance severely limits therapeutic options when dealing with human infections (Heuer *et al.* 2009). Players of great interest in terms of both socio-economic impact and (multiple) drug resistance, are members of the genus *Vibrio*, especially those belonging to the Harveyi clade. Vibriosis is one of the major disease problems in shellfish and finfish aquaculture and can be held responsible for mortality of cultured shrimp worldwide (Chandrakala & Priya, 2017).

Alternative therapeutic strategies to combat the occurrence of antimicrobial resistance are expected in the field of antivirulence therapy, the inhibition of virulence factors. Virulence factors are usually gene products, involved either in direct interactions with the host tissues or in concealing the bacterial surface from the host's defence mechanisms (Wu *et al.* 2008). Important and well-documented virulence factors are swimming motility and biofilm formation, contributing to overall bacterial virulence. Regulation of virulence gene expression is achieved by quorum sensing systems, bacterial cell-to-cell communication. Such communication systems depend on the production and secretion of so-called autoinducers, chemical signalling molecules (Mok *et al.* 2003). Tricks to interfere with this regulatory system

might be of crucial importance in the near future (Defoirdt *et al.* 2011; Defoirdt, 2013).

Previous research of our own research group showed that indole signalling is a promising target for antivirulence therapy as high indole levels decrease the virulence of pathogenic vibrios (Zhang *et al.* 2022). Unfortunately, indole is toxic to invertebrates at concentrations needed for full protection and therefore, we need to find indole analogues that have the same antivirulence effect, but show lower toxicity. In this regard, the present study investigated the potency of indole-3-acetic acid, being a naturally occurring plant auxin, as quorum-sensing interfering agent. The compound was tested against twelve strains belonging to the Harveyi clade of vibrios and showed high inter-species and inter-strain variability. The addition of 200 µM indole-3-acetic acid showed a decrease in swimming motility for most of the *Vibrio parahaemolyticus*, *Vibrio harveyi* and *Vibrio campbellii* strains. It showed a slight alteration in biofilm formation behaviour and more importantly, virulence towards a host (*Artemia fransiscana*) was generally decreased in the presence of indole-3-acetic acid. Following more elaborate research and a better characterisation of molecular interactions between quorum sensing and virulence, indole-3-acetic acid could further be tested in real aquaculture settings and might lead to preventing marine vibriosis in the near future.

SAMENVATTING

Als gevolg van de historisch hoge menselijke populatie met een dieet die evolueert richting meer visconsumptie, verhoogt de interesse naar de exploitatie van onze waterige ecosystemen om voor iedereen voldoende voedsel te kunnen blijven voorzien (Office of Aquaculture, 2017). Aangezien klassieke visvangst niet aan deze vraag kan blijven voldoen, heeft aquacultuur een aanzienlijk aandeel van aquatische productie overgenomen en groeide die uit tot de snelst groeiende voedselproducerende sector, met een toenemend aandeel in globale voedselvoorziening en economische groei (Gutiérrez *et al.* 2020). Deze sterke sectorgroei was niet mogelijk geweest zonder het veelvuldig gebruik van antibiotica om infecties te bestrijden. Net zoals voor terrestrische productie, merkt men momenteel de negatieve gevolgen van dit redding systeem.

Aangezien antibioticagebruik sterk selecteert voor resistente bacteriën die via natuurlijke selectie verspreiden terwijl hun antibioticagevoelige concurrenten verwijderd worden, vormt resistentie een enorme uitdaging voor de dierlijke geneeskunde (Defoirdt, 2013; Read & Woods, 2014). Sterker nog, gezien de overlap tussen antibiotische producten algemeen gebruikt in aquacultuur en in humane geneeskunde, beperkt dit therapeutische opties bij het bestrijden van bacteriële infecties bij mensen (Heuer *et al.* 2009). Belangrijke spelers in termen van zowel socio-economische impact als resistentie, zijn leden van het genus *Vibrio*, vooral deze die behoren tot de Harveyi clade. Mariene vibriose is één van de grootste ziekteproblemen bij het kweken van schaaldieren en vinvissen en kan verantwoordelijk worden gesteld voor wereldwijde mortaliteit van garnalen (Chandrakala & Priya, 2017).

Alternatieve strategieën om het voorkomen van antibioticaresistentie tegen te gaan, worden verwacht binnen het gebied van antivirulentietherapie, de inhibitie van virulentiefactoren. Virulentiefactoren zijn over het algemeen genproducten, betrokken bij directe interacties met gastheerweefsels of het bedekken van bacteriële oppervlakken om bescherming te bieden tegen specifieke afweermechanismen van de gastheer (Wu *et al.* 2008). Belangrijke en goed gedocumenteerde virulentiefactoren zijn mobiliteit en het vormen van een biofilm. Beide processen dragen bij aan de algehele virulentie van de bacterie. Regulatie van

de genexpressie van dit soort factoren gebeurt via quorum sensing systemen, bacteriële cel tot cel communicatie, sterk afhankelijk van de productie en secretie van zogenaamde autoinducers, chemische signaalmoleculen (Mok *et al.* 2003). Manieren om the interfereren met dit regulatiemechanisme kunnen in de nabije toekomst belangrijk zijn (Defoirdt *et al.* 2011; Defoirdt, 2013).

Voorgaand onderzoek door onze eigen vakgroep toonde dat indool signalering een veelbelovend doelwit is voor antivirulentietherapie aangezien hoge indoolconcentraties de virulentie van pathogene vibrios verminderen (Zhang *et al.* 2022). De nodige concentraties voor volledige bescherming blijken spijtig genoeg toxisch voor invertebraten en daarom wordt er op zoek gegaan naar indool analogen dat hetzelfde antivirulente effect hebben, maar minder toxiciteit tonen. In deze studie wordt daarom de potentie van indool-3-azijnzuur, een natuurlijk voorkomend plantenhormoon, getest als quorum-sensing interfererende stof. De stof werd getest tegen twaalf bacteriestammen die horen tot de Harveyi clade van vibrios en toonde hoge variatie binnen verschillende soorten en stammen. Het toevoegen van 200 µM indool-3-azijnzuur toonde een vermindering in mobiliteit voor de meeste *Vibrio parahaemolyticus*, *Vibrio harveyi* en *Vibrio campbellii* stammen. Ook toonde het een lichte verandering in biofilmgedrag. Belangrijker nog, virulentie tegenover een gastheer (*Artemia fransiscana*) was over het algemeen verlaagd in de aanwezigheid van indool-3-azijnzuur. Na uitgebreider onderzoek en een betere karakterisering van moleculaire interacties tussen quorum sensing en virulentie, zou indool-3-azijnzuur verder getest kunnen worden in echte aquacultuursystemen en kan het leiden tot het bestrijden van mariene vibriose in de toekomst.

LIST OF FIGURES

LIST OF TABLES

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CHAPTER 1: LITERATURE REVIEW

1. Feeding the world: Aquaculture

We're all familiar with the concept of agriculture, being an umbrella term for activities meeting the nutritional needs of as many individuals as possible. Where agriculture tries to get the best out of farming and husbandry of terrestrial organisms, aquaculture does so for the aquatic ones. Kept species are numerous and involve fish, crustaceans, molluscs and aquatic plants but also some amphibians and reptiles (Metian *et al.* 2020).

With its 332 farmed species reported in 2017, aquaculture is the world's most diverse farming practice in terms of organism variety (Metian *et al.* 2020). This high diversity also holds when looking at farming methods and used environments. Fish can be kept in the classical pond or cage system, but an open water system or even integration with plant crops is possible. Furthermore, these systems are not bound to be applied in the sea or saltwater in general. Environments such as rain-fed natural depressions, rivers and streams, lakes and reservoirs, coastal swamplands and irrigation systems often make a good candidate (Rabanal & Delmendo, 1988).

Another interesting feature of aquaculture production is the way in which the animals are fed. Particularly in the larval stages of cultivable fish and shellfish, live feed organisms play an important role, mimicking the natural situation (Sorgeloos & Kulasekarapandian, 1984). In contrast to the natural environment, where larvae have access to a large variety of plankton organisms, the artificial food web applied in hatcheries consists of only a few species including microalgae, rotifers, the brine shrimp *Artemia* and (to a lesser extent) copepods. Especially *Artemia* popularity can be attributed to its convenience in use as their long-storage and easy-to-transfer cysts can be hatched within 24 hours (Dhondt *et al.* 2013).

Increased interest in exploiting our waters can be related to various trends, mostly related to historically high human population numbers. Moreover, a growing proportion of these people rely on fish for protein, with a doubling of per capita fish consumption worldwide in the period from 1977 to 2017 (Office of Aquaculture, 2017). To meet this demand, the mean global per capita fish production from fisheries and aquaculture has more than tripled in the same period, outpacing growth

in all other livestock sectors. With fisheries only slightly fluctuating around a production of 90 million tonnes annually (Blanchard *et al.* 2017), aquaculture has become the world's fastest growing food producing sector, with an increasing contribution to global food supply and economic growth, as seen in Figure 1 (Gutiérrez *et al.* 2020).

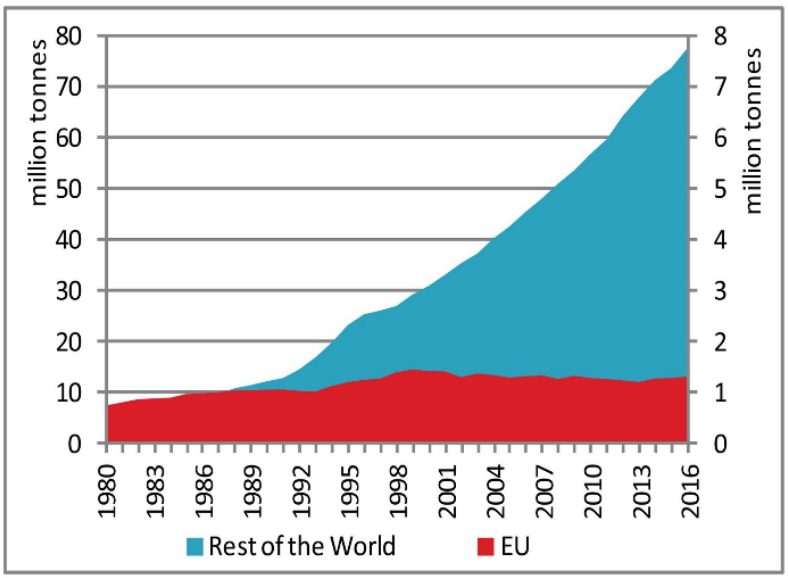


Figure 1. *Evolution of the aquaculture production in the EU (right axis) and rest of the world (left axis) for the period 1980-2016. Source: Gutiérrez et al. 2020*

More people eating more fish increases the risk of depleted global fish populations, already severely compromised by human as well as environmental factors (Office of Aquaculture, 2017). Besides for feeding the world, fish may thus be raised to stock public waters for sport and recreation or to save endangered species (Anders, 1998). It can provide full or supplemental employment and plays an important role in the production of industrial products such as carrageenan from seaweed, fish oils, medicals and pearls (Rabanal & Delmendo, 1988).

2. Diseases in aquaculture

2.1. Introduction

Living in an era where climate change and ecological burden can't be considered distant future anymore, performing aquaculture has no exception when it comes to sustainability issues. By choosing to rely on the complex ecology of the aquatic environment, the production process therefore hangs by a thread in many ways.

One of the main constraints in scaling up aquacultural production, is the emergence and spread of a wide range of bacterial, viral, parasitic and fungal infections. Live production always comprises a risk for loss due to infectious diseases, with aquaculture husbandry practices imposing a higher vulnerability for farmed fish (Moreira *et al.* 2021). After decades of preventing and restoring major economic losses due to disease outbreaks, contributing factors have been studied intensively.

Firstly, performing aquaculture inevitably involves different routes of release or introduction of pathogens. Although movement of diseased fish is never intended, some signals might go undetected, leading to the introduction of infected stocks and associated consequences (Peeler *et al.* 2007). Other ways of diseases finding their way into marine habitats are import of fish products, movement of equipment, the passing by of pathogen infected wild fish and ongoing virulence evolution (Murray & Peeler, 2005). Secondly, keeping and transferring fish in and between controlled or semi-controlled environments, brings stress for the animals, making them more vulnerable (Rehman *et al.* 2017).

Furthermore, higher water temperatures due to climate change, might be a lot less fun than we think. Proof exists that increasing water temperatures influence dynamics of some diseases by altering replication and infection patterns (Karvonen *et al.* 2010). Moreover, in most infected aquatic organisms, an increase in temperature is associated with an increase in mortality rates. For various bacterial species, including *Aeromonas spp.* and *Vibrio spp*., models predict that a temperature increase of 1°C in temperate organisms infected with bacteria could even lead to an increase of mortality of 3.87 to 6 percent (Reverter *et al.* 2020).

2.2. Socio-economic impact

By taking a step forward in terms of intensification and commercialization of aquatic products, the aquaculture industry has been overwhelmed with its share of losses due to disease outbreaks, accounting for an overall 50% of lost production capacity (Assefa & Abunna, 2018). Not only classical fish species are at risk of this unfortunate fate, events in for example molluscs and oysters have caused problems in the past as well (Bondad-Reantaso *et al.* 2005).

As discussed before, certain aquaculture actions facilitate disease transmission. Furthermore, organisms that are no serious threat to their host species in the wild,

may become highly pathogenic when found in aquaculture conditions (Rehman *et al.* 2017). The vast majority of recorded disease outbreaks in the past 50 years are either known or believed to have been caused by transboundary aquatic animal pathogens. These are exotic organisms that have been transported between countries and regions into new geographic areas, where they have flourished (Arthur, 2005).

Since aquaculture production is by a large extent dominated by Asian countries, most of the historical examples can be located in this area. Already in 1932, *Ichthyophthirius multifiliis*, cause of the so-called white spot disease, hit Java. The pathogen found its way there with the transport of infected fish and benefited from a very long drought period (Arthur, 2005). Considerable losses from white spot disease were also observed in European countries, where salmonids and later shrimp farms suffered from it (Bondad-Reantaso *et al.* 2005; Stentiford & Lightner, 2011).

Studies quantifying socio-economic and other impacts of cases similar to that of white spot disease, draw attention to the fact that disease control is crucial when wanting to perform aquaculture in a lasting and sustainable way. Extreme examples can be found in China, where three bacterial species (*Aeromonas hydrophila*, *Yersinia ruckeri* and *Vibrio fluvialis*) were capable of making more than $120 million disappear on an annual basis between 1990 and 1992 (Pridgeon & Klesius, 2021). A few years later in Japan, marine fish diseases accounted for a loss of $114.4 million, also being no exception (Bondad-Reantaso *et al.* 2005).

2.3. Vibriosis in aquaculture

Players of great interest in the field of disease of aquatic organisms, are members of the genus *Vibrio*. The *Vibrio* genus is a genetically and metabolically diverse group of heterotrophic, gram-negative, curved, rod-shaped bacteria that often account for a large fraction of the total bacterial community in natural marine environments (Castillo *et al.* 2018; Nithya & Pandian, 2010). Even though many species are free living, a small group can form pathogenic interactions with eukaryotic hosts, providing huge outbreak risks when triggered by environmental factors frequently stimulated by aquaculture practices (Chandrakala & Priya, 2017).

Vibriosis is one of the major disease problems in shellfish and finfish aquaculture and can be held responsible for mortality of cultured shrimp worldwide (Chandrakala

& Priya, 2017). Depending on the invaded strain characteristics, clinical signs of disease caused by *Vibrio* include lethargy, tissue and appendage necrosis, slow growth, slow metamorphosis, body malformation, bioluminescence, muscle opacity and melanization. Moreover, infected fish show skin discoloration, the presence of red necrotic lesions in the abdominal muscle and bloody blotches at the base of the fins, around the vent and within the mouth (Ina-Salwany *et al.* 2019; Novriadi, 2016).

For the last 10 years, both industry and public health instances are especially worried about the impact of the members of the Harveyi clade, being a rather large subset of the *Vibrio* core group. Although future adjustments are very much possible, eleven *Vibrio* species are currently documented as members of the Harveyi clade: *V. alginolyticus*, *V. parahaemolyticus*, *V. campbellii*, *V. harveyi*, *V. rotiferianus*, *V. natriegens*, *V. azureus*, *V. mytili*, *V. owensii*, *V. communis* and *V. sagamiensis* (Hoffmann *et al.* 2012; Pretto, 2020). Although not all luminescent, strains belonging to these species are usually referred to as luminescent vibrios (Defoirdt *et al.* 2007; Defoirdt *et al.* 2017).

In general, the Harveyi clade contains known pathogens of fishes, crustaceans, corals, molluscs and humans (Hoffmann *et al.* 2012). Next to their isolation from marine and estuarine surface waters and sediments, they can commonly be found as commensals on the surface or within the intestinal flora of marine animals. Strains of interest thrive as opportunistic or primary pathogens of many commercially farmed marine species (Lin *et al.* 2010).

Members of the Harveyi clade share a very high degree of genetic and phenotypic similarity, making it impossible to accurately differentiate certain sister species using traditional methods (Lin *et al.* 2010). Nevertheless, whole-genome sequencing has meanwhile reported a co-occurrence system between multiple Harveyi clade species following a vibriosis outbreak in Pacific white shrimp. Furthermore, the co-occurring species possessed numerous protein secretion systems that may contribute to interspecies competition (Bachand *et al.* 2020).

Growing interest in fighting these bacteria rises not only from ongoing multibillion dollar losses due to severe mortality of livestock but also from their association with human diseases. Examples of socio-economic and other impacts of *Vibrio* related diseases in aquaculture systems worldwide, are given in Table 1 (Novriadi, 2016).

Table 1. *Examples of socio-economic and other impacts of Vibrio related diseases in aquaculture systems. Source: Novriadi, 2016*

Country	*Vibrio* spp. caused disease	Losses and other impacts	Reference
China	*V. fluvialis*	>US$ 120M annual losses between 1990-1992	Wei (2002)
Egypt	*V. anguillarum* *V. alginolyticus* *V. ordalii* *V. harveyi*	Red spot on the ventral and lateral area Swollen and dark skin lesions, necrosis, hemorrhagic areas, exophthalmia and ulcers on the skin surface 50% mortality in Seabass and Seabream	Saad and Atallah (2014)
Indonesia	Luminescent Vibrio	>US$ 100 M in 1991 at shrimp hatcheries	APEC (2000)
Tunisia	*V. parahaemolyticus*	Darkened body color, white nodular skin lesion, and sudden death with haemorrhages in the skeletal muscle of European Seabass	Khouadja et al. (2013)
Mexico	*V. parahaemolyticus*	Acute Hepatopancreatic Necrosis Disease (AHPND) in *L. vannamei* include empty gut, anorexia, lethargy, expanded chromatophores and pale HP with discoloration	Soto-rodriquez et al. (2015)
Thailand	*V. harveyi*	Mass mortalities in *P. monodon*	Groumellec et al. (1995)
Ecuador	*V. harveyi*	Mass mortalities in *P. monodon*	Groumellec et al. (1995)
Japan	*V. carchariae*	Mass mortalities in Japanese abalone *Haliotis diversicolor*	Nishimori et al. (1998)
India	*V. harveyi*	Tail rot, erythemia, and as white patches on the body of seahorses, *Hippocampus kuda*	Raj et al. (2010)
India	*V. parahaemolyticus* *V. alginolyticus* *V. anguillarum* *V. vulnificus*	Poor growth, lethargic movements, red discoloration, and mortality in *Penaeus monodon*	Thakur et al. (2003)
Italy	*V. alginolyticus* *V. anguillarum* *V. harveyi* *V. ordalii* *V. salmonicida* *V. vulnificus*	Mass mortalities in bivalves farm located in Mar Piccolo in Taranto	Cavallo et al. (2012)
West coast of North America	*V. tubiashii*	Reduce the bivalve shellfish larval and seed production. One hatchery in their study estimated a 59% loss in 2007 production.	Elston et al. (2008)

2.4. Antibiotics and antibiotic resistance

Humanity's answer to keeping our beloved fish alive and well in this literal pool of threats, has always been the extensive use of antibiotics, without which such great sector growth could not have been achieved. Besides for therapeutic use, in regions not yet subjected to the strict regulations western countries have set up, animal feed

is frequently supplemented with low concentrations of antibiotic compounds to promote growth (Zhao *et al.* 2021). As a result of common sense and many study outcomes, it has now become very clear that this rescue system comes with its own problems.

Although very specific biological processes can filter out many antibiotic compounds (Koch *et al.* 2021; Shao & Wu, 2020), a considerable fraction is inevitably released in the environment, where it can accumulate. The pressure on watery ecosystems varies greatly according to investigated substance and organism sample, with mostly traces of sulfonamides found in surface water. According to the same Chinese freshwater study, quinolones were widely distributed in sediments and aquatic plants, whereas both quinolones and macrolides could be found in the animals, therefore also creating dietary risks (Li *et al.* 2012; Song *et al.* 2017).

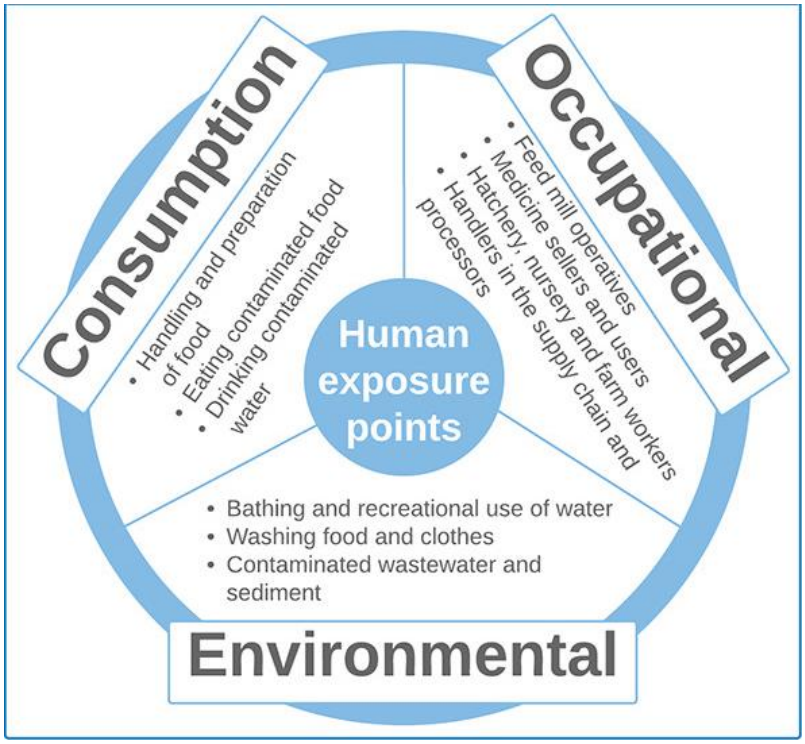


Figure 2. *Systems approach to identify routes of human exposure to antibiotics and antibiotic-resistant bacteria in aquaculture. Source: Brunton et al. 2019*

Human and animal health hazards go beyond this spread and ingestion effect, as microbes tend to fight back in a very persistent way. An overview of these routes of human exposure to antibiotics and antibiotic-resistant bacteria in aquaculture, is given in Figure 2 (Brunton *et al.* 2019). The accumulation of residual antibiotics strictly selects for local bacterial communities that can cope with their formerly toxic

environment, the well-known phenomenon of antibiotic resistance. These bacteria acquire tricks from random chromosomal mutations or horizontal gene transfer and have gained the ability to (i) vary the in- and efflux of antibiotics, (ii) modify the antibiotics or (iii) modify their targets (Petchiappan & Chatterji, 2017; Shen *et al.* 2020). General mechanisms of antibiotic resistance in bacteria are shown in Figure 3 (Kumar & Pal, 2018).

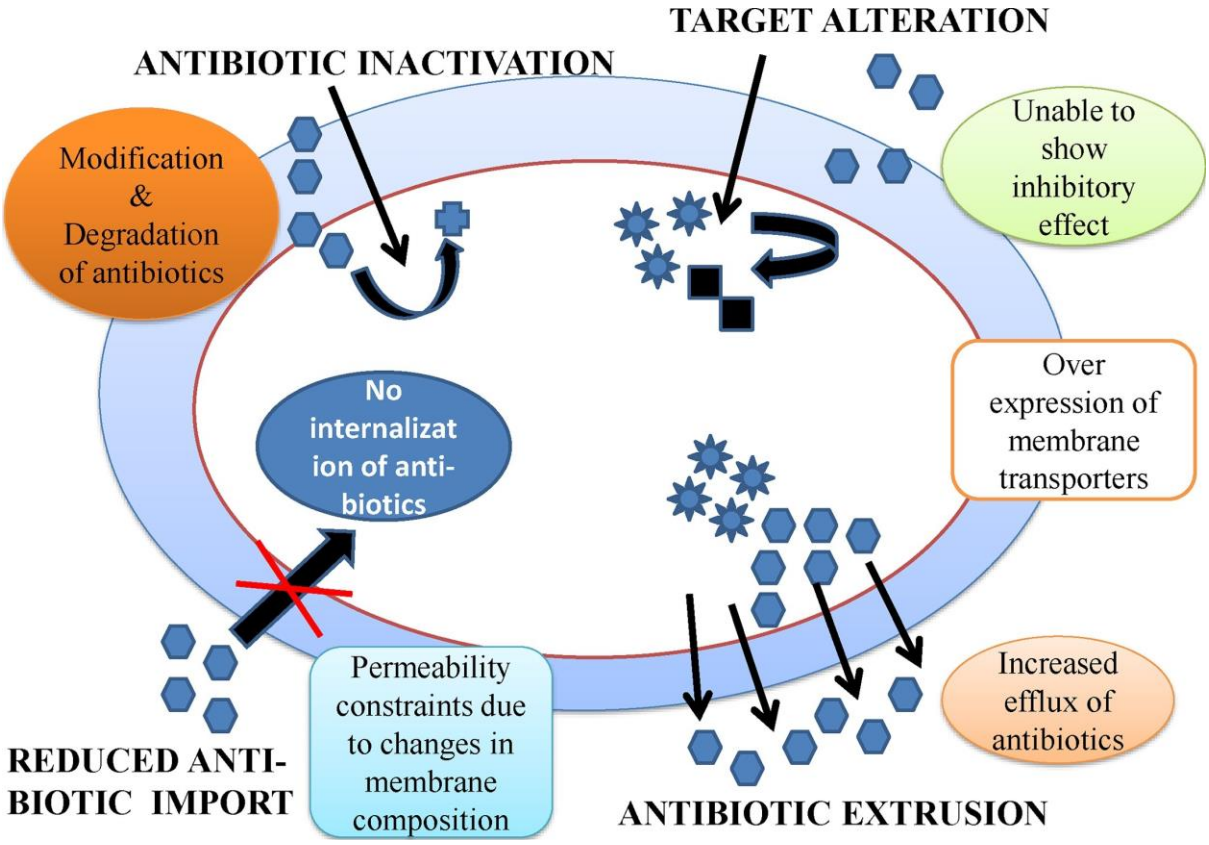


Figure 3. *Mechanisms of antibiotic resistance in bacteria. Source: Kumar & Pal, 2018*

Transmittance of both antibiotic-resistant bacteria and antibiotic-resistance genes from animal production to humans, presents a major challenge for modern medicine (Singer *et al.* 2003). From aquaculture reservoirs specifically, transfer can happen in both direct and indirect ways. Drug-resistant pathogenic bacteria finding their way into human hosts do occur, but more importantly, antibiotic-resistance genes from bacteria in the aquatic environment can disseminate by horizontal gene transfer and reach human pathogens (Heuer *et al.* 2009). Considering the overlap between antimicrobial agents commonly used in aquaculture and in human medicine, occurrence of this (multiple) resistance severely limits therapeutic options when dealing with human infections (Heuer *et al.* 2009). An overview including the

importance of resistance against different drug classes for human medicine, is given in Table 2 (Defoirdt *et al.* 2011).

Table 2. *The different classes of antibiotics used in aquaculture, their importance for human medicine and examples of (multi)resistant pathogenic bacteria isolated from aquaculture settings. Source: Defoirdt et al. 2011*

Drug class	Importance for human medecine[a]	Example	Resistant bacteria	Multiple[b] resistance?	Isolated from	Reference
Aminoglycosides	Critically important	Streptomycin	*Edwardsiella ictulari*	Yes	Diseased striped catfish (*Pangasianodon hypophthalmus*), Vietnam	[6]
Amphenicols	Important	Florfenicol	*Enterobacter* spp. and *Pseudomonas* spp.	Yes	Freshwater salmon farms, Chile	[7]
Beta-lactams	Critically important	Amoxicillin	*Vibrio* spp., *Aeromonas* spp. and *Edwardsiella tarda*	Yes	Different aquaculture settings, Australia	[8]
Beta-lactams	Critically important	Ampicillin	*Vibrio harveyi*	Yes	Shrimp farms and coastal waters, Indonesia	[9]
Fluoroquinolones	Critically important	Enrofloxacin	*Tenacibaculum maritimum*	Yes	Diseased turbot (*Scophthalmus maximus*) and sole (*Solea senegalensis*), Spain and Portugal	[10]
Macrolides	Critically important	Erythromycin	*Salmonella spp.*	Yes	Marketed fish, China	[11]
Nitrofurans	Critically important	Furazolidone	*Vibrio anguillarum*	Yes	Diseased sea bass and sea bream, Greece	[12]
Nitrofurans	Important	Nitrofurantoin	*Vibrio harveyi*	Yes	Diseased penaeid shrimp, Taiwan	[13]
Quinolones	Critically important	Oxolinic acid	*Aeromonas* spp., *Pseudomonas* spp. and *Vibrio* spp.	Yes	Pond water, pond sediment and tiger shrimp (*Penaeus monodon*), Philippines	[14]
Sulphonamides	Important	Sulphadiazine	*Aeromonas* spp.	Yes	Diseased katla (*Catla catla*), mrigel (*Cirrhinus mrigala*) and punti (*Puntius* spp.), India	[15]
Tetracyclines	Highly important	Tetracycline	*Aeromonas hydrophila*	Yes	Water from mullet and tilapia farms, Egypt	[16]
Tetracyclines	Highly important	Oxytetracycline	*Aeromonas salmonicida*	Yes	Atlantic salmon (*Salmo salar*) culture facilities, Canada	[17]

[a] On the basis of World Health Organisation Expert Consultations on 'Critically Important Antimicrobials for Human Medicine' [18•].
[b] Resistance to antibiotics belonging to different classes in at least one of the isolates.

3. Virulence mechanisms of bacterial aquaculture pathogens

3.1. Introduction

To overcome emerging antibiotic resistance worldwide, researchers seek and find alternatives every day. Before going over to identification of targets for novel drugs and design of new vaccines, it is of crucial importance to understand bacterial pathogenesis and their interactions with the host (Wu *et al.* 2008). This leads us to the concept of virulence mechanisms or virulence factors. Realizing that seemingly diverse pathogens share virulence traits increases the likelihood that novel compounds can be developed to inhibit such processes (Finlay & Falkow, 1997).

In general, the term virulence refers to a quantitative measure of the pathogenicity or the intensity of the disease caused by the pathogen (Chen *et al.* 2005). In order for this pathogen to cause disease in a susceptible host, virulence factors are needed.

These are usually gene products, involved either in direct interactions with the host tissues or in concealing the bacterial surface from the host's defence mechanisms. Bacterial virulence mechanisms can be classified into several groups, depending on mechanisms of virulence and function (Wu *et al.* 2008) and an overview is given in Figure 4 (Defoirdt, 2014).

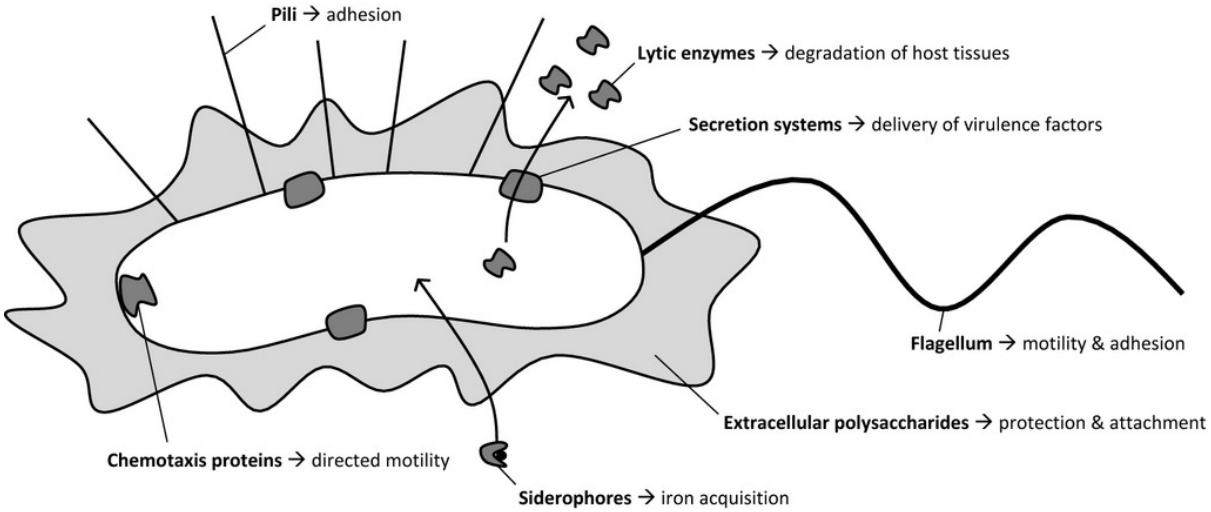


Figure 4. *Schematic overview of different virulence factors produced by pathogenic bacteria. Source: Defoirdt, 2014*

3.2. Swarming and swimming motility

Motility structures are vital in all three domains of life. Also for microbes, it's crucial to enable colonization of appropriate environments (Tsai *et al.* 2020). Six different types of surface translocation have been recognized so far, where we will speak of two. Unlike many virulence factors that have no direct macroscopic appearance, swarming and swimming motility have been observed since the very beginning of microbiological practices (Henrichsen, 1972).

Although both powered by rotating flagella, there are some fundamental differences between swimming and swarming motility, as shown in Figure 5 (Ha *et al.* 2014; Kearns, 2010). Whereas swimming motility is described as an unicellular process (Ha *et al.* 2014), swarming motility is a form of cooperative multicellular behaviour (Young *et al.* 1999). Also clearly different, is the micromorphological pattern, as seen in Figure 6 (Ha *et al.* 2014). During swarming motility, this pattern is highly organized in whirls and bands. The movement is continuous and regularly follows the long axis of the cells, with flagella being predominantly aggregated in bundles during the movement. For swimming motility, which only takes place when the film of surface

fluid is sufficiently thick, the micromorphological pattern is much more organized. The cells move individually and at random (Henrichsen, 1972).

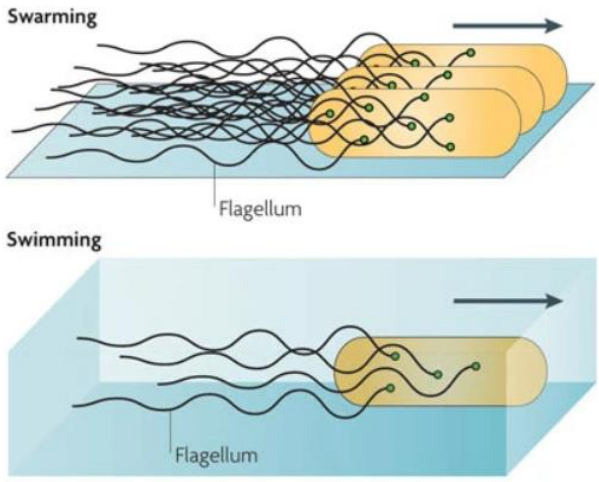


Figure 5. *Illustration of swarming and swimming motility in bacteria. Source: Kearns, 2010*

Figure 6. Occasional swarming motility (left) may interfere with interpreting swimming motility (right). Source: Ha et al. 2014

Next to their flagella, the so-called chemotaxis system is of crucial importance for both swarming and swimming motility of motile bacterial cells. Bacterial chemotaxis is known to be relatively simple, with a high sensitivity and wide dynamic range and robustness (Sourjik & Berg, 2004). In essence, cells respond to a changing environment by moving towards a beneficial gradient in terms of temperature, chemicals or electric fields. Furthermore, they avoid dangerous situations by moving away from toxic gradients (Karmakar, 2021; Taktikos *et al.* 2013). In response to these conditions, bacteria adjust the durations of their run phases, becoming longer

when moving towards an attracting source and shorter when moving towards a repelling source (Taktikos *et al.* 2013).

3.3. Production of extracellular polysaccharides and biofilm formation

Virulence of gram-negative bacterial cells benefits from the fact that their surface is covered by a variety of extracellular polysaccharides (extracellular polymeric substances, EPS) (Ayers *et al.* 1979). Major organic fractions of EPS include carbohydrates, proteins, glycoproteins, glycolipids, extracellular DNA and humic substances (Flemming *et al.* 2007).

Extracellular polysaccharides can either be secreted as a capsule or as a loose slime. Capsular polysaccharides (EPS characterized by a high molecular weight) tend to surround the bacterial cell, stimulate attachment to host cells and play an important role in immune evasion as encapsulated pathogens show an increased resistance to phagocytosis and complement-mediated killing (Ruwandeepika *et al.* 2012).

Another group of EPS, the exopolysaccharides, form a loose slime outside the cells that represents the biofilm matrix (Flemming *et al.* 2007). Dealing with multiple bacterial species and a mixture of biological compounds (consisting of about 80% EPS), biofilm formation is a complex cyclic multi-step process as shown in Figure 7 (Abebe, 2020; Khatoon *et al.* 2018). In addition to motility and adhesion factors such as flagella and pili, the biofilm formation in vibrios strongly depends on the presence of this intercellular matrix (Vanmaele *et al.* 2015).

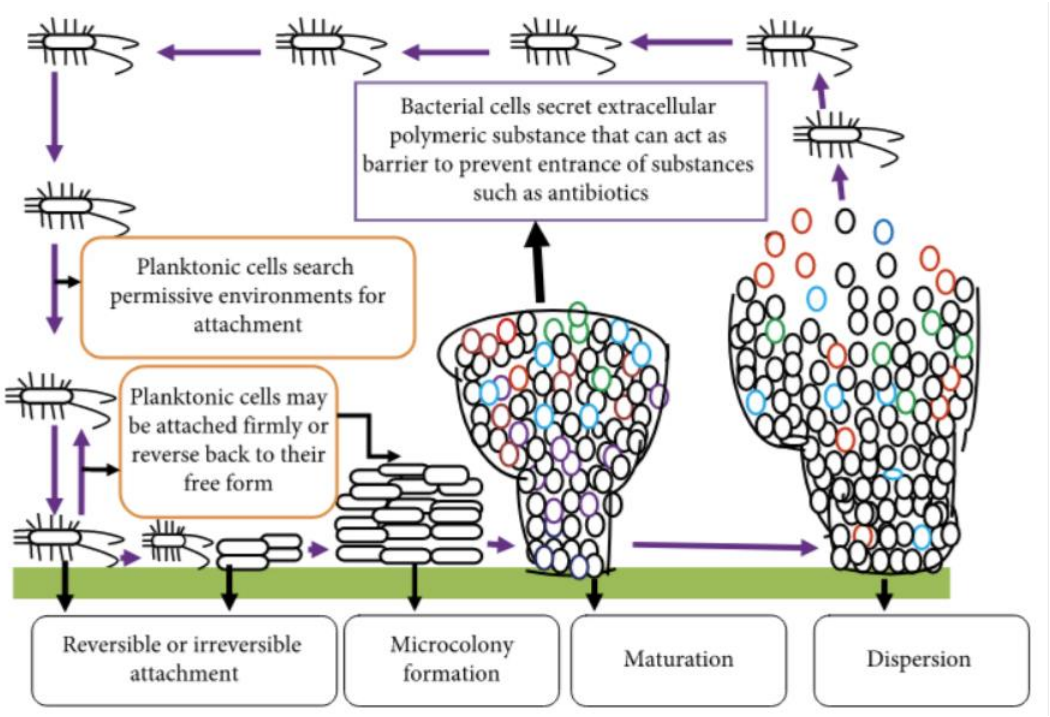


Figure 7. *Biofilm formation and structure.* ***Initial or Reversible Attachment:*** *turning point from planktonic life to biofilm mode. Bacterial structures such as fimbriae, pili and flagella give strength to the (very weak) interaction between bacteria and the surface of attachment.* ***Irreversible attachment:*** *the cell starts an irreversible adhesion and accumulates as multi-layered cell clusters. EPS play an important role in this consolidation of attachment.* ***Microcolony formation:*** *coordinated community growth. Simultaneous aggregation and growth of microorganisms, accompanied by the production of EPS.* ***Biofilm maturation:*** *if conditions suitable for sufficient growth and differentiation, biofilm develops into spatially well-arranged, three-dimensional mature biofilm structures with fluid filled channels for the diffusion of nutrients, oxygen and essential substances.* ***Dispersion:*** *bacterial cells re-enter their planktonic state. Detached bacterial cells will seek new surfaces to attach and start up a new round of biofilm formation. Source: Abebe, 2020*

Microorganisms growing in a biofilm tend to grow and survive better due to their ability to exist in a dormant state, making them tolerant to antibiotic treatment, which only acts on active cells (Lewis, 2005). Furthermore, they benefit from protection against predators and drying (Donlan & Costerton, 2002). The persistence and survival of *V. harveyi* in shrimp hatcheries, for instance, has been attributed to its ability to form biofilms with resistance to both disinfectants and antibiotics (Karunasagar *et al.* 1994). The multiple mechanisms that may contribute to community resistance within the static structure of a biofilm, are shown in Figure 8 (Vega & Gore, 2014).

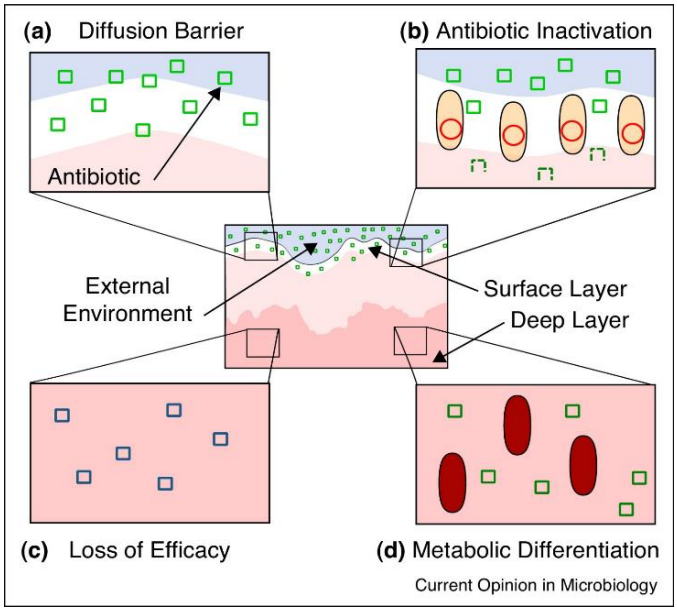


Figure 8. *Mechanisms of collective resistance in biofilms.* ***(a)*** *The matrix itself may act as a diffusion barrier, preventing the drug (green squares) from reaching its target.* ***(b)*** *Enzymatic inactivation by bacteria near the surface of the film (white layer) can protect sensitive bacteria deeper withing the structure. In the deep layers of a biofilm (dark pink layer), diffusion gradients will produce a hostile environment where* ***(c)*** *the drug itself may lose efficacy (blue squares) and* ***(d)*** *the member bacteria of the biofilm may enter altered metabolic states (red cells) inimical to antibiotic action. Source: Vega & Gore, 2014*

3.4. Production of lytic enzymes

Lytic enzymes are produced by many pathogenic bacteria and often play a central role in their pathogenesis. By causing damage to host tissues, the enzymes allow the pathogen to obtain nutrients and to spread through the tissues. The most well-known lytic enzymes produced by vibrios belonging to the Harveyi clade include haemolysins, proteases, lipases and chitinases (Ruwandeepika *et al.* 2012).

Haemolysins are among the major exotoxins identified in Harveyi clade vibrios. Next to showing haemolytic activity against erythrocytes (Mizuno *et al.* 2019), haemolysins work their magic on host cell membranes, involving either a pore-forming protein or a phospholipase enzyme (Sun *et al.* 2007). Moreover, it's often the combined action of the enzyme isoforms that contribute to the efficiency and specificity of haemolysin-mediated lysis, especially in some pathogenic vibrios such as *V. harveyi* VIB645 strain (Montanchez & Kaberdin, 2020).

Proteases constitute a second important group of lytic enzymes and have been found to digest a wide range of substrates, transforming high molecular weight polypeptides into shorter chains for an easier uptake and utilization. In pathogenic

bacteria, however, they may act as virulence factors (Salamone *et al.* 2019). As is the case for haemolysins, proteases have been linked to virulence towards both shrimp and fish. Reported proteases in Harveyi clade vibrios include metalloproteases (proteases that need a metal ion to function), serine proteases (proteases in which serine serves as the nucleophilic amino acid at the active site), cysteine proteases (proteases with a nucleophilic cysteine thiol at the active site), collagenases (proteases that are able to degrade collagen), caseinases (proteases that are able to degrade casein) and gelatinases (proteases that are able to degrade gelatin) (Ruwandeepika *et al.* 2012). In the search of exploring sources of enzymes and other biomolecules for biomedical applications, members of the genus *Vibrio* are among the most promising proteases-producing bacteria (Salamone *et al.* 2019).

Lipases on the other hand, are enzymes that are active at the water-lipid interface and break down long chain triacylglycerols into fatty acids and glycerol molecules. Although they may potentially cause damage to host tissues, very little is known about their involvement in the pathogenesis of Harveyi clade vibrios (Ruwandeepika *et al.* 2012).

In order to successfully infect their host, bacteria also have to be capable to adhere to host surfaces, more specifically the mucosal ones. This brings us to chitinases that, together with chitin-binding proteins, pili and flagella, appear to be involved in mucus adhesion next to their lytic enzyme activity breaking down chitin (Beachey, 1981). All of these adhesion mechanisms have been reported in Harveyi clade vibrios, but their actual role in infection has not yet been confirmed (Ruwandeepika *et al.* 2012).

3.5. Siderophores and iron acquisition

Iron is an essential trace element for nearly all microorganisms. However, bioavailability is limited in most marine areas (Sigel & Payne, 1982). Moreover, certain host factors further deprive microbes from iron, forcing them to secrete siderophores to cope with this shortage. A few iron-targeting host defence mechanisms are shown in Table 3 (Ganz, 2018). Siderophores are small, high-affinity compounds that serve to solubilize and transport iron into the bacterial cell under conditions of iron limitation. Different types exist, with vibrioferrin-mediated

iron uptake being reported in *Vibrio parahaemolyticus* and *Vibrio alginolyticus* (Ruwandeepika *et al.* 2012).

Table 3. *Iron-targeting host defence mechanisms. Source: Ganz, 2018*

Tissue	Molecule	Primary function
Mucosal epithelia	Lactoferrin	Iron chelator secreted by epithelial cells
	Lipocalin-2	Siderophore binder secreted by epithelial cells
Plasma and extracellular fluid	Transferrin	Iron-transporting protein, tightly binds iron
	Interleukin-6	Cytokine that induces hepcidin
	Hepcidin	Iron-regulatory hormone, degrades ferroportin
	Ferroportin	Cellular iron exporter to extracellular fluid
Phagocytes	Lactoferrin	Iron chelator secreted by neutrophils
	Lipocalin-2	Siderophore binder secreted by neutrophils
	Nramp-1	Depletes iron and manganese from phagosomes

3.6. Bioluminescence

The potency of a range of organisms to emit certain light quanta is a remarkable natural phenomenon (Burtseva *et al.* 2020). Luminescent bacteria are predominantly present in marine environments, either free living or host associated. In symbiotic associations, the ecological significance for the host has long been recognized. These higher organisms benefit from luminescence for a variety of purposes, including attraction of prey, intraspecies communication and escape from predators. Even though underlying biochemical structures are meanwhile well understood, it is not certain what specific benefit the luminescent bacteria derive from this property, meaning bioluminescence cannot (yet) be called a virulence factor (Brodl *et al.* 2018; Engebrecht *et al.* 1983).

Apart from their obvious presence in “light organs” in fish, luminescent bacteria and their close non-luminescent relatives were found to be abundant in the digestive system of fish. In this way, it may be suggested that we’re dealing with intestinal bacteria that benefit from the attraction of the organisms that will ingest them. Support for this view can be found in the fact that strains of luminous bacteria survive passage through the gut tracts of fish (Burtseva *et al.* 2020; Nealson *et al.* 1979).

3.7. Type III secretion system

To ensure that produced virulence factors can manipulate host cell function, they must reach their target inside eukaryotic host cells. Most Gram-negative bacteria make use of a bacterial protein injection machinery called the type III secretion system (T3SS) (Osei-Adjei *et al.* 2017). T3SS consists of multiple components that form needle-like pores through the inner and outer membranes, eventually delivering effector proteins from the cytoplasm of bacteria directly into the host cell cytoplasm. After successful transfer, effector proteins can perform a variety of functions, including modifying host signalling and immune response that result in disease progression (Ruwandeepika *et al.* 2015; Waters *et al.* 2010).

4. Regulation of virulence gene expression: quorum sensing systems

4.1. Introduction

As many of the previously discussed virulence mechanisms are costly metabolic products not needed throughout the entire bacterial lifespan, their production is limited in space and time. Well-described in certain members of the Harveyi clade of vibrios is bacterial cell-to-cell communication called Quorum Sensing (QS). A non-exhaustive list of reported associations between this regulatory system and specific virulence factors, not all being evenly understood or clarified, is given in Figure 9. Many of the shown mechanisms for *V. harveyi* and *V. parahaemolyticus* are also applicable for *V. campbellii*, being closely related with *V. harveyi* (Ruwandeepika *et al.* 2012).

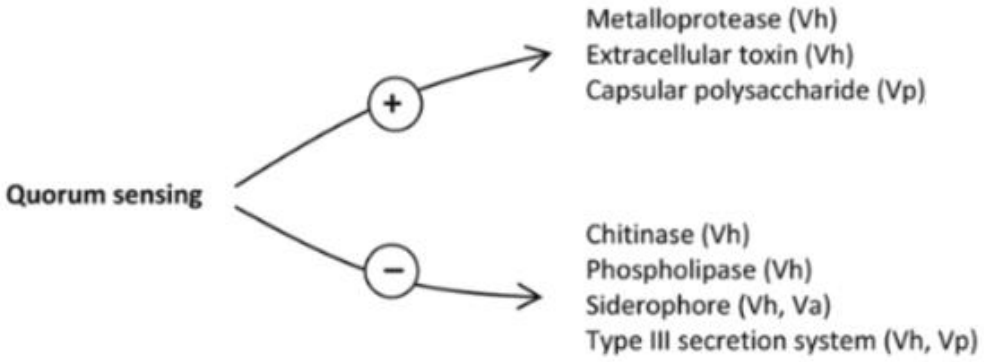


Figure 9. *Reported associations between quorum sensing and specific virulence factors for Vibrio harveyi and Vibrio parahaemolyticus. (+) Positive regulation; (-) Negative regulation; (Va) Vibrio anguillarium; (Vh) Vibrio harveyi; (Vp) Vibrio parahaemolyticus. Adapted from: Ruwandeepika et al. 2012*

4.2. Three-channel quorum sensing in *Vibrio harveyi*

The system of microorganisms sensing population density relies on the secretion and detection of so-called autoinducers, these are chemical signalling molecules. Considering many virulence factors going to waste when expressed by an individual bacterium, this trick effectively stimulates virulence and is referred to as quorum sensing (QS) (Mok *et al.* 2003). For luminescent vibrios, most information has been gained on the QS system of *V. harveyi*, but similar mechanisms can be found in strains belonging to *V. campbellii* and *V. parahaemolyticus* (Ruwandeepika *et al.* 2012).

Vibrio harveyi makes use of a three-channel quorum sensing system, mediated by the Harveyi Autoinducer 1 (HAI-1), the Autoinducer 2 (AI-2) and the Cholerae Autoinducer 1 (CAI-1). Signal molecule 1 (HAI-1) is known to be an N-acylated homoserine lactone (AHL), whereas signal system 2 (AI-2) consists of a furanosyl borate diester. CAI-1 has more recently been identified as (S)-3-hydroxytridecan-4-one (Defoirdt *et al.* 2008; Higgins *et al.* 2007; McDougald *et al.* 2003). HAI-1, AI-2 and CAI-1 are synthesised by respectively LuxM, LuxS and CqsA enzymes within the *V. harveyi* cell. The autoinducers are detected at the cell surface by membrane-bound, two-component receptor proteins (LuxN, LuxQ and CqsS) that feed a shared phosphorylation/dephosphorylation signal transduction cascade, as seen in Figure 10 (Defoirdt *et al.* 2010).

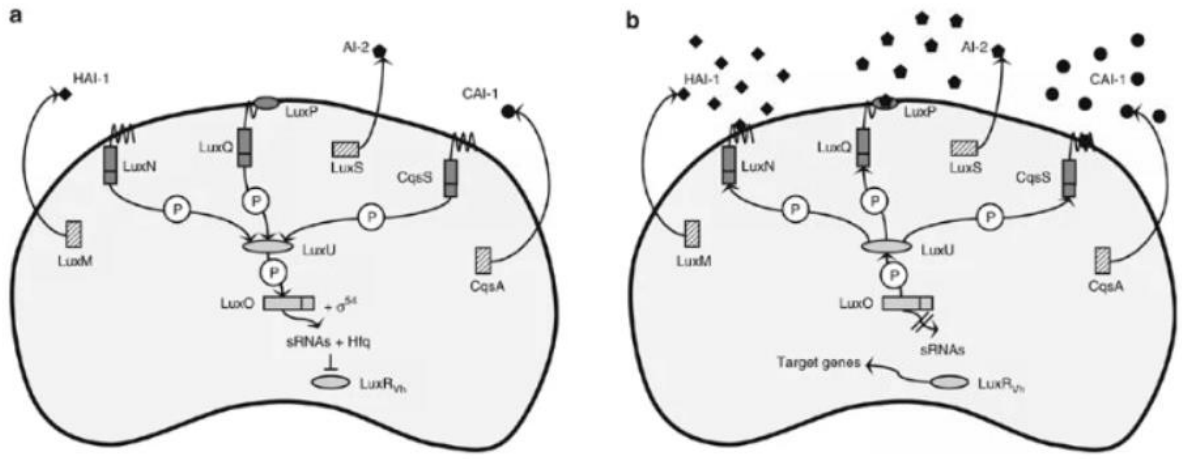


Figure 10. *Quorum sensing in Vibrio harveyi. (a) In the absence of autoinducers, the receptors autophosphorylate and transfer phosphate to LuxO via LuxU. Phosphorylation activates LuxO, which together with σ^{54} activates the production of five small regulatory RNAs (sRNAs). These sRNAs, together with the chaperone Hfq, destabilize the mRNA encoding the transcriptional regulator LuxRvh. The LuxRvh protein is thus not produced in the absence of autoinducers. (b) In the presence of high concentrations of the autoinducers, the receptor proteins switch from kinases to phosphatases, resulting in dephosphorylation of LuxO. Dephosphorylated LuxO is inactive and therefore, the sRNAs are not formed and the transcriptional regulator LuxRVh is produced. Source: Defoirdt et al. 2010*

4.3. Impact of quorum sensing on virulence

Being a transcriptional regulator, an active LuxR protein can bind promotors of various genes, including those of certain virulence factors (some associations already briefly shown in Figure 9), and activate or inactivate them (Defoirdt *et al.* 2008). By making use of specific mutations important for the QS pathway, many of the virulence factors can meanwhile be associated with this cell-to-cell communication system (Natrah *et al.* 2011).

Surprisingly, it has been found that in the presence of autoinducers (so at high cell densities), quorum sensing represses the Type III secretion system in both *V. harveyi* and *V. parahaemolyticus*, having a similar Type III secretion system (Henke & Bassler, 2004). Also repressed by the QS system are chitinase A, three phospholipase genes and siderophores (Mok *et al.* 2003; Natrah *et al.* 2011).

By assessing the swimming motility of various QS mutants compared to the wild type strain, flagellar motility has on the other hand been found to be positively regulated by QS (Yang & Defoirdt, 2015). Also haemolytic activity and V_{hp} metalloprotease can be attributed to positive regulation by quorum sensing (Hong *et al.* 2016).

4.4. Indole and indole-3-acetic acid

The emerging picture that molecules other than traditionally defined autoinducers integrate into QS circuits, has meanwhile been confirmed (Henares *et al.* 2012). Furthermore, next to self-signalling pathways, it has now become clear that prokaryotes and eukaryotes also signal one another through inter-species communication (Lee & Wood, 2007). Here, the role and functionalities of indole come into play. Indole is a natural metabolite produced by over 85 bacterial species. It's a degradation product which results from the action of the enzyme tryptophanase upon tryptophan and has meanwhile been considered to be a QS molecule (Howard *et al.* 2019; Melander *et al.* 2014; Rasmussen & Givskov, 2006).

Hence indole is an early described feature of *Escherichia coli* metabolism, early considerations about the associations between indole and bacterial physiology result from studying *E. coli* (Kim & Park, 2013). Importantly, it has been illustrated how non-indole producer *Pseudomonas aeruginosa* shows less quorum-regulated competition factors when co-cultured with *E. coli*, stating that also extracellular indole affects virulence in these bacteria (Chu *et al.* 2012). For multiple other non-indole-producing microorganisms, varying effects could meanwhile be observed, stating a very species- and strain-specific effect, as seen in Table 4 (Lee & Wood, 2015; Mueller, 2009; Yang *et al.* 2017; Zarkan *et al.* 2020).

Table 4. *Roles of indole in non-indole-producing microorganisms. Adapted from: Lee & Wood, 2015*

Bacteria	**Phenotypic change**	**Mechanism**	**Refs**
Acinetobacter oleivorans	Inhibition of biofilm formation and motility	Inhibition of QS regulator folding	Kim & Park, 2013
Agrobacterium tumefaciens	Increase of biofilm formation and antibiotic tolerance	Change of biofilm-, stress-, and efflux-related genes	Lee *et al.* 2015
Bdellovibrio bacteriovorus	Reduction of predation	Downregulation of flagellar and ribosome assembly genes	Dwidar & Mitchell, 2015
Chromobacterium violaceum, Pseudomonas chlororaphis, Serratia marcescens	Inhibition of QS-regulated pigmentation	QS inhibition	Hidalgo-Romano *et al.* 2014
Cyanobacterial *microsystis aeruginosa*	Inhibition of cyanobacterial blooms	Formation of periphyton biofilms	Wu *et al.* 2011
Pseudomonas putida	Increase of antibiotic tolerance	Activation of efflux pump	Molina-Santiago *et al.* 2014
Salmonella enterica serovar Typhimurium	Increase of antibiotic tolerance	Oxidative stress response	Vega *et al.* 2013
Salmonella enterica serovar Typhimurium	Increase of antibiotic tolerance and decrease of motility	Activation of efflux pump and suppression of flagellar genes	Blair *et al.* 2013 Nikaido *et al.* 2012

Also for *Vibrio* bacteria, virulence was proven to be altered when challenged with an extracellular concentration of indole. Originally, this was shown for *V. campbellii* BB120 (Yang *et al.* 2017). Meanwhile, positive associations were confirmed for *V. cholerae* SIO (Yang *et al.* 2017), *V.* NB10 (Li *et al.* 2014; Yang *et al.* 2017), *V. tasmaniensis* LGP32 and *V. crassostreae* J2-9 (Zhang *et al.* 2022). A more elaborate study conducted by our own research group assessed the effect of indole in 12 different strains belonging to the Harveyi clade of vibrios. Indole reduced the virulence of all strains towards gnotobiotic brine shrimp larvae. Additionally, indole significantly decreased biofilm formation in all of the strains. Swimming and swarming motility was decreased in eight and five of the strains, respectively.

Moreover, in the AHPND-causing strains (Acute Hepatopancreatic Necrosis Disease) *V. parahaemolyticus* M0904 and *V. campbellii* S01, the mRNA levels of the pirA and pirB toxin genes were downregulated. Results of this study confirm the inter-species and inter-strain variability (Zhang *et al.* 2022).

In challenging the increasing antibiotic resistance worldwide, a full understanding of these indole signalling pathways might thus be of importance (Defoirdt *et al.* 2013; Tomberlin *et al.* 2017). While antibiotic compounds bring along selective pressure while drastically disrupting pathogen-host symbioses, targeting virulence with indole does not put life or death pressure on the target bacteria (Cegelski *et al.* 2008; Tang & Zhang, 2014). Since the first discoveries in *E. coli*, it was assumed that indole was acting on the SdiA protein (Kim & Park, 2013). It has meanwhile become clear that SdiA does not respond to indole (Sabag-Daigle *et al.* 2012), leading to a model where indole acts on two sensor kinases, BaeS and CpxA (Hirakawa *et al.* 2005). For *V. cholerae*, indole was suggested to interact with DksA (Howard *et al.* 2019; Mueller, 2009).

In vitro, a concentration of 200 µM indole showed to be optimal to intervene with QS regulated virulence factors. However, this concentration seems to be toxic to invertebrates, as high mortality among brine shrimp larvae was observed during the experiments (Yang *et al.* 2017). In this way, an interesting journey lies ahead not only for indole applications. Its derivatives gain more and more attention as possible antivirulence agents, hoping to solve the toxicity problem (Melander *et al.* 2014). Interestingly, aquatic animals cultured in the presence of high levels of microalgae (producers of high levels of indole analogues), do seem to suffer from less disease incidence. In this way, similarities between the effect of indole and these analogues may be suspected (Yang *et al.* 2017). Indeed, for indole-3-acetic acid, a well-known phytohormone (auxin, Figure 11), evidence has been accumulating that even microorganisms that do not naturally produce the auxin, make use of it as a signalling molecule steering microbial behaviour (Melander et al. 2014; Yang *et al.* 2017).

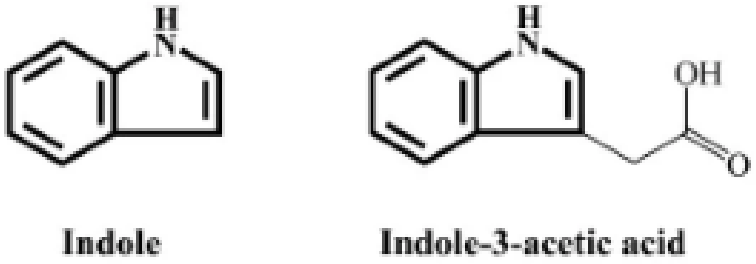


Figure 11. *Indole and indole-3-acetic acid. Indole motifs are in bold. Adapted from: Lee & Wood, 2007*

Also when looking at Harveyi clade vibrios, striking similarities between the effect of indole and indole-3-acetic acid could be found. Whereas indole-3-acetic acid had no effect on motility of *V. campbellii*, pretreatment of the pathogen with indole-3-acetic acid decreased biofilm formation and exopolysaccharide production to the same extent as was observed for indole. Furthermore, mortality in challenged brine shrimp larvae was found to decrease (Yang *et al.* 2017). In the interest of fighting marine vibriosis, it might thus be of great importance to decide on a sufficient but non-toxic concentration of indole-3-acetic acid to be used in aquaculture systems.

CHAPTER 2: OBJECTIVES

Due to the emergence and spread of antibiotic resistance in human as well as animal pathogens, alternatives for the use of antibiotic compounds are needed. One such promising non-antibiotic possible therapeutic compound is indole. Originally for *Vibrio campbellii* strain ATCC BAA-1116, it was shown that indole offers protection in challenge tests with gnotobiotic brine shrimp larvae, known model organisms for vibriosis in shrimp (Yang *et al.* 2017). However, the optimal protective concentration of indole (200 µM) appeared to be toxic to the larvae. Therefore, in a second experiment, bacterial pretreatment was proposed. *Vibrio campbellii* was incubated in the presence of 200 µM indole, after which the cultures were washed before inoculation into the brine shrimp rearing water. By performing challenge tests in this way, a significant increased survival was observed. Indole also decreased biofilm formation, exopolysaccharide production and motility of *V. campbellii* (Yang *et al.* 2017).

A more elaborate study conducted by the same research group, investigated the effects of indole on twelve different bacterial strains belonging to the Harveyi clade of vibrios, being important aquaculture pathogens (Hoffman *et al.* 2012; Zhang *et al.* 2022). Also here, it was proven that for different pathogenic strains, indole pretreatment decreased certain characteristic virulence factors and was able to protect brine shrimp larvae in the challenge tests (Zhang *et al.* 2022).

All of the forementioned observations, make indole gain more and more attention as potential therapeutic agent. However, implementing bacterial pretreatment makes results only valuable for research purposes. For this thesis, the use of indole-3-acetic acid was therefore proposed as an alternative. Indole-3-acetic acid is a very common phytohormone containing the indole structure. The effect of 200 µM indole-3-acetic acid was tested on growth, swimming motility, biofilm formation and overall virulence of different bacterial strains belonging to the Harveyi clade. Taking into account practical applications, for the challenge tests, indole-3-acetic acid was added directly into the brine shrimp rearing water. There were no bacterial pretreatments in most of the experiments.

CHAPTER 3: MATERIALS AND METHODS

3.1. Bacterial strains, culture conditions and chemicals

Bacterial strains used in this study are listed in Table 5. These strains were all preserved with glycerol (50%) at -80°C. Bacterial strains were cultured in Luria-Bertani medium containing 35 g/L of instant ocean salt (LB_{35}) at 28°C under constant agitation (120 min^{-1}). For plating experiments, the right amount of agar was dissolved in this LB_{35} liquid medium. Used artificial seawater contained 35 g/L of instant ocean synthetic sea salt from Aquarium Systems (France). Indole-3-acetic acid, purchased from Sigma-Aldrich (Belgium), was dissolved in sterile water at 200 mM and stored at 4°C until use. Indole-3-acetic acid was used at 200 µM in all of the experiments. Control groups received the same volume of sterile water (i.e., 1µL/mL).

Table 5. *Bacterial strains used in this study. LMG, Laboratory of Microbiology Collection (Ghent University, Ghent, Belgium); CAIM, Collection of Aquacultural Important Micro-organisms (CAID/Mazatlan Unit for Aquaculture, Mazatlan, Mexico). Source: Zhang et al. 2021*

Strain	Relevant features and/or synonyms	References
Vibrio parahaemolyticus		
M0904	AHPND-causing strain; isolated from the hepatopancreas of diseased shrimp (Penaeus vannamei), Mexico	Soto-Rodriguez et al. (2015)
CAIM 170	Isolated from the hemolymph of diseased shrimp (Penaeus spp.), Mexico	Defoirdt et al. (2006)
Vibrio harveyi		
E022	=STD3-101; isolated from diseased shrimp (Penaeus vannamei) larvae, Ecuador	Robertson et al. (1998)
VIB 571	Isolated from sea bass (Dicentrarchus labrax), Spain	Zhang et al. (2001)
VIB 645	Isolated from sea bass (Dicentrarchus labrax), Tunisia	Zhang et al. (2001)
LMG 22893	=CAIM148; isolated from the hemolymph of diseased shrimp (Penaeus spp.), Mexico	Defoirdt et al. (2006)
Vibrio campbelli		
S01	=20130629003S01; AHPND-causing strain; isolated from a shrimp (Penaeus vannamei) farm, China	Dong et al. (2017a)
LMG 21361	= CAIM415 = Z1; isolated from seawater from shrimp (Litopenaeus spp.) broodstock tank, Mexico	Soto-Rodriguez et al. (2003); Gomez-Gil et al. (2004)
LMG 21362	= CAIM333 = M1; isolated from seawater from shrimp (Litopenaeus spp.) broodstock tank, Mexico	Soto-Rodriguez et al. (2003); Gomez-Gil et al. (2004)
LMG 22888	= CAIM416 = Z2; isolated from seawater from shrimp (Litopenaeus spp.) broodstock tank, Mexico	Soto-Rodriguez et al. (2003); Gomez-Gil et al. (2004)
LMG 22889	= CAIM417 = Z3; isolated from seawater from shrimp (Litopenaeus spp.) broodstock tank, Mexico	Soto-Rodriguez et al. (2003); Gomez-Gil et al. (2004)
LMG 22890	= CAIM395 = STD3-131; isolated from diseased shrimp (Litopenaeus spp.) postlarvae, Ecuador	Soto-Rodriguez et al. (2003); Gomez-Gil et al. (2004)

3.2. Growth assay

To assess the impact of indole-3-acetic acid on growth of *Vibrio* bacteria, preserved strains were inoculated (1%) and grown overnight in liquid LB_{35} broth at 28°C under constant agitation (120 min^{-1}). After transfer to fresh liquid medium (1%), strains were cultured for 6 more hours. Bacterial cultures were diluted to OD_{600} of 0.1 and 1 µL of sterile water or indole-3-acetic acid (200 mM) was each time added to 1 mL of the control and test group, respectively, reaching a final indole-3-acetic acid concentration of 200 µM. After sufficient mixing, 200 µL of each sample was transferred into the wells of a 96 well plate, each treatment having 4 replicates. The plate was put into a 28°C plate reader for 24h. Cell densities were measured spectrophotometrically (600 nm) every hour and logarithmic growth curves were obtained using the software of RStudio. Manipulations were performed in a laminar flow hood in order to maintain sterility.

3.3. Quantification of swimming motility

Quantification of swimming motility was determined on LB_{35} soft agar plates containing 0.3% agar. Preserved strains were inoculated (1%) and grown overnight in liquid LB35 broth at 28°C with constant agitation (120 min^{-1}). After transfer to fresh liquid medium (1%), strains were cultured for 6 more hours and diluted to OD_{600} of 1.

After autoclaving, the LB_{35} soft agar was cooled down to approximately 50°C and indole-3-acetic acid (200 mM) was added to half of it at 1/1000, reaching a final concentration in the plates of 200 µM. The same volume of sterile water was added to the control agar. Subsequently, the agar was poured into Petri plates and the plates were air-dried with the lid open at room temperature for 15 min. Five µL aliquots of the strain suspensions were applied to the centre of the soft agar plates (six replicate plates per treatment). The plates were incubated upright at 28°C for 48h and swimming motility halo diameters were measured. Manipulations were performed in a laminar flow hood in order to maintain sterility.

3.4. Crystal violet biofilm assay

To assess the impact of indole-3-acetic acid on biofilm formation of *Vibrio* bacteria, a crystal violet assay was performed. Preserved strains were inoculated (1%) and grown overnight in liquid LB_{35} broth at 28°C under constant agitation (120 min^{-1}).

After dilution to OD_{600} of 0.01, 1 mL of the culture suspension was each time transferred into a 1.5 mL tube. Indole-3-acetic acid was added to half of the tubes in order to reach a final concentration of 200 µM. The same amount of sterile water was added to the control groups. 200 µL aliquots of these dilutions were pipetted into the wells of a polystyrene 96 well plate with 4 replicates each. Sterile medium served as negative controls. The plates were covered with a lid and cultured without agitation at 28°C for 24h.

After measuring planktonic cell density at 600 nm, unattached cells were washed away with Phosphate Buffered Saline (PBS) for three times. After short air-drying of the plates, 200 µL methanol was added per well for 20 min to fix the remaining attached bacteria. Methanol was removed and the plates were air-dried overnight. In the dry plates, 200 µL of 1% crystal violet solution was added for 15 min to each well to stain the biofilm. Plates were washed with tap water and plates were then rinsed with running water until the washings were free of the stain. Plates were air-dried overnight again.

In the dry plates, 200 µL of 95% ethanol was added to each well to dissolve the bound crystal violet. To have a homogeneous purple colour, pipetting up and down was performed during the 30 min reaction time. After this, absorbance was measured at 570 nm. Manipulations were performed in a fume hood.

3.5. Axenic hatching of brine shrimp larvae

Five hundred milligrams of high-quality hatching cysts of *Artemia fransiscana* (EGVR Type; INVE Aquaculture) were hydrated in 45 mL of filtered and autoclaved artificial seawater. After one hour, 1.65 mL of NaOH (32%) and 25 mL of NaOCl (50%) were added to facilitate decapsulation. The process was stopped after 2 minutes by adding 35 mL of autoclaved $Na_2S_2O_3$ (10 g/L). Aeration with filtered (0.22 µM) incoming air was provided during the whole reaction.

From here on, manipulations were performed in a laminar flow in order to maintain sterility. Cysts were washed with at least 2L of filtered and autoclaved artificial seawater, using a 100 µM sieve. The cysts were then re-suspended in a bottle containing 1L of filtered, autoclaved seawater and hatched for 28h at 28°C with sterile aeration (0.22 µM) and constant illumination (2000 lux).

3.6. Brine shrimp challenge tests

After axenic hatching, brine shrimp larvae were counted and 30 of them were each time transferred into 30 mL of filtered, autoclaved seawater in tubes. Cultured LVS3 bacteria were washed with seawater, autoclaved and after dilution to OD_{600} of 1, added into each tube as feed at 10^7 cells/mL (300 µL bacterial culture to 30 mL seawater).

Preserved Vibrio bacteria were inoculated (1%) and grown overnight in liquid LB_{35} broth at 28°C. After transfer to fresh liquid medium (1%), strains were cultured for 6 more hours and diluted to OD_{600} of 1. Bacterial cultures were inoculated into the brine shrimp rearing water at different concentrations. To determine these concentrations, bacterial pathogen concentrations started at 10^7 cells/mL and were gradually lowered by performing preliminary challenge tests until sufficient but not too much mortality in the samples. We aimed at reaching a survival rate in the treated group of at least 40%. After the addition of pathogens, indole-3-acetic acid was added into the rearing water of the test group at a concentration of 200 µM, each having 3 replicates. Control groups received the same volume of sterile water. Finally, the tubes were put on a rotor and kept for 2 days at 28°C under constant illumination (2000 lux).

After counting of survival of the larvae, the concentration of bacteria in the rearing water of each group was measured using flow cytometry. Pooled rearing water from control and treated groups was transferred into separate wells of a 96 well plate. The final volume in each well was 200 µL. Bacterial cultures were diluted multiple times in Phosphate Buffered Saline (PBS) and stained with SYBR Green I (SG). Plates were then put in the Accuri C6 Flow Cytometer and bacterial cell count was measured using the dilution for which an event rate between 200 and 2000 events/second was ensured.

3.7. Statistics

Data analysis was carried out using the RStudio statistical software (Version 4.1.3.). Since for small sample sizes, normality is hard to conclude, normality of data was assumed. Variances of control and test sample were compared using an F test with a significance level of 0.01. For groups with equal variances, student's *t*-tests were performed. For experiments with unequal variances, Welch *t*-tests were performed to

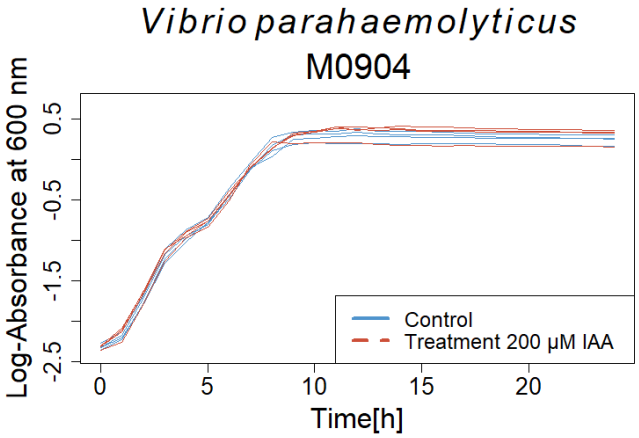
compare the treatments receiving indole-3-acetic acid to those without indole-3-acetic acid. A significance level of 0.05 was chosen to indicate statistically significant differences. A significance level of 0.01 was chosen to indicate statistically highly significant differences.

CHAPTER 4: RESULTS

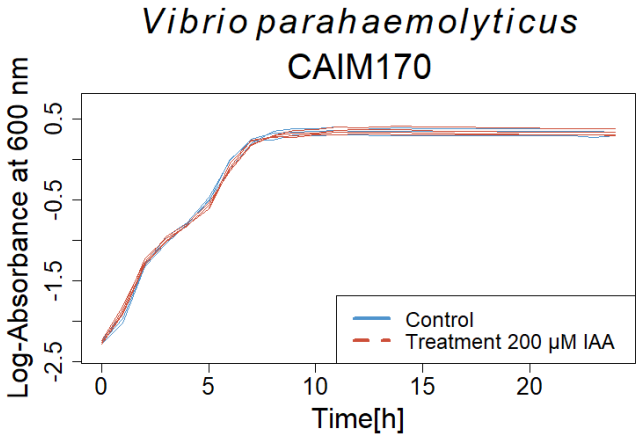
4.1. Impact of indole-3-acetic acid on growth of the vibrios

To check whether indole-3-acetic acid has an adverse effect on the growth of *Vibrio parahaemolyticus*, *V. campbellii* and *V. harveyi* strains, spectrophotometric measurements were done at 600 nm for 24h in presence or absence of indole-3-acetic acid. Obtained growth curves for each strain are shown in Figure 12. As seen from the graphs, no notable difference in growth of the pathogen in the presence of indole-3-acetic acid is observed when compared to cultures to which only sterile water was added. Indole-3-acetic acid thus has no bacteriostatic or bacteriocidal effect on any of the bacterial strains used in this study.

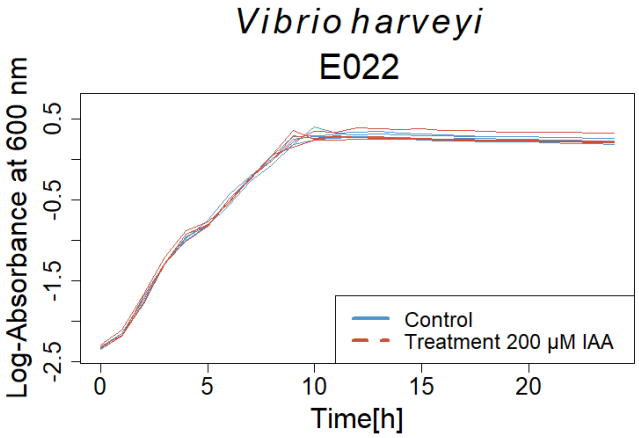
(a)



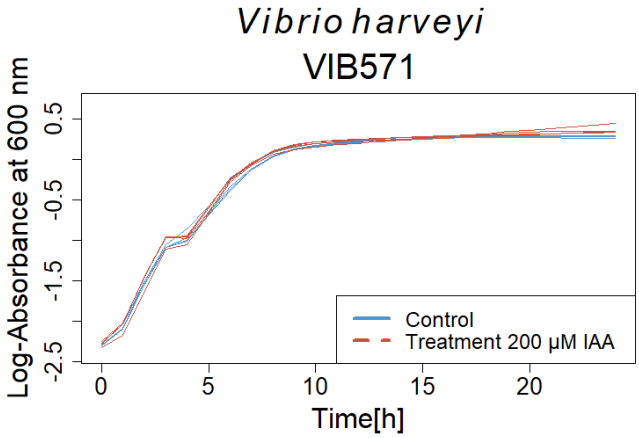
(b)



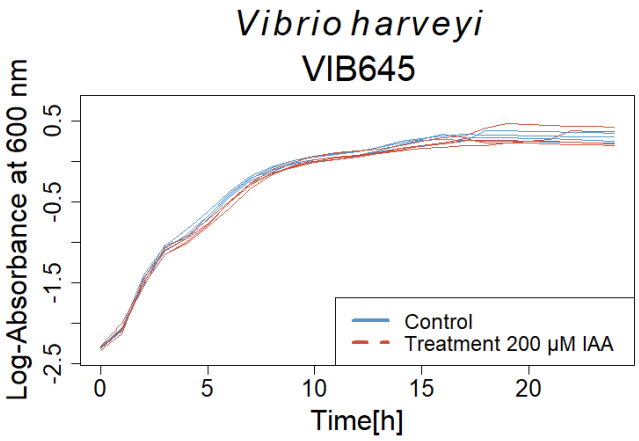
(c)



(d)



(e)


Vibrio harveyi
VIB645
Log-Absorbance at 600 nm
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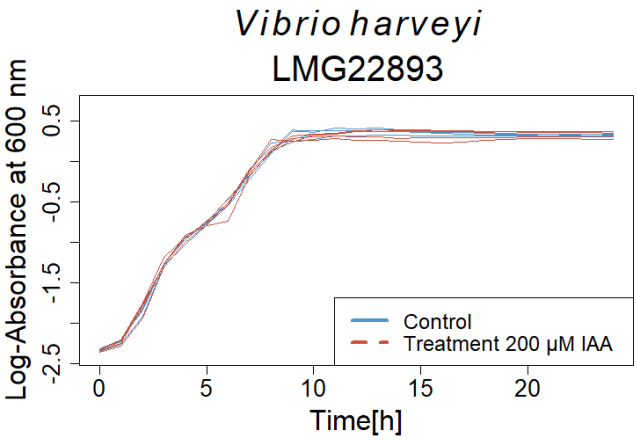
-0.5
-1.5
-2.5
0
5
10
15
20
Time[h]
Control
Treatment 200 µM IAA

(f)


Vibrio harveyi
LMG22893
Log-Absorbance at 600 nm
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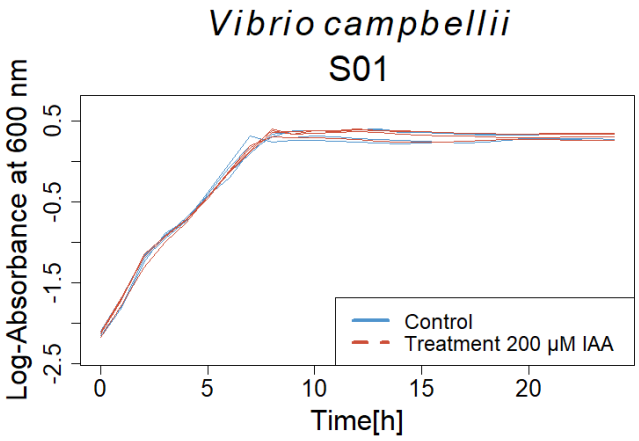
-2.5
0
5
10
15
20
Time[h]
Control
Treatment 200 µM IAA

(g)


Vibrio campbellii
S01
Log-Absorbance at 600 nm
0.5
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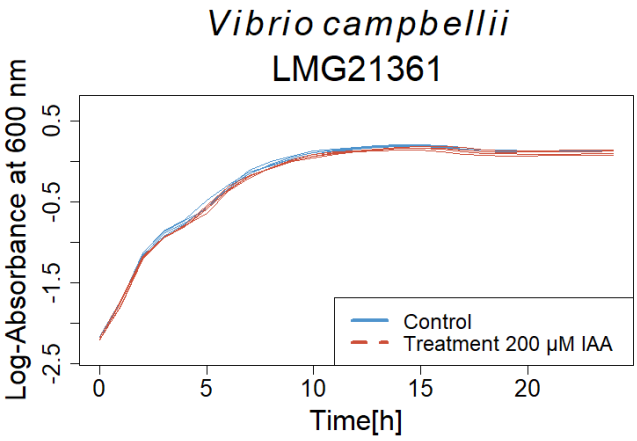
-1.5
-2.5
0
5
10
15
20
Time[h]
Control
Treatment 200 µM IAA

(h)


Vibrio campbellii
LMG21361
Log-Absorbance at 600 nm
0.5
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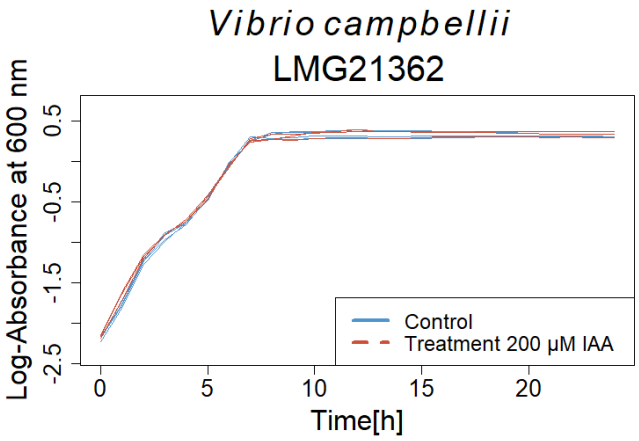
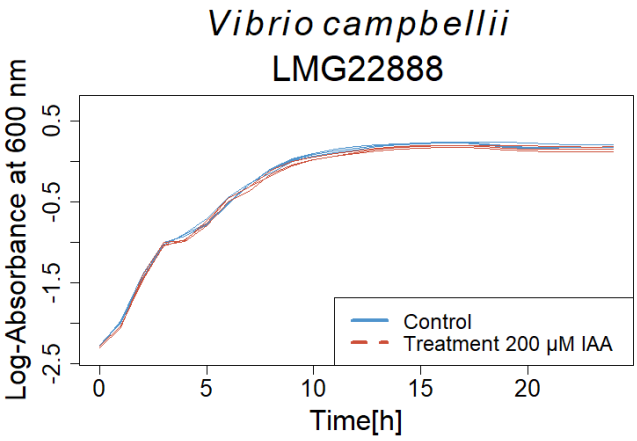
-1.5
-2.5
0
5
10
15
20
Time[h]
Control
Treatment 200 µM IAA

(i)


Vibrio campbellii
LMG21362
Log-Absorbance at 600 nm
0.5
-0.5
-1.5
-2.5
0
5
10
15
20
Time[h]
Control
Treatment 200 µM IAA

(j)


Vibrio campbellii
LMG22888
Log-Absorbance at 600 nm
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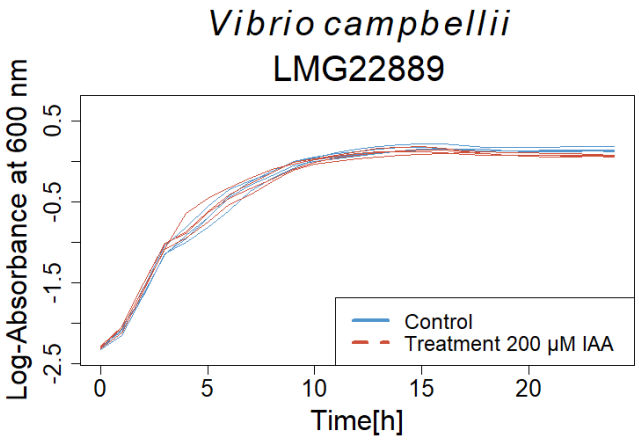
-1.5
-2.5
0
5
10
15
20
Time[h]
Control
Treatment 200 µM IAA

(k)



(l)

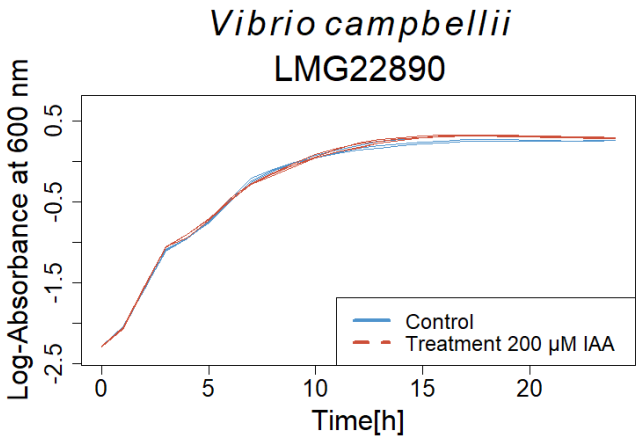


Figure 12. *Impact of indole-3-acetic acid on growth of Harveyi clade Vibrio strains. (a) Vibrio parahaemolyticus M0904; (b) Vibrio parahaemolyticus CAIM170; (c) Vibrio harveyi E022; (d) Vibrio harveyi VIB571; (e) Vibrio harveyi VIB645; (f) Vibrio harveyi LMG22893; (g) Vibrio campbellii S01; (h) Vibrio campbellii LMG21361; (i) Vibrio campbellii LMG21362; (j) Vibrio campbellii LMG22888; (k) Vibrio campbellii LMG22889; (l) Vibrio campbellii LMG228890*

4.2. Impact of indole-3-acetic acid on swimming motility of the vibrios

To observe motility behaviour of *Vibrio parahaemolyticus*, *V. harveyi* and *V. campbellii* strains in the presence of 200 µM indole-3-acetic acid, swimming motility was determined on LB_{35} soft agar plates. For each bacterial strain, average swimming motility halo diameters of the control and test group were measured. An F test was performed to compare variances. Assuming normality of the data, *t*-tests were performed to compare the treatments receiving indole-3-acetic acid to those without indole-3-acetic acid. Results are shown in Figure 13.

Indole-3-acetic acid was found to decrease swimming motility in most of the strains: *V. parahaemolyticus* strain CAIM170 (p=0.0043) and M0904 (p=0.0032); *V. campbellii* strain LMG21362 (p=0.042), LMG22888 (p=0.019), LMG22889 (p=0.0021), LMG22890 (p=0.030) and S01 (p=0.00066); *V. harveyi* strain LMG22893 (p=0.0026) and VIB571 (p=0.0015). Indole-3-acetic acid did not decrease swimming motility in *V. campbellii* strain LMG21361 (p=0.46); *V. harveyi* strain E022 (p=0.060) and VIB645 (p=0.70).

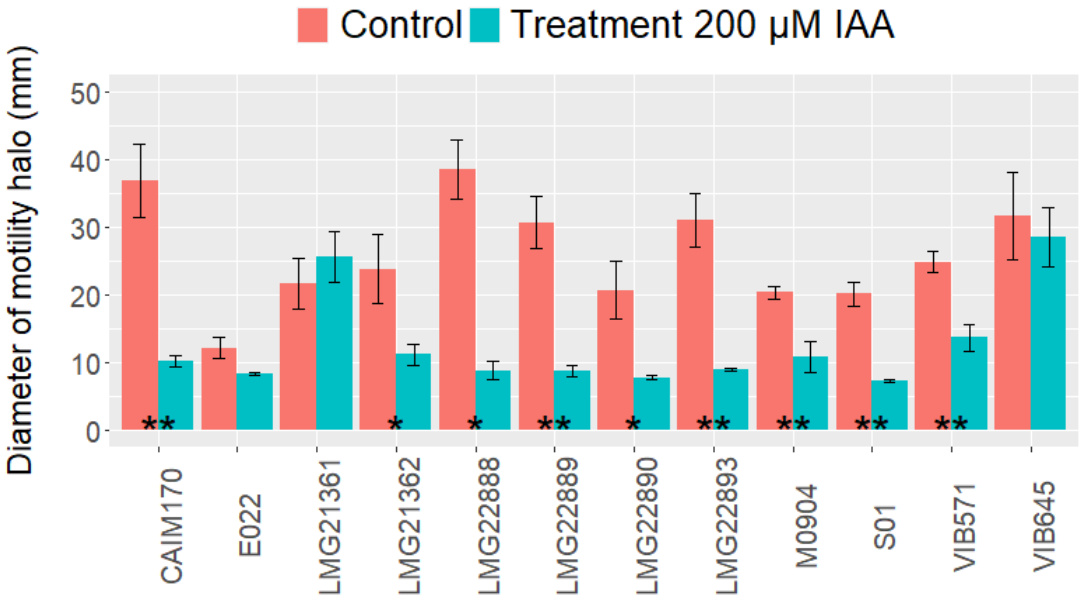


Figure 13. *Impact of indole-3-acetic acid on swimming motility of Harveyi clade Vibrio strains. The bars represent the relative average halo diameter of 6 replicate cultures. The error bars represent the standard error for each group. Single asterisks indicate statistically significant differences when compared with the corresponding treatment without indole-3-acetic acid ($p<0.05$). Double asterisks indicate highly statistically significant differences when compared with the corresponding treatment without indole-3-acetic acid ($p<0.01$).*

4.3. Impact of indole-3-acetic acid on biofilm formation of the vibrios

To observe biofilm formation behaviour of *Vibrio parahaemolyticus*, *V. harveyi* and *V. campbellii* strains in the presence of 200 µM indole-3-acetic acid, a crystal violet assay was conducted. Bacteria cultured in the presence of indole-3-acetic acid were transferred into a 96-well plate. The control group received the same volume of sterile water.

With the available data, the ratio of biofilm formed within each of the bacterial cultures (OD_{570}/OD_{600}) was calculated. For each strain, the obtained OD_{570} values were divided by the mean of the OD_{600} values for this strain. An F test was performed to compare variances. Assuming normality of the data, *t*-tests were performed to compare the treatments receiving indole-3-acetic acid to those without indole-3-acetic acid. Results are shown in Figure 14.

Indole-3-acetic acid was found to decrease biofilm formation in two of the strains: *V. parahaemolyticus* strain CAIM170 ($p=0.0011$) and *V. harveyi* strain VIB645 ($p=0.0041$). Indole-3-acetic acid did not decrease biofilm formation in *V. parahaemolyticus* strain M0904 ($p=0.28$); *V. harveyi* strain LMG22893 ($p=0.29$),

E022 (p=0.72), VIB571 (p=0.23); *V. campbellii* strain LMG21361 (p=0.91), LMG21362 (p=0.30), LMG22889 (p=0.58), LMG22888 (p=0.44), LMG22890 (p=0.92) and S01 (p=0.29).

Figure 14. *Impact of indole-3-acetic acid on biofilm formation of Harveyi clade Vibrio strains. The bars represent the average biofilm ratio (OD570/OD600) of 4 replicate cultures. The error bars represent the standard error for each group. Double asterisks indicate highly statistically significant differences when compared with the corresponding treatment without indole-3-acetic acid (p<0.01).*

4.4. Impact of indole-3-acetic acid on survival of brine shrimp larvae: toxicity test

To check whether an indole-3-acetic acid concentration of 200 µM does not impact survival of brine shrimp larvae, a toxicity test was performed in triplicate. After axenic hatching, shrimp larvae were counted and transferred to filtered, autoclaved seawater. Cultured, washed and autoclaved LVS3 bacteria were added into each tube as feed.

Indole-3-acetic acid was added into the rearing water of the test group at a concentration of 200 µM. Control groups received the same volume of sterile water. Finally, the tubes were put on a rotor and kept for 2 days at 28°C under constant illumination (2000 lux). For the control group, the average *Artemia* survival was 82.22 ± 10.72%. For the test group, the average *Artemia* survival was 78.89 ±

6.94%. There was no significant difference in survival between both groups (p=0.67). Indole-3-acetic acid thus has no adverse effect on brine shrimp larvae when added at a concentration of 200 µM.

4.5. Impact of pathogen concentration on survival of brine shrimp larvae: pathogenicity test

To determine the bacterial pathogen concentration to be added during the challenge experiments, preliminary challenge test were performed without the addition of indole-3-acetic acid. We aimed at a survival rate not less than 10%, preferably even over 25%. After axenic hatching, shrimp larvae were counted and transferred to filtered, autoclaved seawater. After feeding them LVS3 bacteria, 3 replicate cultures of these brine shrimp larvae were challenged for 2 days with varying pathogen concentrations. Results are shown in Table 6.

Bacterial pathogen concentrations started at 10^7 cells/mL, seemingly fine for all of the *V. harveyi* strains. For the remaining strains, challenge tests were performed with a bacterial pathogen concentration of $1.5x10^6$ cells/mL. This concentration seemed fine for most of the remaining strains, except for *V. parahaemolyticus* CAIM170 and *V. campbellii* LMG22888. For these two strains, pathogen concentration was lowered to $0.5x10^6$ cells/mL and challenge tests in triplicate were performed again, to determine whether this concentration was usable in further experiments.

Table 6. *The impact of added bacterial pathogen concentration on the average Artemia survival and standard deviation after 2 days of challenge with this pathogen. Values are all rounded to the closest integer.*

Strain	**Average survival of Artemia (%)**		
	10^7 cells/mL	**$1.5x10^6$ cells/mL**	**$0.5x10^6$ cells/mL**
Vibrio parahaemolyticus			
M0904	6 ± 2	29 ± 3	
CAIM170	7 ± 1	9 ± 2	10 ± 4
Vibrio harveyi			
E022	61 ± 3		
VIB571	52 ± 2		
VIB645	63 ± 2		
LMG22893	54 ± 4		
Vibrio campbellii			
S01	8 ± 3	29 ± 1	

LMG21361	7 ± 3	34 ± 1	
LMG21362	4 ± 1	30 ± 1	
LMG22888	1 ± 2	6 ± 2	13 ± 1
LMG22889	12 ± 2	26 ± 2	
LMG22890	9 ± 2	23 ± 2	

4.6. Impact of indole-3-acetic acid on the virulence of vibrios towards gnotobiotic shrimp larvae

To assess the impact of indole-3-acetic acid on virulence of vibrios towards a host, *Artemia fransiscana* larvae were used as model organisms. For practical reasons, the following experiments were conducted in 3 groups of 4 strains each. Three control tubes, containing only *Artemia* and their feed, were added per group.

After axenic hatching, shrimp larvae were counted and transferred to filtered, autoclaved seawater. After feeding them LVS3 bacteria, these brine shrimp larvae were challenged for 2 days with the pathogens, with and without indole-3-acetic acid added in their rearing water. Added pathogen concentration differed and was determined based on the previous test. An overview of used pathogen concentrations in the brine shrimp rearing water for each bacterial strain, is given in Table 7. For each group, rearing water was then diluted and cell count was measured using flow cytometry. Results from the challenge tests are shown in Figure 15. Bacterial concentrations in the brine shrimp rearing water after 48h are shown in Figure 16.

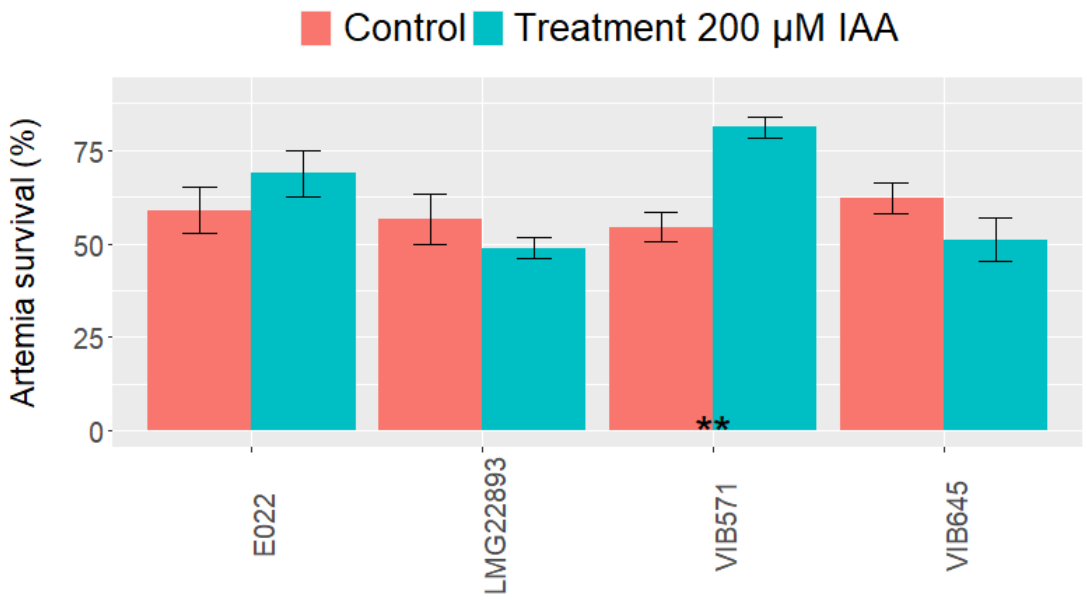
Table 7. *Used pathogen concentration in brine shrimp challenge tests for each bacterial strain*

Strain	**Used pathogen concentration (cells/mL)**
Vibrio parahaemolyticus	
M0904	$1.5x10^6$
CAIM170	$0.5x10^6$
Vibrio harveyi	
E022	10^7
VIB571	10^7
VIB645	10^7
LMG22893	10^7
Vibrio campbellii	

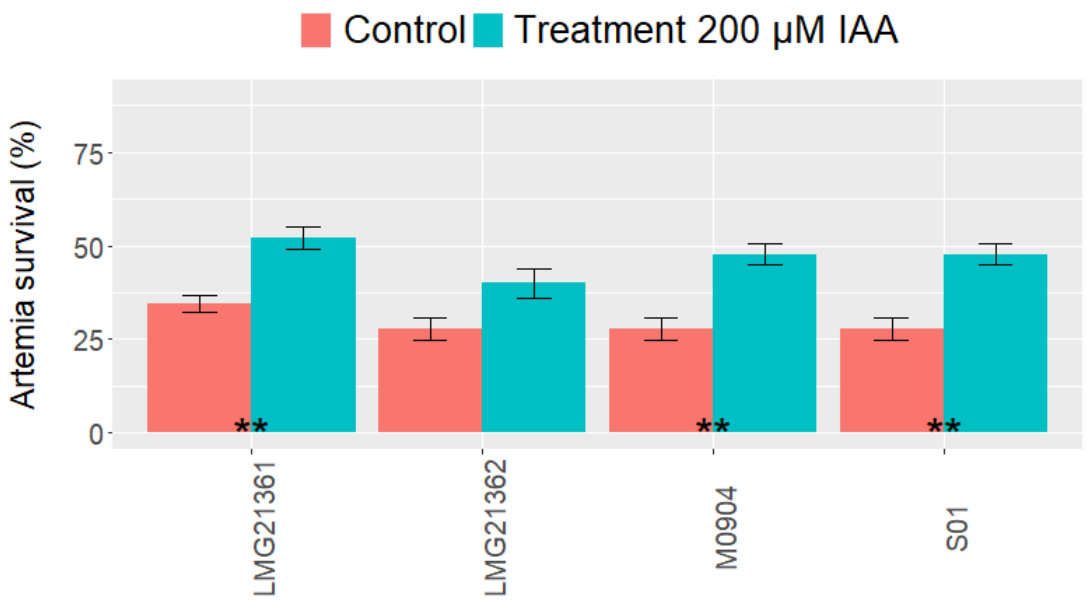
S01	$1.5x10^6$
LMG21361	$1.5x10^6$
LMG21362	$1.5x10^6$
LMG22888	$0.5x10^6$
LMG22889	$1.5x10^6$
LMG22890	$1.5x10^6$

Indole-3-acetic acid was found to increase *Artemia* survival in half of the strains: *V. parahaemolyticus* strain M0904 (p=0.0086); *V. harveyi* strain VIB571 (p=0.0058); *V. campbellii* strain LMG21361 (p=0.0086), S01 (p=0.0086), LMG22889 (p=0.0072), LMG22890 (p=0.020). Indole-3-acetic acid did not increase *Artemia* survival in *V. parahaemolyticus* strain CAIM170 (p=0.77); *V. harveyi* strain E022 (p=0.32), LMG22893 (p=0.35) and VIB645 (p=0.20); *V. campbellii* strain LMG21362 (p=0.065), LMG22888 (p=0.051). As seen from the graph, no notable difference in bacterial cell density in the presence of indole-3-acetic acid is observed when compared to the tubes to which only sterile water was added. Protection offered by indole-3-acetic acid is thus no consequence of growth inhibition of the pathogens.

(a)


Control
Treatment 200 µM IAA
Artemia survival (%)
75
50
25
0
**
E022
LMG22893
VIB571
VIB645

(b)


Control
Treatment 200 µM IAA
Artemia survival (%)
75
50
25
0
**
**
**
LMG21361
LMG21362
M0904
S01

(c)

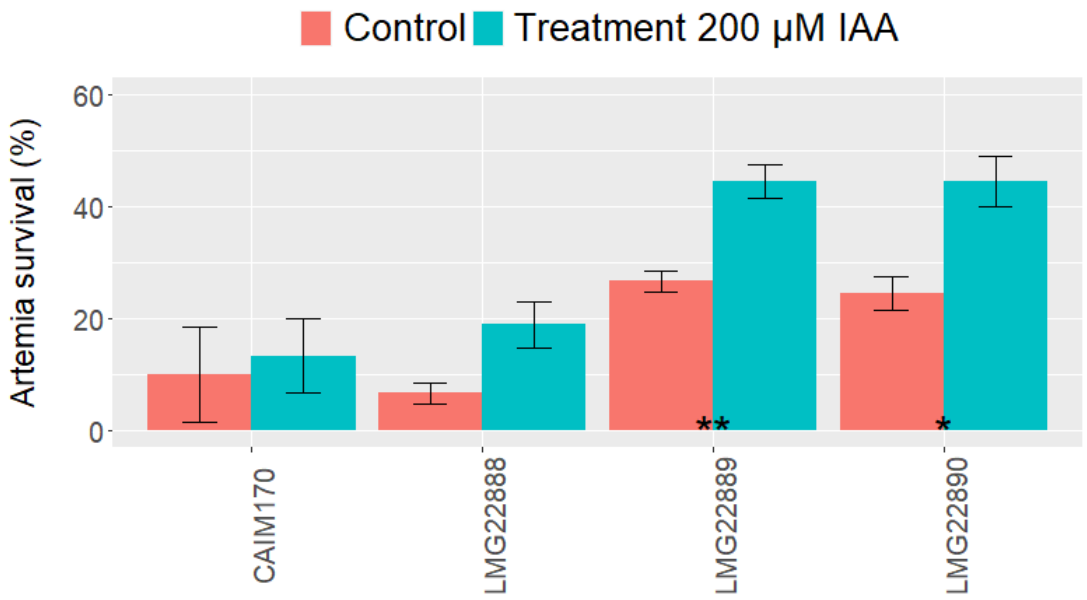


Figure 15. *Impact of indole-3-acetic acid on virulence of Harveyi clade Vibrio strains towards gnotobiotic brine shrimp larvae. The bars represent the percentage average survival of 3 replicate tubes. The error bars represent the standard error for each group. Single asterisks indicate statistically significant differences when compared with the corresponding treatment without indole-3-acetic acid ($p<0.05$). Double asterisks indicate highly statistically significant differences when compared with the corresponding treatment without indole-3-acetic acid ($p<0.01$). The average survival of Artemia cultured in the same way without addition of pathogens was (a) 82.22 ± 8.39 %, (b) 77.78 ± 11.7 % and (c) 66.67 ± 15.28 %.*

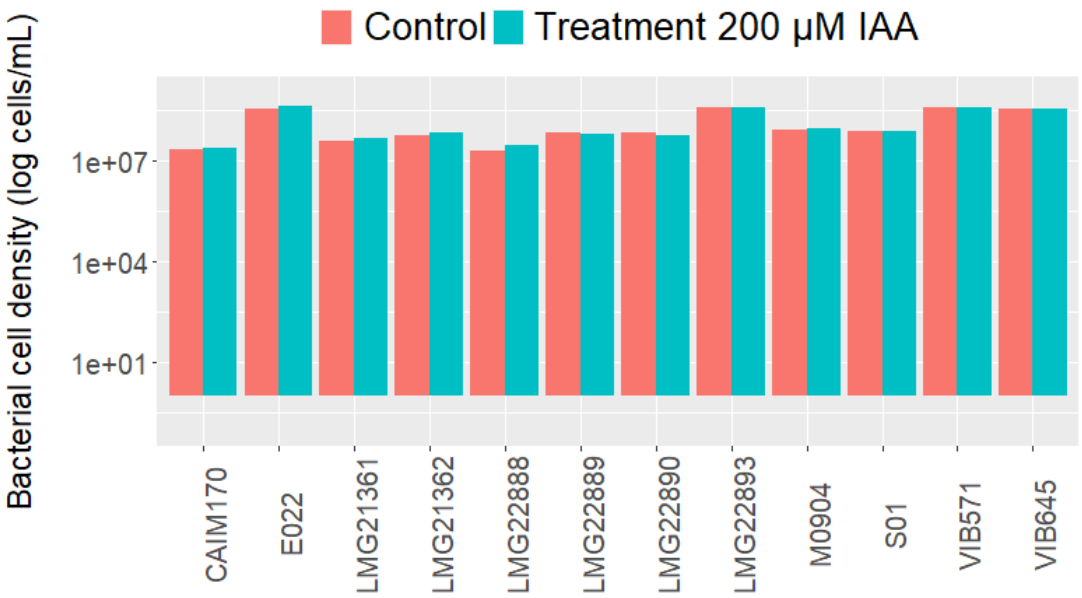


Figure 16. *Impact of indole-3-acetic acid on bacterial cell density of Harveyi clade Vibrio strains in rearing water of gnotobiotic shrimp larvae. The bars represent the bacterial cell count, obtained by flow cytometry, in the pooled rearing water of 3 tubes.*

CHAPTER 5: CONCLUSION AND DISCUSSION

In this study, we found that indole-3-acetic acid showed protection of brine shrimp larvae against different pathogenic strains belonging to the species *V. harveyi*, *V. campbellii* and *V. parahaemolyticus*. Furthermore, we found that indole-3-acetic acid significantly decreased swimming motility in most of the Harveyi clade *Vibrio* strains. However, biofilm formation was only decreased in 2 of the strains. Indole-3-acetic acid showed no toxicity against our model organisms (*Artemia fransiscana*) and had no impact on growth of the Harveyi clade *Vibrio* strains.

In the search of ensuring adequate food supplies for the world's rapidly growing population, it might be of importance to look at the ocean's contribution, mainly aquaculture practices. However, as for terrestrial animal production, emerging antibiotic resistance puts severe pressure on the sector. The evolution of resistance is driven by antibiotic use, strictly selecting for resistant bacteria that spread by natural selection while removing their drug-sensitive competitors (Read & Woods, 2014). The incidence of antibiotic resistance was compared in bacteria isolated from different pond environments and cultured shrimp from ponds. Some of these reservoirs had not used any antimicrobials, while others did previously use antimicrobial compounds. The lowest incidence of antibiotic resistance was observed in ponds that had not used any antimicrobials. Interestingly, most of the bacteria isolated from all sample and pond types were vibrios, with *V. harveyi* being most commonly isolated (Tendencia *et al.* 2001). Next to environmental isolation, *Vibrio* species could also be collected from diseased marine fishes. Among them, 72.86% belonged to the Harveyi clade, the most important pathogen clade in aquatic organisms (Deng *et al.* 2020). Also for this core group of *Vibrio* spp., multiple antibiotic resistance was shown (Zhu *et al.* 2018; Ruwandeepika *et al*. 2012).

Increasing antibiotic resistance in pathogenic microorganisms forms a huge challenge for human as well as animal medicine (Defoirdt, 2013). More responsible use of classical antibiotic compounds will only partly diminish the problem, as they still bring along selective pressure while drastically disrupting pathogen-host symbioses (Cegelski *et al.* 2008; Schroeder *et al.* 2017; Tang & Zhang, 2014). To end the era of fighting fire with fire, researchers are therefore exploring alternative ways to control bacterial diseases. Since the great expansion of knowledge and

expertise in terms of bacterial virulence, novel therapeutic strategies are expected in the field of antivirulence therapy, the specific inhibition of functions required to infect the host (virulence factors). Tricks to interfere with these virulence factors and more importantly, their regulation, might be of crucial importance in the near future (Defoirdt *et al.* 2011; Defoirdt, 2013). An interesting track is that of quorum sensing, bacterial cell-to-cell communication. Being one of the most intensively studied targets for antivirulence therapy, we can meanwhile map past achievements and learn from them (Fleitas Martinez *et al.* 2019; Defoirdt, 2018). Quorum sensing is highly dependent on the secretion and detection of chemical signalling molecules, so-called autoinducers. For *V. harveyi*, for example, three autoinducers are described in the quorum sensing system. However, more and more data confirm the emerging picture that molecules other than traditionally defined autoinducers integrate into quorum sensing circuits (Henares *et al.* 2012). Multiple examples of quorum-sensing interfering agents have meanwhile been discovered for Gram-negative pathogens, including enzymes, antibodies and natural compounds such as indole (Defoirdt, 2018). The interest of this study goes to the interference of quorum sensing via the indole signalling pathway by using indole-3-acetic acid, a naturally occurring plant auxin (Fu *et al.* 2015; Guo *et al.* 2019; Spaepen *et al.* 2007). The impact of indole-3-acetic acid was assessed on different virulence traits of twelve Harveyi clade *Vibrio* strains. The impact of indole-3-acetic acid was tested on swimming motility and biofilm formation of the strains. Virulence towards a host was assessed during challenge test experiments using *Artemia fransiscana* larvae as a model. These larvae were challenged with the pathogens for 48h, with or without indole-3-acetic acid and results were compared.

Indole-3-acetic acid is considered the most common plant hormone. As a signal molecule, it is fundamental for plant growth and development (Duca *et al.* 2014; Fu *et al.* 2015; Guo *et al.* 2019). Next to synthesis by the plant itself, plant-associated soil bacteria (such as *Pseudomonas*, *Azospirillum*, *Agrobacterium* and *Rhizobium*) produce indole-3-acetic acid in order to communicate with their host plant. Best documented are the phytopathogenic interactions such as tumours, galls and hairy roots, where bacterial indole-3-acetic acid disturbs the auxin balance which inhibits plant development (Bianco *et al.* 2006; Fu *et al.* 2015). It has meanwhile been shown that also bacteria inhabiting fresh and marine waters, cyanobacteria and even fungi

are capable of producing indole-3-acetic acid (Patten *et al.* 2013). Although indole and its derivatives gain more and more popularity in the research field, there doesn't exist a lot of studies on the impact of indole-3-acetic acid on virulence factors for marine bacteria. Since it is a plant hormone, most information on bacterial associations is gathered for soil bacteria, mostly for the model organism *Agrobacterium tumefaciens*. In order to compare our results to existing literature, we are thus forced to focus mainly on other indole derivatives and other bacterial species. The great inter-strain and interspecies variability for this antivirulence research field, has to be kept in mind.

In a first experiment, we investigated the impact of indole-3-acetic acid on growth of the Harveyi clade *Vibrio* strains. Indole-3-acetic acid had no bacteriostatic or bacteriocidal effect on the twelve Harveyi clade *Vibrio* strains used in this study. Our observations align with results for indole and *V. campbellii*, where growth was not altered after bacterial treatment with 200 µM indole (Yang *et al.* 2017). When looking at indole-3-acetic acid, specifically, our results confirm the observations of Liu & Nester, 2006. While showing significant growth inhibition of most plant-associated bacteria in the presence of 200 µM indole-3-acetic acid, growth was never inhibited for bacteria occupying other ecological niches (*Acidovorax temperans*, *Bacillus subtilis*, *Enterococcus faecalis*, *Staphylococcus epidermidis*, *Pseudomonas aeruginosa*, *Enterobacter aerogenes*, *Serratia marescens*, *Salmonella typhi*) (Liu & Nester, 2006). The effect of indole-3-acetic acid on growth is not limited to the bacterial kingdom, as also data for the yeast *Saccharomyces cerevisiae* are available. In this latter study, a concentration-dependent effect was shown. For *S. cerevisiae*, indole-3-acetic acid added at low concentrations enhanced filamentation and growth. However, when using higher concentrations, indole-3-acetic acid arrested growth in all stages of the cell cycle. At an indole-3-acetic acid concentration of 250 µM, growth was the half-maximum obtainable in the absence of the drug. Interestingly, this concentration-dependent effect was only illustrated for indole-3-acetic acid, showing that *Saccharomyces* distinguishes indole-3-acetic acid from other closely related compounds such as indole-3-pyruvic acid and indole-3-acetamide (Prusty *et al.* 2004). Summarized for the growth experiment, as indole-3-acetic acid does not kill the pathogens or inhibit their growth, the application of it decreases the risk of resistance insurgence by not exerting selective pressure on the

microorganism population (Buroni & Chiarelly, 2020). Therefore, its therapeutic potential and effect on important virulence factors could further be investigated.

The potency of a pathogen to cause infection, starts with invasion of the host, requiring chemotactic motility (O'Toole *et al.* 1996). In the aquatic environment as well as during host colonization, motility, originating from the synthesis of flagella that rotate and propel the bacteria (Echazarreta & Klose, 2019), plays an important role in the lifestyle of *Vibrio* spp. (Khan *et al.* 2020). Next to the association between flagellar motility and colonization and adhesion, it plays a role in several other biological and cellular processes such as chemotaxis, biofilm formation and overall virulence (Khan *et al.* 2020; Yang & Defoirdt, 2015). For *V. campbellii* BB120, swimming motility was proven to be closely related to the quorum sensing (QS) system. By studying behaviours of quorum-sensing *V. campbellii* mutants, it was demonstrated that (i) QS positively regulates motility by affecting flagellar biosynthesis, (ii) LuxR is involved in the regulation of motility and (iii) flagellar motility significantly affects virulence of *V. campbellii* in a gnotobiotic brine shrimp model (Yang & Defoirdt, 2015). Manipulation of motility via quorum sensing interference by naturally derived or chemically synthesized compounds could thus be a potential treatment for preventing *Vibrio*-associated infections (Khan *et al.* 2020; Yang & Defoirdt, 2015).

In a second experiment, we assessed the impact of 200 µM indole-3-acetic acid on swimming motility of the strains. We found that indole-3-acetic acid significantly decreased swimming motility in nine of the strains (CAIM170, LMG21362, LMG22888, LMG22889, LMG22890, LMG22893, M0904, S01, VIB571). Although specific effects for specific strains do not align with findings for indole, the overall effect of indole-3-acetic acid is found to be similar to the effect reported for indole (Zhang *et al.* 2022). Moreover, indole decreased swimming motility of other bacteria such as *V. cholerae*, *E. coli* and *P. aeruginosa*, whereas it had no effect on motility of *V. anguillarum* (Melander *et al.* 2014; Yang *et al.* 2017). Indole decreasing swimming motility was also documented for *Salmonella enterica* serovar Typhimurium, where it was shown that indole represses the expression of genes involved in bacterial flagella biosynthesis, flagella motor activity and chemotaxis. Interestingly, although it reacts on indole, *Salmonella enterica* serovar Typhimurium does not naturally produce indole (Melander *et al.* 2014). While indole and several halogenated indoles

also inhibited swimming motility in *A. tumefaciens* (Ahmed *et al.* 2022), indole-3-acetic acid seemed to have no effect on swimming motility for this bacterium when used at a concentration of 200 µM (Lee *et al.* 2015). For several other plant-associated bacteria (*Pseudomonas putida*, *Rhizobium etli*, *Bradyrhizobium japonicum*, *Pseudomonas syringae*), however, there is growing evidence for a role of indole-3-acetic acid in the modulation of bacterial motility and chemotaxis, as even chemoreceptors for indole-3-acetic acid are meanwhile known for these species (Rico-Jiménez *et al.* 2022).

Next to and being closely related with swimming motility, biofilm formation of bacterial strains contributes to their virulence (Ruwandeepika *et al.* 2012). As antibiotic tolerance of bacteria is drastically enlarged by their capacity to live in surface adherent structures, great interest goes to manipulating this bacterial biofilm feature (Lewis, 2005; Schroeder *et al.* 2017). In a third experiment, we therefore assessed the impact of indole-3-acetic acid on biofilm formation of the strains by conducting a crystal violet assay. We found that indole-3-acetic acid significantly decreased biofilm formation in only two of the strains (*V. parahaemolyticus* CAIM170 and *V. harveyi* VIB645). This does not align with findings for indole, where the addition of indole decreased biofilm formation in most of the strains (Zhang *et al.* 2022). On the other hand, results align with the majority of similar studies for other bacteria. For *Acinetobacter baumannii*, for example, indole-3-acetic acid did not impact biofilm formation (Lin *et al.* 2018). Indole-3-acetic acid seemed to even stimulate biofilm formation for *Candida tropicalis* (Miyagi *et al.* 2020), *E. coli* (Bianco *et al.* 2006; Melander *et al.* 2014), *P. aeruginosa* and *A. tumefaciens* (Plyuta *et al.* 2013). Furthermore, an inverse relation between motility and biofilm formation has been reported before, confirming their very complex association (Verstraeten *et al.* 2008). In order for bacteria to reach collective resistance in biofilms, their initial reversible surface attachment involves a turning point from planktonic life to biofilm mode (Abebe, 2020; Vega & Gore, 2014). Later on, motility is also involved during dispersion of bacteria from mature biofilms, re-entering their planktonic state (Abebe, 2020; Verstraeten *et al.* 2008). The discrepancy between results on swimming motility and biofilm formation might be due to this bacterial decision-making between motility and biofilm formation at certain stages, crucial for bacterial colony survival (Verstraeten *et al.* 2008). Furthermore, also for vibrios, the biofilm formation ability

strongly depends on the presence of produced exopolysaccharides (Fazli *et al.* 2014; Vanmaele *et al.* 2015), which has not been investigated in this research. In investigating the response of *Xylella fastidiosa* to Zinc, for example, the phenomenon of increased biofilm formation was correlated with increased exopolysaccharide production in stationary-phase cells (Navarrete *et al.* 2014). Conducting a more dose-dependent research in order to find the tipping point between planktonic and sessile lifestyle, next to considering exopolysaccharide production for the Harveyi clade *Vibrio* strains, might give us some more insight into what's behind the obtained results regarding biofilm formation.

In aquaculture practices, in order to mimic the natural situation, particularly larval stages of cultivable fish and shellfish are fed with live organisms (Sorgeloos & Kulasekarapandian, 1984). Very popular is the implementation of *Artemia*, showing great convenience in use (Dhondt *et al.* 2013). Furthermore, brine shrimp larvae are known model organisms for vibriosis and other crustacean diseases (Chan *et al.* 2021; Zhang *et al.* 2022). In all of the following experiments, *Artemia fransiscana* larvae were therefore used as model organisms, as was done in the study investigating the impact of indole on the virulence of Harveyi clade vibrios (Zhang *et al.* 2022). In a fourth experiment, we assessed the toxicity of indole-3-acetic acid towards these gnotobiotic brine shrimp larvae. While the addition of 200 µM indole induced mortality of brine shrimp larvae, we found that a concentration of 200 µM indole-3-acetic acid had no adverse effect the on survival of the brine shrimp larvae.

In a final experiment, we assessed the impact of indole-3-acetic acid on the virulence of vibrios towards gnotobiotic brine shrimp larvae. Indole-3-acetic acid significantly increased *Artemia* survival in half of the strains belonging to *V. parahaemolyticus*, *V. harveyi* and *V. campbellii* (LMG21361, LMG22889, LMG22890, M0904, S01, VIB571). Importantly, indole-3-acetic acid showed no effect on bacterial growth in the brine shrimp rearing water, indicating that the offered protection by indole-3-acetic acid was not due to growth inhibition of the pathogens. This data however suggests that indole-3-acetic acid shows less protection of brine shrimp larvae than indole, which led to an increased *Artemia* survival in eleven out of the twelve Harveyi clade *Vibrio* strains (Zhang *et al.* 2022). A similar study performed with *V. anguillarum* NB10 and sea bass (*Dicentrarchus labrax*) larvae also reported a decrease in

virulence in the presence of indole (Li *et al.* 2014). Also for the bivalve model pathogens *V. tasmaniensis* LGP32 and *V. crassostreae* J2-9, indole decreased virulence towards mussel (*Mytilus edulis*) larvae (Zhang *et al.* 2022). Next to using another compound, the discrepancy in experimental procedures could lie at the basis of these different outcomes for indole and indole-3-acetic acid. Because of the indole toxicity to brine shrimp larvae, challenge tests were performed using bacterial pretreatment. Our study was the first to add 200 µM of the compound directly into the *Artemia* rearing water, therefore overcoming the need for bacterial pretreatment, since these results are only valuable for research purposes.

Since it's the *Vibrio* spp. ruining the economy of the aquaculture industries (Arunkumar *et al.* 2020), the importance of fighting marine vibriosis cannot be underestimated. Furthermore, antibiotic resistance as shown for the Harveyi clade of vibrios, should be overcome in order to protect animal as well as human medicine (Defoirdt *et al.* 2011). Our research might be a stepping stone towards both applications, seen the impact of exogeneous indole-3-acetic acid on multiple important characteristics of Harveyi clade *Vibrios*. Next to showing an overall therapeutic potential for indole-3-acetic acid, however, our study once again proved that the link between quorum sensing and virulence is different for different *Vibrios* (Islam *et al.* 2022). On the other hand, despite the presence of distinct quorum sensing systems in different microorganisms, the fundamental processes are similar (Tang & Zhang, 2014). Although said that antivirulence therapy would have fewer side effects towards non-target bacteria than conventional antibiotics (Vale *et al.* 2016), the similarity in QS systems for a variety of bacteria, fungi and archaea could however impose an effect of indole-3-acetic acid on marine species other than *Vibrios*. In order to develop an effective indole-3-acetic acid treatment, effects on bacterial characteristics of symbiotic, free-living and other pathogenic marine organisms should be evaluated and taken into consideration.

Although some antivirulence drugs have meanwhile been approved and many are in clinical trials, none are widely used in the clinical setting at present. Their promises and challenges thus still have to be verified in the real world (Dickey *et al.* 2017). One such promise is that antivirulence therapy is expected to outcompete antibiotic therapy in terms of resistance insurgency. Although it may considerably weaken the selective pressure on microorganisms, antivirulence therapy can unfortunately only

delay resistance to quorum sensing interfering agents (Tang & Zhang, 2014). For the example of brominated furanone C-30, the best-characterized compound that inhibits QS, a rapid arise in resistance was indeed observed (Garcia-Contreras *et al.* 2013). Not only for *Pseudomonas aeruginosa* but also for bacteria responsible for chronic cystic fibrosis, a mutation that increased the efflux of C-30 was shown. Interestingly, the C-30 resistant mutant even showed more pathogenicity compared to the wild type towards *Caenorhabditis elegans* in the presence of C-30 (Maeda *et al.* 2012). The combination of antivirulence agents together with antibiotic compounds was therefore proposed for this C-30 and for gallium (a siderophore quencher). This first systematic analysis of antivirulence-antibiotic combinatorial treatment suggested that such combinations have the potential to be (i) effective in treating infections and (ii) limiting the spread of antibiotic resistance (Rezzoagli *et al.* 2020). Also for indole-3-acetic acid, future applications should be evaluated in real aquaculture setting. Next to the effect of indole-3-acetic acid on non-target organisms, the toxicity of the auxin to non-target organisms should be considered. Another important feature will be resistance emergence, which might lead to the need for also conducting combinatorial studies with antibiotic compounds such as ciprofloxacin, colistin, meropenem and tobramycin in order to get the greatest therapeutic potential out of this auxin.

All of our experimental results were obtained by using synthetic indole-3-acetic acid, purchased from Sigma-Aldrich (Belgium). The auxin was thereby dissolved in sterile water to reach the desired concentration for all of the experiments. Interestingly, aquatic animals cultured in the presence of high levels of microalgae (putative producers of high levels of indole-3-acetic acid), do seem to suffer from less disease incidence (Yang *et al.* 2017). Creating an ecosystem where virulence of *Vibrio* bacteria is altered via co-cultivation with these indole-3-acetic acid producing microalgae, could thus lead to a more sustainable approach to control diseases. Next to plants and microalgae, a variety of bacterial species are capable of producing indole-3-acetic acid. This has for a long time been proven for microorganisms that are commonly associated with plant surfaces (such as *Pseudomonas*, *Azospirillum*, *Agrobacterium* and *Rhizobium*), using the auxin in order to communicate with their host plant (Bianco *et al.* 2006; Fu *et al.* 2015; Zhang *et al.* 2022). A multitude of other bacteria, however, have meanwhile been shown to

also produce indole-3-acetic acid. These bacteria include pink-pigmented facultative methylotrophic bacterial cultures (Omer *et al.* 2004) and multiple *Streptomyces* strains (*S. violaceus*, *S. scabies*, *S. griseus*, *S. exfoliates*, *S. coelicolor*, *S. lividans*) (Manulis *et al.* 1994). Interestingly, many species of the genus *Vibrio* were recently shown to be indole-3-acetic acid producers (Gutierrez *et al.* 2009). Corresponding pathways are best well-documented for *Vibrio natriegens*, being the fastest-growing non-pathogenic marine bacterium known to date (Hoff *et al.* 2020; Thoma & Blombach, 2021). Next to investigating the effect of indole-3-acetic acid produced by microalgae, co-cultivation of pathogenic vibrios with bacterial species such as *Vibrio natriegens* could be tested, leading us to a probiotic approach to cure marine vibriosis. In aquaculture, several types of probiotic bacterial strains in aquafeed show unique and beneficial properties. These involve bacteria belonging to the species of *Bacillus*, *Lactococcus*, *Lactobacillus*, *Pseudomonas*, *Enterococcus*, *Aeromonas*, *Alteromonas*, *Bifidobacterium*, *Clostridium*, *Phaeobacter*, *Pseudoalteromonas*, *Rhodosporidium*, *Roseobacter* and *Streptomyces* (Yilmaz *et al.* 2022). For *Vibrio parahaemolyticus*, feeding pacific white shrimp (*Litopenaeus vannamei*) with different probiotic strains (*Vibrio alginolyticus* UTM 102, *Bacillus subtilis* UTM 126 and others) reduced disease (Balcázar *et al.* 2007). A similar effect for fish and shrimp was observed for *Streptomyces* species, where the role of indoleacetic acid as growth-promoting hormone was discussed (Tan *et al.* 2016).

To conclude, although high inter-species and inter-strain variability, our study showed a clear therapeutic potential of the plant auxin indole-3-acetic acid, interfering with quorum sensing systems of Harveyi clade *Vibrios*. The addition of 200 µM indole-3-acetic showed a decrease in virulence factors such as swimming motility and biofilm formation for *Vibrio parahaemolyticus*, *Vibrio harveyi* and *Vibrio campbellii* strains. Furthermore, virulence towards a host was altered in the presence of indole-3-acetic acid. Following more elaborate research and a better characterisation of molecular interactions between quorum sensing and virulence, synthetic or natural indole-3-acetic acid could be tested in real aquaculture settings and might lead to preventing marine vibriosis in the future.

CHAPTER 6: REFERENCES

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