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MINIREVIEW ON SUSTAINABLE ANTIVIRULENCE STRATEGY FOR AQUACULTURE

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ABSTRACT

The increasing occurrence of antibiotic-resistant bacteria is one of the major challenges currently faced by the aquaculture sector. Ineffective applications of antibiotics to treat bacterial diseases, leading to the need for alternative strategies to address the problem. The antivirulence approach is a highly promising strategy that aims to stop pathogenic bacteria from causing harm to the host by disrupting their virulence mechanisms. This approach involves understanding the mechanisms of bacterial pathogenicity that can be developed into new therapeutic methods. There have been numerous advancements in combating bacterial infections, such as disrupting host-pathogen communication and inhibiting quorum sensing (QS). Antivirulence therapy offers a significant advantage as it specifically targets bacterial virulence without imposing excessive pressure on bacterial growth, reducing the risk of resistance development. This review outlines the limitations of antibiotic use and presents current insights into bacterial pathogenicity mechanisms and antivirulence strategies in aquaculture. It particularly highlights the impact of host-pathogen signaling via catecholamines, stress hormones, and QS mechanisms in certain aquaculture-pathogenic bacteria. The influence of host stress hormones on pathogen growth and virulence is noteworthy. Quorum sensing (QS) is known to regulate the expression of certain virulence genes in response to bacterial density by releasing and detecting a small signal molecule called autoinducers. This review further explains various strategies to interfere with QS mechanisms, including inhibiting signal molecule biosynthesis, using QS antagonists, chemical inactivation, or biodegradation of QS signals. These promising strategies have been considered as the first step and proof of concept of antivirulence strategies to prevent disease outbreaks in aquaculture.

KEYWORDS: antibiotic resistant; quorum quenching; quorum sensing; vibriosis; virulence factors

ABSTRAK: Reviu Mini Strategi Antivirulensi Berkelanjutan untuk Akuakultur

Meningkatnya jumlah bakteri yang resisten terhadap antibiotik merupakan salah satu tantangan besar yang saat ini dihadapi oleh sektor akuakultur. Penerapan antibiotik yang

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tidak efektif untuk mengobati penyakit bakterial, menyebabkan perlunya strategi alternatif untuk mengatasi masalah tersebut. Pendekatan antivirulensi adalah strategi yang sangat menjanjikan yang bertujuan untuk menghentikan bakteri patogen dalam menyebabkan kerusakan pada inang dengan mengganggu mekanisme virulensinya. Pendekatan ini melibatkan pemahaman mekanisme patogenisitas bakteri yang dapat dikembangkan menjadi metode terapi baru. Terdapat banyak perkembangan dalam melawan infeksi bakteri, seperti mengganggu komunikasi inang-patogen dan menghambat quorum sensing *(QS). Terapi antivirulensi menawarkan keuntungan yang signifikan karena secara spesifik menargetkan virulensi bakteri tanpa memberikan tekanan berlebihan pada pertumbuhan bakteri, sehingga mengurangi risiko berkembangnya resistensi. Reviu ini menguraikan keterbatasan penggunaan antibiotik dan menyajikan wawasan terkini mengenai mekanisme patogenisitas bakteri dan strategi antivirulensi dalam budidaya perikanan. Reviu ini terutama menyoroti dampak sinyal patogen inang melalui katekolamin, hormon stres, dan mekanisme QS pada bakteri patogen tertentu dalam akuakultur. Pengaruh hormon stres inang terhadap pertumbuhan dan virulensi patogen patut diperhatikan.* Quorum sensing *(QS) diketahui mengatur ekspresi gen virulensi tertentu sebagai respons terhadap kepadatan bakteri dengan melepaskan dan mendeteksi molekul sinyal kecil yang disebut autoinduser. Reviu ini lebih lanjut menjelaskan berbagai strategi untuk mengganggu mekanisme QS, termasuk menghambat biosintesis molekul sinyal, menggunakan antagonis QS, inaktivasi kimia, atau biodegradasi sinyal QS. Strategi yang menjanjikan ini telah dianggap sebagai langkah pertama dan bukti dari konsep strategi antivirulensi untuk mencegah wabah penyakit pada budidaya perikanan.*

KATA KUNCI:** faktor virulensi;* **quorum quenching;* **quorum sensing***; resistan antibiotik; vibriosis***

INTRODUCTION

Aquaculture refers to the farming of aquatic animals and plants in environments that can be natural or controlled, including marine, brackish or freshwater settings. This activity includes the production process, such as breeding in hatchery to rearing and harvesting market-size products in ponds, tanks, cages or raceways (Food and Agriculture Organization, 2024). Aquaculture fulfills multiple roles, including the cultivation of aquatic species for human consumption, ornamental species for the aquarium trade, and other species used in pharmaceutical, nutritional, and biotechnology products. Aquaculture stands as the fastest-growing sector in animal food production (Anderson *et al.*, 2017) and plays a vital role in the economic development of both developed and developing nations. With a global fish production of around 179 million tonnes and a first-sale value of approximately USD 401 billion in 2018, aquaculture contributed 82 million tonnes valued at USD 250 billion, showcasing its significant economic impact (Food and Agriculture Organization, 2020). As global capture fishery production remains unchanging while the human population continues to grow, aquaculture is the key to meeting the increasing demand for safe and high-quality aquatic food in the future.

Despite the rapid growth of the global intensive aquaculture industry, it continues to grapple with significant challenges in controlling infectious bacterial diseases. The aquatic environment, unlike the terrestrial environment, provides an ideal breeding ground for pathogenic bacteria, posing a threat to the health of aquatic species. This translates to highly unpredictable survival rates, especially in the early stages of aquaculture species (Irsath *et al.*, 2023). Infectious diseases caused by viral, bacterial, and eukaryotic pathogens significantly hamper aquaculture production, leading to industry-wide losses exceeding US$6 billion annually. Notably, sectors like shrimp farming suffer severe economic and social impacts, with total losses surpassing 40% of global capacity (Stentiford *et al.*, 2017).

In aquaculture, the impact of infectious diseases is significant, causing both economic losses and animal welfare problems. To combat this issue, farmers often rely on chemical compounds as antimicrobial agents to treat bacterial diseases. These agents include disinfectants, anthelmintic agents, and commonly used antibiotics (Danner & Merrill, 2006; Schar *et al.*, 2020; Zhou *et al.*, 2020). Antibiotics play a crucial role in treating various bacterial infections (Defoirdt, 2014; Thiang *et al.*, 2021). However, the emergence and spread of antibiotic-resistant bacteria and resistance genes, as well as the presence of antimicrobial residues in aquaculture products and the environment, have raised concerns about the use of antibiotics in this industry. The challenge of diseases caused by antibiotic-resistant bacteria is also significant. Therefore, it is paramount to develop alternative methods and techniques to control pathogenic bacteria for the sustainable development of the aquaculture sector.

ANTIBIOTICS PROBLEM IN AQUACULTURE

In the realm of aquaculture, antibiotics are essential for fighting bacterial diseases, just as they are in human medicine and terrestrial animal production. These applications are categorized as therapeutic, prophylactic or metaphylactic. Table 1 lists various classes of antibiotics utilized in aquaculture, along with examples of pathogenic bacteria in aquaculture exhibiting (multi)resistance. Instead of directly injection into the adult aquaculture animal, the use of antibiotics in aquaculture is commonly added to the feed, which is then delivered to the animal by placed in the rearing water, whereas in some cases, antibiotics may be added directly to the water. Almost every aquaculture farmer uses antibiotics to protect their culture animals from diseases. A wide range of antibiotics, encompassing over ten different types including chloramphenicol, trimethoprim, gentamicin, tetracyclines, tiamulin, quinolones, and sulfonamides, were deployed (Schar *et al.*, 2020). The common antibiotics used are also different in different countries; for instance, oxytetracycline, oxolinic acid, chloramphenicol, furazolidone, nitrofurans, and erythromycin (Suprapto *et al.*, 2015), and oxytetracycline, florfenicol, trimethoprim-sulfamethoxazole, sarafloxacin, and enrofloxacin (Prena *et al.*, 2020).

The increase in bacterial diseases in intensive aquaculture farming has led to an increase in the use of antibiotics. The use of antimicrobials in the aquaculture industry has been documented, which may contribute to the rise of antimicrobial resistance, carrying potential consequences for animal-, human-, and ecosystem health (Defoirdt *et al.*, 2011; Schar *et al.*, 2020). The overuse of antibiotics in aquaculture has led to harmful effects in many farms worldwide. The most common way antibiotics are given to aquaculture animals is by mixing them with specially formulated feed. However, this method is not very effective because fish and other aquaculture animals do not effectively break down antibiotics. As a result, about 75 percent of the antibiotics fed to the animals are excreted into the water (Burridge *et al.*, 2010). These leftover antibiotics can be ingested by wild fish and shellfish, and the rest can remain in the sediment. These leftover antibiotics can lead to the selection of antibiotic-resistant bacteria, changing the composition of the sediment's microflora (Cabello, 2006). A study found that *Vibrio harveyi* strains with multiple resistances caused mass mortality in *Penaeus monodon* larvae (Karunasagar *et al.*, 1994). The resistance gene determinants in aquatic antibiotic-resistant bacteria have the potential to be transmitted to terrestrial bacteria through horizontal gene transfer, including to human and animal pathogens (Ishida *et al.*, 2010; Miller & Harbottle, 2018; Sørum, 2006;). This has been observed in *Salmonella enterica* serotype Typhimurium and *Vibrio cholerae*

Table 1. Various classes of antibiotics used in aquaculture

Class of antibiotic	**Mode of Action**	**Examples**	**Country**	**References**
Aminoglycosides*	Inhibiting bacterial protein synthesis by binding to bacterial ribosomes	Streptomycin	Vietnam	Dung *et al*. (2008)
		Neomycin	China	Liu *et al*. (2017)
	Inhibit protein synthesis	Kanamycin	Greece, Italy, France, and Egypt	El-Gohary *et al*. (2020); Pepi *et al*. (2021)
Glycopeptides*	Inhibit bacterial cell wall biosynthesis	Teicoplanin	China	Zhao *et al*. (2024)
Ansamycins*	Inhibit bacterial RNA synthesis	Rifamycin	China, the Philippines, and Vietnam	Lulijwa *et al*. (2020)
Amphenicols**	Inhibit protein synthesis	Florfenicol, Thiamphenicol and florfenicol, amphenicols	Chile, South Korea, Turkey, China, Viet Nam, Chile, Korea, and Portugal	Jang *et al*. (2018)
	Interfering with bacterial protein synthesis	Chloramphenicol	Chile	Saavedra *et al*. (2018)
Beta-lactams*	Inhibit bacterial cell wall biosynthesis	Amoxicillin	Australia	Algammal *et al*. (2022)
		Ampicillin	Vietnam, Thailand, Malaysia, Indonesia	Suyamud *et al*. (2024); Teo *et al*. (2000)
Fluoroquinolones**		Enrofloxacin	Spain, and Portugal	Avendano-Herrera *et al*. (2008)
Macrolides**	Inhibit the synthesis of protein by bacteria	Erythromycin	China	Broughton *et al*. (2009)
Nitrofurans**	binds bacterial DNA which leads to the gradual inhibition of monoamine oxidase	Furazolidones	Greece	Smith & Christofilogiannis (2007)
	inhibit the citric acid cycle, the synthesis of DNA, RNA, and protein	Nitrofurantoin	Taiwan	Liu *et al*. (1997)
	Inhibit bacterial cell wall biosynthesis	Furazolidone, nitrofurantoin, nitrofurazone and furaltadone	China, Vietnam, Korea and Portugal.	Bondad-Reantaso *et al*. (2023)
Quinolones*	Interfere with bacterial DNA replication and transcription	Oxolinic acid, enrofloxacin, ciprofloxacin, norfloxacin, nalidixic acid, ofloxacin, levofloxacin, enoxacin, sarafloxacin and flumequine	China, Philippines, Vietnam, South Korea, Egypt, Thailand, and Brazil	Lulijiwa *et al*. (2020); Tendencia & de la Pena (2001)
Sulphonamides**	Prevent bacterial growth and multiplication	Sulphadiazine	India	Das *et al*. (2009)
Tetracyclines**	Inhibit the synthesis of protein by bacteria	Tetracycline	China, Vietnam, South Korea, Thailand, Brazil, and Malaysia	Algammal *et al.* (2022); Lo *et al.* (2014); Shah *et al.* (2014); Suyamud *et al.*, 2024
		Oxytetracycline	Canada	Food and Drug Administration (2022); McIntosh *et al.* (2008)
		Doxycycline	Brazil, Finland, Chile, Taiwan Province of China, Vietnam, Bangladesh, Korea, South Africa, Tunisia, and Portugal.	Bondad-Reantaso *et al.* (2023)

*Commonly act as bactericidal agents, causing bacterial cell death

**Commonly act as bacteriostatic agents; restrict growth and multiplication

(Cabello, 2006; Defoirdt *et al.*, 2011; Miller & Harbottle, 2018; Sørum, 2006).

Another significant issue is the difficulty in determining the current dose of antibiotics used in aquaculture due to variations in distribution and registration systems across different countries (Burridge *et al.*, 2010). In 1994, about 500-600 metric tons of antibiotics were used in shrimp farm production in Thailand (Moriarty, 1999). Antibiotic use varies greatly between countries, with Norway using 1 g per metric ton of production and Vietnam using up to 700 g per metric ton (Smith, 2008). It is crucial to address the limited data availability that hinders our understanding of antibiotic usage and content in the aquaculture sector. Research by Heuer *et al.* (2009) and Smith (2008) highlights the challenges in obtaining a complete overview of this issue. The presence of residual antibiotics in commercialized aquaculture products due to this problem creates unpredictability and poses a risk to human health. Overuse of antibiotics in aquaculture has been linked to the unnoticed intake of these substances by humans consuming aquaculture animals such as fish and shrimp (Cabello, 2006). This unnoticed intake can lead to allergies and toxicity, which are difficult to diagnose due to the lack of information about antibiotic content in the aquaculture products.

From an aquatic environment perspective, the effect of excessive usage and large residual amounts of antibiotics on the normal flora and plankton in the aquatic environment can result in changes in the diversity of the aquatic microbiota by eutrophication because of high input of N, C, and P from non-ingested feed and feces in the water. Moreover, the heavy use of antibiotics is also capable of altering ecological equilibrium at the microorganism level, such as indicated by algal blooms and anoxic environments that have a big influence on the higher levels of consumers such as fish and humans (Cabello, 2006).

Those cumulative issues led to a significant restriction in the use of antibiotics in the aquaculture industry in numerous countries (Cabello, 2006; Defoirdt *et al.*, 2011). This restriction is not only strict regulations including prescription and proscription on the use of antibiotics, but also on the presence of antibiotic residues in aquaculture products. However, some countries that lack adequate regulation on antibiotic usage in aquaculture often face a problem in the worldwide trade of their aquaculture product. Their export product was rejected because the antibiotic residues were over the limit of maximum residue levels (MRLs) that are applied by many importing countries that have more strict regulations (Scalia-Bruce, 2023).

Intensive studies are underway to find alternative methods to protect aquaculture animals from pathogenic bacteria due to the negative impact of using antibiotics in this sector. One alternative strategy to replace antibiotic usage in aquaculture is by preventing the pathogenic bacteria from attacking the host without the need to kill them, called the antivirulence strategy. This strategy targets non-essential pathways of bacterial metabolism; therefore, it does not pose a strong pressure on the pathogen, making it unlikely to develop resistance. Indeed, this strategy needs a comprehensive understanding of the virulence mechanism by which pathogenic bacteria cause disease in aquaculture animals (Defoirdt, 2014).

VIRULENCE MECHANISM OF AQUACULTURE PATHOGENS

Virulence Factors

Infection by pathogenic bacteria triggers the activation of diverse virulence factors that are crucial for the pathogen to invade and harm its host. These essential virulence factors include gene products that play a role in adhesion, motility, host tissue degradation, toxin secretion, iron acquisition, and defense against host immunity (Defoirdt, 2014). Some studies showed that several factors have an influence on virulence factor expression, such as regulated by quorum sensing mechanism, bacterial cell-to-cell communication (Natrah *et al.*, 2011; Yang & Defoirdt, 2015), and increased by the presence of catecholamine stress hormones in media containing serum (Pande *et al.*, 2014; Yang *et al.*, 2014). Virulence factors play a critical role in the infectious cycle of pathogenic bacteria. These factors facilitate the entry of the pathogen into the host, as well as the establishment and multiplication of bacterial cells. They also help the bacteria evade host defenses, cause damage to host tissues and cells, and eventually exit the host.

The virulence factors of pathogenic bacteria have different mechanisms to infect their target hosts. Some important mechanisms, including bacterial motility, adhesion, production of lytic enzymes, chemotaxis, biofilm formation, siderophores, production of extracellular polysaccharides, iron acquisition, and secretion systems, have been reported (Defoirdt, 2014). Bacterial motility, adhesion, and chemotaxis play crucial roles in successfully infecting a host by colonizing and adhering to the host surface (Yang & Defoirdt, 2015). The production of extracellular polysaccharides (EPS) and biofilm formation enhances bacterial resistance to phagocytosis, providing protection from antimicrobial agents (Chen *et al.*, 2010). Moreover, the production of lytic enzymes, such as hemolysins, proteases, lipases, and chitinases, is essential for breaking down host tissues and enabling the pathogen to obtain nutrients and spread through tissues (Finlay & Falkow, 1997). Additionally, the iron acquisition mechanism is vital for thriving within the iron-repleted environment of a host. Many pathogenic bacteria can acquire iron via siderophores, and the secretion system is instrumental for transporting virulence factors out of the cell (Defoirdt, 2014).

Regulations

Understanding how bacteria regulate their pathogenicity is crucial for developing effective alternative treatments for bacterial diseases in aquaculture. Identifying the gene products responsible for bacterial pathogenicity and how these virulence factors are controlled is essential. Pathogenicity is not strictly species-dependent but rather a characteristic of specific bacterial strains, with some being highly virulent and others not. The relationship between the presence of virulence genes and bacterial pathogenicity is not always clear. In pathogenic bacteria, the production of virulence factors is tightly regulated, involving mechanisms such as cell-to-cell communication (quorum sensing) and ToxR, and is also influenced by host factors (Natrah *et al.*, 2011; Ruwandeepika *et al.*, 2012).

Quorum Sensing

Quorum sensing is a crucial regulatory mechanism in bacteria. It allows them to coordinate gene expression based on their population density using small signal molecules called autoinducers. This system was initially discovered in luminous marine bacteria such as *Vibrio fischeri* and *Vibrio harveyi* (Fuqua *et al.*, 1996; Miller & Bassler, 2001), but similar systems were later found in many other bacteria (Bassler, 1999). Quorum sensing systems can use N-acyl-homoserine lactones (AHLs) as signal molecules, or multi-channel signaling (Defoirdt *et al.*, 2011). AHLs of different species typically contain invariable lactone rings connected to variable acyl side chains with four carbons and 18 carbons. This acyl chain can have an oxo or hydroxyl substitution at the third position (Figure 1). Some aquaculture pathogens like *Yersinia ruckeri*, *Aeromonas hydrophila*, *Edwardsiella tarda*, *Aeromonas salmonicida*, and *Vibrio anguillarum* use AHLs as signal molecules, while the multi-channel quorum sensing system is found in vibrios such as *Vibrio harveyi* and *Vibrio vulnificus* (Milton, 2006; Natrah *et al.*, 2011).

The quorum sensing system in *Vibrio campbellii* strains BB120 (=ATCC BAA-1116) is one of the most intensively studied (Lin *et al.*, 2010) (Figure 2). This bacterium uses three different signals: Harveyi Autoindicer-1 (HAI-1), Autoinducer-2 (AI-2), and Cholerae Autoinducer 1 (CAI-1) (Cao & Meighen, 1989; Chen *et al.*, 2002; Higgins *et al*., 2007).

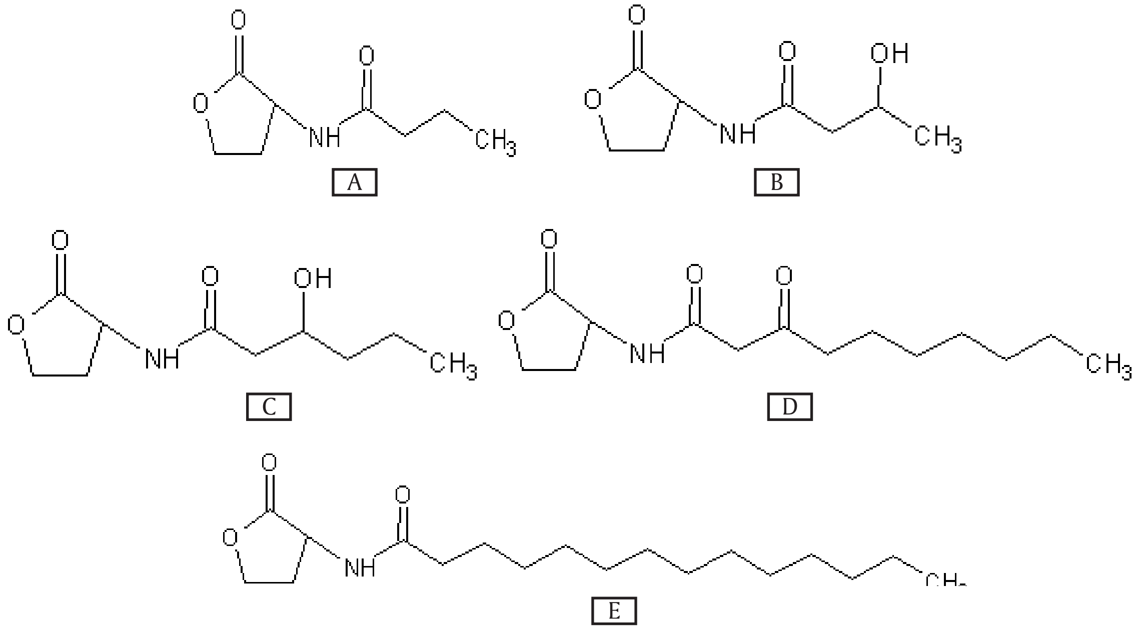


Figure 1. Chemical structures of different AHL molecules produced by different aquaculture pathogenic bacteria species: a. N-butanoyl-L-homoserine lactone produced by *Aeromonas hydrophila* and *Aeromonas salmonicida*, b. N-(3-hydroxybutanoyl)-L-homoserine lactone produced by *Vibrio campbellii*, c. and d. N-(hydroxyhexanoyl)-L-homoserine lactone and N-(oxododecanoyl)-L-homoserine lactone, both produced by *Vibrio anguillarum*, and e. N-tetradecanoyl-L-homoserine lactone

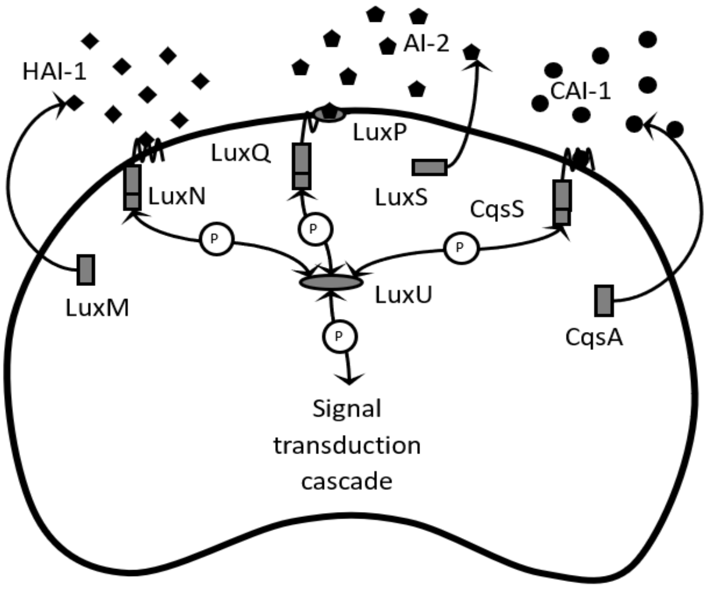


Figure 2. Quorum sensing in *Vibrio harveyi*. The LuxM, LuxS, and CqsA enzymes synthesize the autoinducers HAI-1, AI-2, and CAI-1, respectively. These autoinducers are detected at the cell surface by the LuxN, LuxQ, and CqsS two-component receptor proteins, respectively. Detection of AI-2 by LuxQ requires the periplasmic protein LuxP. The receptors feed a common phosphorylation or dephosphorylation signal transduction cascade regulating the expression of target genes. Ⓟ denotes phosphotransfer

Discoveries in the detection of autoinducers by surface district membrane-bound, two-component receptor proteins have led to a better understanding of how these proteins initiate a signal transduction cascade controlling the production of the transcriptional regulator protein LuxR (Taga & Bassler, 2003). The significant role of quorum sensing in regulating the expression of various virulence factors in pathogenic bacteria and influencing virulence towards different hosts *in vivo* has garnered substantial attention and research efforts focused on developing techniques to disrupt quorum sensing (Defoirdt *et al.*, 2008; Pande *et al.*, 2013) are underway.

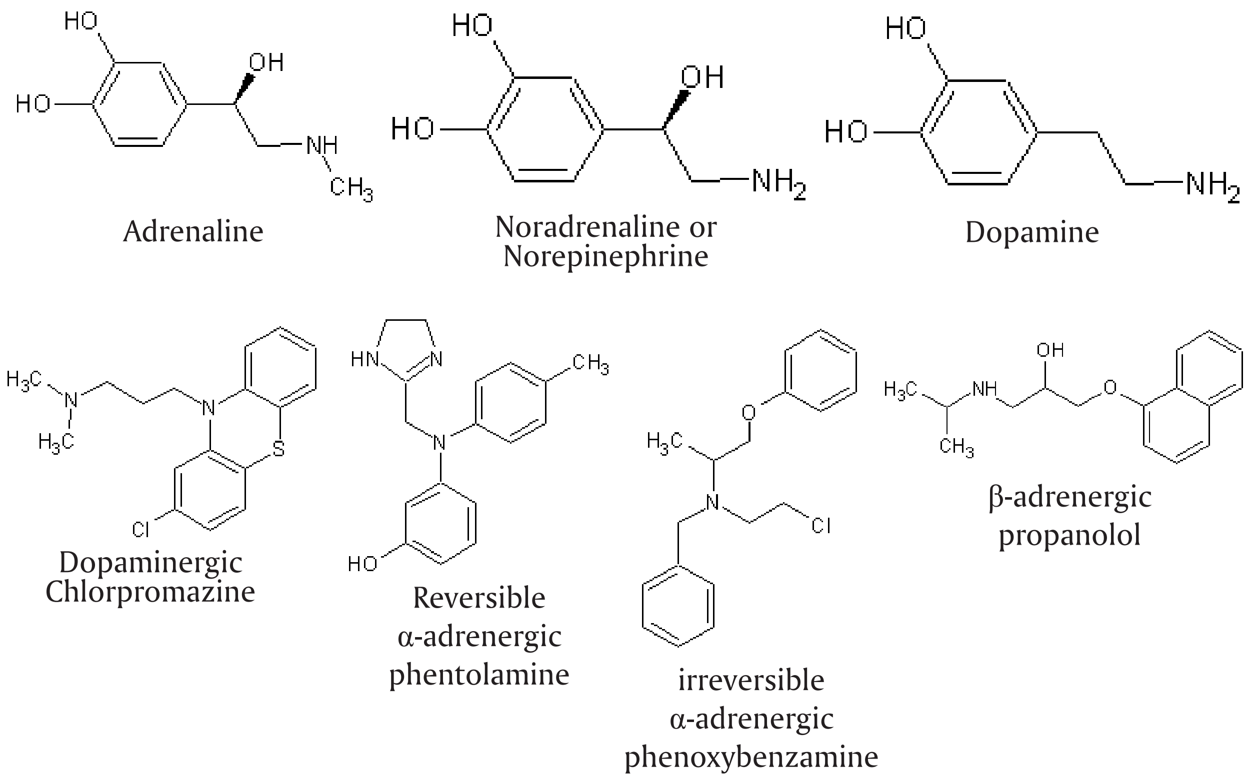
Host Factors

Consideration of host factors is crucial in understanding the expression of virulence in bacterial infections. Several metabolism products and stress levels of the host play a significant role in the success of bacterial infections. It has been established that host stress can diminish the activity of the host defense system, thus influencing the outcome of host-microbe interactions (Vicente-Santos *et al.*, 2023). Moreover, new findings indicate that infectious bacteria have evolved specialized detection systems to identify stress hormones produced by their host, which may have a potential link to the heightened virulence of pathogens (Sarkodie *et al.*, 2019).

Catecholamines Stress Hormones

Research has primarily honed in the impact of stress hormones such as adrenaline (epinephrine), noradrenaline (norepinephrine), and dopamine on the growth and virulence of pathogenic bacteria in the host gut tissues (Freestone *et al.*, 2008). It has been demonstrated that these hormones can influence the growth, motility, biofilm formation, and/or virulence of intestinal pathogens like *Escherichia coli* and *Salmonella* spp. (Verbrugghe *et al.*, 2012). Notably, catecholamines have been shown to enhance the growth and virulence of the human pathogenic *Vibrio parahaemolyticus* in serum-based media (Nakano *et al.*, 2007). This suggests that the response of bacteria to catecholamines may be important in the virulence of aquaculture pathogenic bacteria, as the host organisms produce these hormones. Norepinephrine and dopamine significantly induced virulence in two aquaculture pathogenic bacteria, *V. campbellii* and *V. anguillarum*, by increasing motility and growth in media containing serum (Pande *et al.*, 2014). The addition of serum accurately mimics the iron-limited host environment, where transferrin, a high-affinity ferric-iron-binding protein, regulates iron availability (Freestone *et al.*, 2008). Current literature supports the mechanism wherein catecholamines create complexes with transferrin, leading to the reduction of ferric iron (Fe3+) to ferrous iron (Fe2+). This process weakens the bond between iron and transferrin, making iron available for bacterial use (Freestone, 2013; Lyte, 2014). In addition, some studies reported that catecholamines enhanced the motility of *E. coli*, *Salmonella typhimurium*, *Campylobacter jejuni*, and the common aquaculture pathogen *E. tarda* and *V. harveyi* (Bearson & Bearson, 2008; Cogan *et al.*, 2007; Kendall *et al.*, 2007; Wang *et al.*, 2011; Yang *et al.*, 2014).

The research has found that the antagonist of eukaryotic catecholamine receptors can counteract some of the effects of catecholamines (Pande *et al.*, 2014) (see Figure 3). The eukaryotic dopamine receptor antagonist effectively neutralized the motility-inducing effect of dopamine in the aquaculture pathogen *V. campbellii*. Additionally, the α-adrenergic receptor antagonists phentolamine and phenoxybenzamine successfully counteracted the motility-inducing effect of norepinephrine. In contrast, the β-adrenergic receptor antagonist propranolol had minimal to no impact (Pande *et al.*, 2014). Other research have shown that both α-adrenergic and β-adrenergic receptor antagonists were capable of blocking the response of *E. coli* O157:H7 to norepinephrine and epinephrine (Sperandio *et al.*, 2003). Moreover, the virulence of *V.*



Adrenaline

Noradrenaline or Norepinephrine

Dopamine

Dopaminergic Chlorpromazine

Reversible α-adrenergic phentolamine

irreversible α-adrenergic phenoxybenzamine

β-adrenergic propanolol

Figure 3. Chemical structure of the catecholamine hormones (adrenaline, noreadrenaline or norepinephrine, and dopamine) and eukaryotic receptor antagonist (chlorpromazine, phentolamine, phenoxybenzamine, and propranolol)

campbellii was found to increase towards the larvae of giant freshwater prawn *Macrobrachium rosenbergii* when they were pre-treated with catecholamines, without affecting the growth of surviving larvae. Nevertheless, the effects of catecholamine receptor antagonists on *in vivo* virulence were less definitive compared to the *in vitro* experiments (Pande *et al.*, 2014). These findings highlight the potential of receptor antagonists in modulating the effects of catecholamines and merit further exploration for their potential applications.

Mucin, Bile Salts, and Cholesterol

The expression of virulence factors in bacteria can be influenced by various host factors, including stress hormones, mucin, bile salts, and cholesterol (Defoirdt, 2014; Li *et al.*, 2014). Studies have shown that these host factors can increase the virulence factors of *V. anguillarum*, including protease activity, flagellar motility, biofilm formation, and exopolysaccharide production, without affecting the growth of the bacterium towards gnotobiotic sea bass (*Dicentrarchus labrax*) larvae (Li *et al.*, 2014). Additionally, bile has been found to trigger the production of virulence factors such as type III secretion system-related protein, hemolysins, and capsular polysaccharide in *V. parahaemolyticus* (Hsieh *et al.*, 2003).

Interference Virulence Factors

Several innovative methods have been developed to combat bacterial infections in aquaculture without relying on antibiotics, by targeting virulence factors. This antivirulence strategy involves disrupting the regulation of virulence factor expression, impacting multiple virulence factors simultaneously, or specifically inhibiting a particular virulence factor (Defoirdt, 2014). Efforts have been made to interfere with virulence regulatory mechanisms, such as inhibiting bacterial quorum sensing with quorum sensing disrupting agents (Defoirdt *et al.*, 2012; Lu *et al.*, 2022; Natrah *et al.*, 2012; Pande *et al.*, 2013; Zhou *et al.*, 2020), and interfering with bacterial detection of host catecholamines stress hormones by the QseC receptor (Rasko *et al.*, 2008). Additionally, specific virulence factors have been inhibited by blocking bacterial secretion systems with acylated salicylaldehyde hydrazones and thiazolidinones (Baron, 2010), and inhibiting bacterial pili formation, known as pilicides, with bicyclic 2-pyridones (Clatworthy *et al.*, 2007). Nonetheless, this strategy is yet to be tested against aquaculture pathogens and requires further exploration.

Quorum Sensing Inhibition

The increasing understanding of bacterial pathogenesis has led to efforts to inhibit bacterial cell-to-cell communication mechanisms known as quorum quenching. Quorum sensing inhibition, which is a key area of study in antivirulence strategies, can be achieved through various methods including inhibiting signal molecule biosynthesis, using quorum sensing antagonists, chemically inactivating and enzymatically degrading quorum sensing signal molecules, and using quorum sensing agonists (Defoirdt *et al.*, 2004; Kalia, 2013). Further exploration of these different methods is needed to identify the most effective approach for treating bacterial diseases across various fields, including aquaculture.

Researchers have successfully reduced the production of quorum sensing signal molecules by using substrate analogs (Defoirdt *et al.*, 2004). For example, S-adenosylcysteine, an analog of S-adenosylmethionine, has been found to decrease the activity of *Pseudomonas aeruginosa* LuxI RhlI by up to 97% (Parsek *et al.*, 1999). S-adenosylmethionine, a substrate for the homoserine lactone moiety utilized by homologs of *V. fischeri* LuxI protein, plays a crucial role in the biosynthesis of Gram-negative AHL signal molecules (Whitehead *et al.,* 2001). This research highlights the potential use of S-adenosylmethionine analogs as specific inhibitors of quorum sensing, offering a targeted approach without disrupting essential processes in prokaryotic and eukaryotic organisms (Defoirdt *et al.*, 2004).

Quorum sensing antagonists can effectively block the transmission of signal molecules, offering a promising strategy to control virulence factors in aquaculture pathogens. One type of quorum sensing antagonist is long-chain natural AHLs produced by bacteria. These AHLs have been shown to reduce the production of virulence factors in aquaculture pathogens such as *A. hydrophila* and *A. salmonicida*, and protect burbot (*Lota lota*) larvae from infection by these pathogens (Natrah *et al.*, 2012). Another type of quorum sensing antagonists is synthetic quorum sensing inhibitory AHL analogs, such as halogenated furanones, brominated thiophenones, and cinnamaldehyde (Benneche *et al.*, 2011; Brackman *et al.*, 2008; Janssens *et al.*, 2008) (Figure 4). Furanones specifically disrupt AHL-mediated quorum sensing by interacting with the LuxR-type AHL receptor, reducing the amount of LuxR available as a transcriptional regulator (Menefield *et al.*, 2002). Furanones and thiophenones have also been shown to block the multichannel quorum sensing systems of vibrios by decreasing the DNA-binding activity of the master regulator LuxRvh (Defoirdt *et al.*, 2007). Studies have demonstrated the protective effect of these antagonists against vibrios in both fish and crustacean larvae (Defoirdt *et al.*, 2006; Pande *et al.*, 2013). However, it's crucial to note that while these compounds offer significant benefits, caution should be exercised as halogenated furanones can be toxic to higher organisms at specific concentrations.

A further method is by chemical inactivation of quorum-sensing molecules. The quorum sensing signal can be chemically inactivated by yielding the cognate acyl homoserine via alkaline hydrolysis at pH ≥ 8

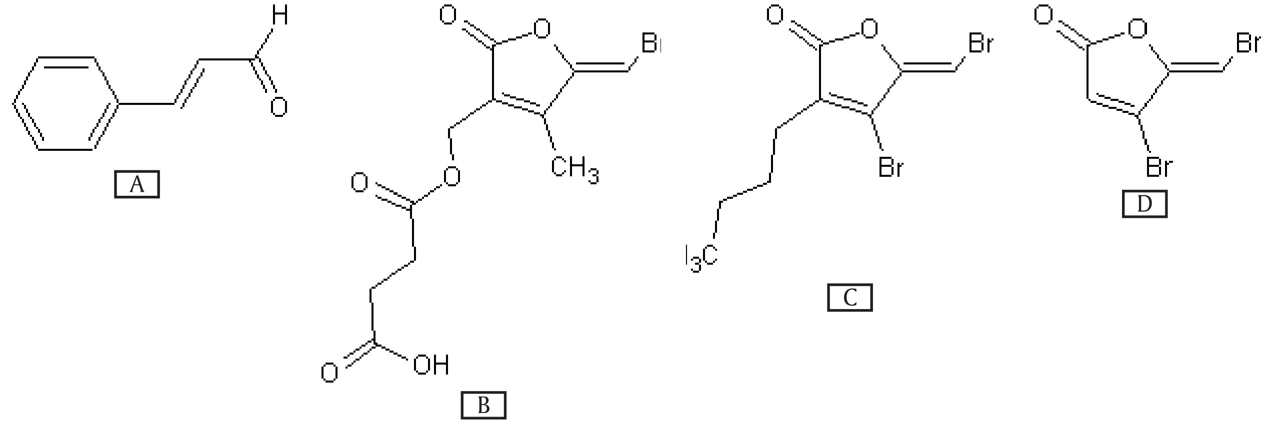


Figure 4. Chemical structure of some quorum sensing-disrupting compounds. a. Cinnamaldehyde, b. The brominated thiophenone (*Z*)-4-((5-bromomethylene)-2-oxo-2,5-dihydrothiophen-3-yl)-4-oxo-butanoic acid, c. The natural furanone (*5Z*)-4-bromo-5-(bromomethylene)-3-butyl-2(*5H*)-furanone, produced by the red marine algae *Delisea pulchra*, and d. The synthetic derivative (*5Z*)-4-bromo-5-(bromomethylene)-2(*5H*)-furanone

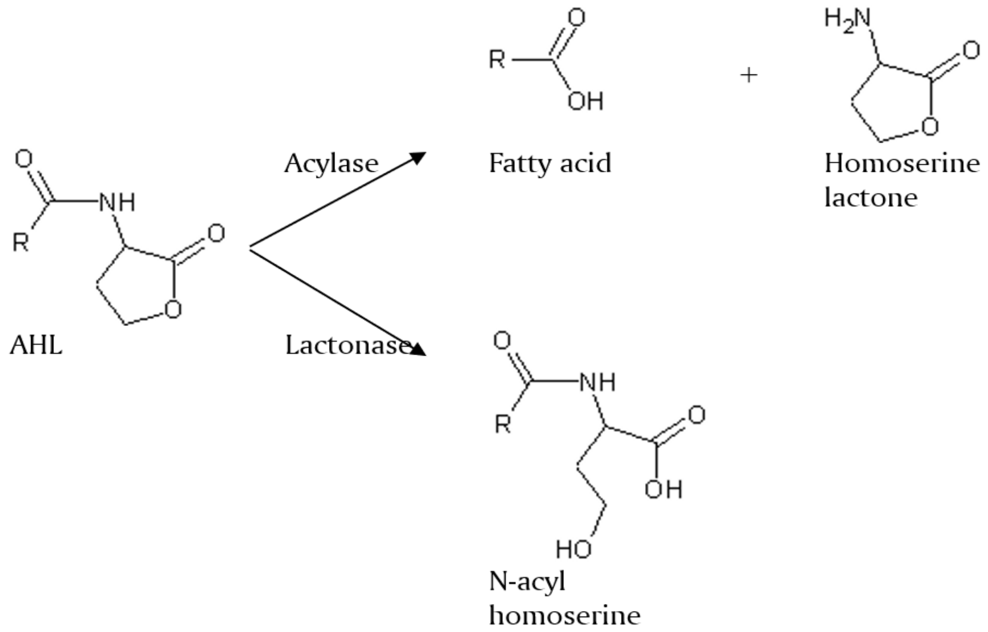


Figure 5. Degradation of AHL molecules by the action of acylase and lactonase enzyme

(Decho *et al.*, 2011), and by oxidized halogen antimicrobials for 3-oxo-substituted AHLs (Michels *et al.*, 2000). The signal inactivated by oxidizing indicated that treating culture water with strong oxidizing agents, such as ozone, for removing the signal molecules of pathogen quorum sensing system might be useful as an anti-infective strategy in aquaculture (Defoirdt *et al.*, 2004).

Quorum sensing can be effectively disrupted through enzymatic breakdown of signal molecules. This method is commonly utilized to interfere with AHLs quorum sensing signals (Kalia & Purohit, 2011). Enzymatic degradation, based on the AHL structure, can be facilitated by deaminase lactonase, acylase, and decarboxylase (Hong *et al.*, 2012).

The lactonase enzyme plays a crucial role in breaking down AHLs by cleaving the ester bond of the lactone ring, which are important for binding with specific transcriptional regulators (Dong *et al.*, 2000). This enzyme is encoded by the AHL-inactivating activity (AiiA) gene and is found in various *Bacillus* species (Dong *et al.*, 2002). Similarly, the AHL acylase enzyme contributes to a significant reduction in the effectiveness of the signaling molecule by breaking the peptide bond of the lactone ring, releasing a fatty acid, and homoserine lactone (Fast & Tipton, 2012).

The use of AHL-degrading enrichment culture to break down AHL-signal molecules has been found beneficial in certain aquaculture animals (Cam *et al.*, 2009; Nhan *et al.*, 2010; Tinh *et al.*, 2007;). AHL-degrading enrichment cultures can be developed by using media that contain AHLs as the primary carbon and/or nitrogen source. Pure strains of AHL-degrading *Bacillus* sp. have been isolated from this enrichment culture (Defoirdt *et al.*, 2011). Therefore, bacteria with the ability to break down quorum sensing signal molecules may be useful as a new type of probiotics for aquaculture.

Future Perspectives

In our comprehensive review, we provided an insightful analysis of antibiotic challenges and offered compelling alternative strategies for effectively controlling bacterial infections in aquaculture. The antivirulence strategy stands out as a particularly promising method for combating diseases caused by aquaculture pathogenic bacteria. One of its key advantages lies in its significantly lower potential for bacterial resistance development compared to antibiotics. This is due to the fact that the selective pressure exerted is confined to the pathogens through specific killing by phages or targeted disruption of quorum sensing in specific environments. This is a marked contrast to the broad impact of conventional antibiotics, which also affect harmless and beneficial bacteria (Defoirdt *et al.*, 2011).

To ensure success, aquaculture's antivirulence strategy requires precise targeting of specific pathogens in their respective environments. Hence, a comprehensive understanding of the virulence mechanisms of aquaculture pathogenic bacteria is imperative. Recent scientific breakthroughs have unveiled promising antivirulence strategies, such as disrupting bacterial cell-to-cell communication and host-pathogen signaling. Further exploration of these mechanisms holds great potential for advancing disease treatment. A deeper grasp of this knowledge could pave the way for innovative biocontrol methods to combat bacterial diseases and infections, offering a sustainable alternative to antibiotics in aquaculture.

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