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MARINE BACTERIA AS POTENTIAL PROBIOTICS IN AQUACULTURE

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ABSTRACT

Marine microbes are known for their bioactive compounds in various industries. Similarly, marine bacteria are vital in sustainability of aquaculture around the world. Despite its essential role in synthesis of bioactive compounds, commercial use of marine bacteria as probiotics in aquaculture industry is the neglected sector in the world. Few developed countries are utilizing the probiotics in aquaculture industry while rest of the world has not considered it as an option. Probiotics can target wide spectrum of issues in aquaculture industry from reducing pollutants to a source of disease treatment that can be exploited accordingly. Marine bacteria are selected via rigorous processes to isolate potential probiotic. These probiotics are known for targeting various sites for competition, chemicals present in the environment, produce inhibitory substances to prevent fungal, bacterial, viral infection, augment stress conditions, and boost immunity. In this review we will highlight the importance of marine bacteria as potential source of probiotics in aquaculture industry. We aimed to highlight the challenges faced by aquaculture industry to emphasize the gravity of this issue. Finally, we stress on the advantages on marine probiotics and the methods that can be employed to identify potential probiotics. Marine bacteria are identified via series of processes involving pre-experimental screening in which the marine bacteria are isolated, cultured and tested for its activities in favor of host health. Second is experimental screening in which the microbe is delivered to aquaculture host and the results are observed. If the results are substantially robust in terms of improving health, the probiotic is approved for final screening tests. Finally, in post-experimental screening, the marine bacteria are identified to its strain level and assessed whether it has the potential to be used as probiotics and can be produced as mass culture. Further research is essential to identify promising bacteria and effectively utilize them in the aquaculture culture industry. It is crucial to aware farmers regarding the economic importance of aquaculture at an industrial scale to boost economy but produce quality seafood.

Keywords: Aqua culture, Probiotics, Screening methods, Mode of action of probiotics Economy

INTRODUCTION

About 3.5 billion years ago, life began in the sea, where evolutionary processes equipped marine organisms with adapted mechanisms to survive in hostile environments. The diverse microbes in marine biosphere consist of unique structural, functional, and metabolic properties. Taxonomic classification cataloged more than 1.2 million marine microbial species **(Mora *et al.*, 2011).** While the active molecules produced by terrestrial organisms have been studied for a long time. Marine environment has accommodated variety of organisms that evolved to particular conditions, thereby inculcated with the broad range of distinctive active bio-compounds—promising resources in various industries such as: nutraceuticals, pharmaceuticals, agrochemicals, enzyme production, agrichemicals, cosmeceutical, food industries **(Prabha et al., 2020).** These bioactive compounds are utilized in different sectors for benefit of human life are worth multibillion dollars of market value. Marine bacteria are ubiquitous in seawater, sediments **(Rehman *et al.*, 2018),** aquatics animals like sponges, corals **(Pereira *et al* 2017),** oysters **(Kang *et al.*, 2018)** and fishes **(Augustine & Joseph, 2018)** etc. Therefore, marine bacteria and its products can be utilized as probiotics in aquaculture industry **(Das *et al.*, 2008).**
The term “probiotics” was first introduced in 1965 to explain the phenomenon in which one organism secrete substances that promotes the growth of another organism. Later the definition of probiotics was changed as “living organisms, which, when administered in adequate amounts confer a health benefit on the host” **(FAO/WHO, 2001).** There are two sources for probiotics based on its origin as terrestrial, and aquatic or marine based. *Pseudoalteromonas* spp. isolation from eukaryotic hosts such as: sponges **(Ivanova *et al.*, 1998),** mussels **(Ivanova *et al.*, 1996; 1998)** or *Staphylococcus saprophyticus* SBPS 15 which is found in sediments of ocean **(Mani *et al.*, 2016).** Though, marine probiotics have been utilized in livestock **(Prieto *et al.*, 2014),** and aquaculture **(Das *et al.*, 2008).**
Previously, research has been focused on the exploitation of microbial products in various industries. However, researchers, industrialists, aqua-culturists are unaware of the potential marine bacteria possesses in nutraceutical industry. Previously, marine bacteria were focus of various studies involving bioactive compounds. However, the use of marine probiotics has hardly been given any credit, and hardly summarized in prospective to its economic worth for developing countries. This review will focus to describe importance of aquaculture, its components, and its applications. However, this domain has not been explored yet, therefore this topic will be make strong inferences from the data available on probiotics, followed by the advantages of marine probiotics such as improve aquatic environment around the aquatic organism, improve stress conditions, interrupt quorum sensing among pathogens, boost host immunity, produce enzymes and nutrients to boost health of fishes. Finally, various screening experiments will be highlighted to identify potential marine probiotics **(Prabha *et al.*, 2020).**

AQUACULTURE

Aquaculture is the farming of aquatic organisms. This farming process is regulated under completely controlled or semi controlled conditions. There are different kind of aquaculture including fish farming, oyster farming, shrimp farming, algaculture, mariculture, and nurturing of ornamental fish **(FAO, 2019).** Aquaculture can be employed for many purposes such as raising the fishes for commercial purpose, for protecting endangered species, for obtaining commercially sustainable crop in a pond or coastal waters. Approximately, 580 species are involved in the aquaculture farming worldwide depicting the biodiversity within and among species **(Kumar & Deshmukh, 2020).** Aquaculture attained great attention as an economic activity worldwide due to overfishing of wild population.
Bacteria can be isolated from anywhere in the marine environment as long as it acts as probiotic—benefits the aquaculture community—and fulfill the requirement of the selection criteria. Once the target bacteria have been isolated it is necessary to test its phenotypic traits beneficial for the host. The bacteria can produce antimicrobial compounds that can prevent the specific infection against the pathogenic organism, or they can produce extracellular enzymes that can improve the host health. This way their potential is proved as a probiotic. Moreover, to ensure bacterial adjustment in new environment, the probiotic is introduced in the environment where the aquaculture farm is developed. The probiotic undergoes series of observation to proof its potential through living in

the waters or through colonizing the host as mentioned. If the bacteria grow and show desired results, it is considered potential organism.

Aquaculture industry

Aquaculture was in practice over 3000 years ago. Indo-pacific regions and China have the history of practicing culturing fishes, shell fishes and plants under controlled conditions at least 2000 years B.C **(FAO, 2019).** It is developing part of global agriculture today (Figure 1). According to **FAO (2014a)** statistics, aquaculture achieved 90.4 million tons production worth of 144.4 billion US dollars in 2012 globally. In 2017, 238 billion dollars was estimated from the aquaculture production worldwide **(FAO, 2018).** From aquaculture and fisheries combined, the total capture production of animals was arisen from 25.7% in 2000 to 46.4% in 2017. The production was 80.1 million tons which was raised to 4.9% in comparison with 2016 **(FAO, 2019).** In 2019, 285,359.7 million dollars was estimated in the global aquaculture market, and it is anticipated to attain 378,005.5 million dollars by 2027 **(Kumar & Deshmukh, 2020).** With influx of population, consumer demand for sea food has increase exponentially and commercial aquaculture is promising source to satisfy the demand of 10 billion people by 2050 **(FAO, 2017).** Aquaculture is well established sector in United States, the country produced 1.5 billion dollars' worth of seafood from aquaculture in 2017. Oyster, clams, and Atlantic salmon were U.S top marine aquaculture species worth 186 million dollar, 129 million dollar and 61 million dollars respectively **(Fisheries, 2020).**

Thailand is among growing country in terms of aquaculture production in last few decades. It was ranked among 25 countries in terms of fisheries production in 2018 **(FAO, 2018).** Department of fisheries' statistics estimated that in 2016, Thailand's aquaculture produced more than 0.9 million tons in which 0.5 million tons or 57% were from coastal aquaculture and 0.4 million tons or 43% were from freshwater aquaculture **(DOF, 2018).** Moreover, Thailand probiotics are being investigated in many countries including China **(Wang *et al.*, 2019),** sub-Saharan African countries **(Kaktcham *et al.*, 2018),** India **(Mukherjee *et al.*, 2016),** Pakistan **(Chaudhary *et al.*, 2021)** Egypt **(Desbois *et al.*, 2021)** etc.

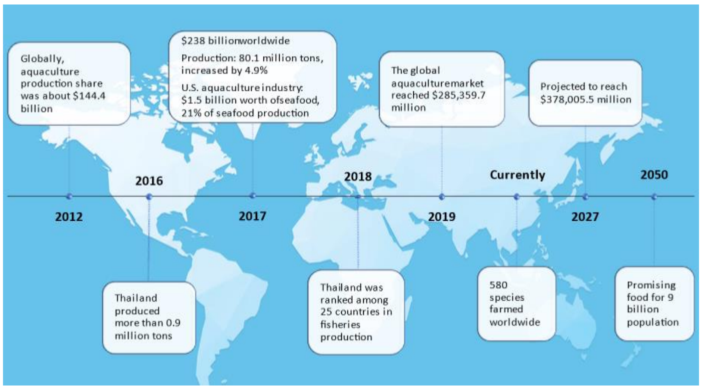

Figure 1 Value of aquaculture worldwide.

Pakistan has recently started to pay attention towards aquaculture so this sector is still in its developing stages but there is great potential for its development in Pakistan (Figure 2). Even though Pakistan has variety of water resources like fresh, brackish, and marine water, only few fishes are cultured in inland waters and earthen ponds with very insignificant inputs. Currently coastal aquaculture has not been initiated where the coastline covers 1,100 km with good potential of aquaculture. The little input in aquaculture sector leads to capture of fishes from wild population which is the major contributor in the production of fishes **(FAO fisheries division, 2009).**

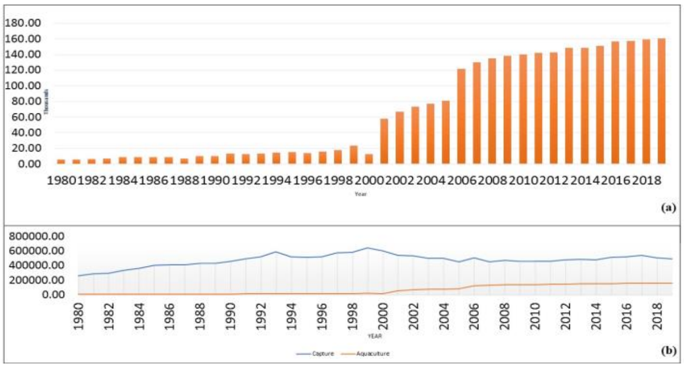

Figure 2 (a) Total aquaculture production (in Tonnes) in Pakistan according to FAO statistics (FAO fisheries division, 2019). (b) Total capture and aquaculture production for the Islamic Republic of Pakistan (tonnes).

The price of fishes is escalating due to limited production from aquaculture with growing population and poverty exacerbating the situation. Pakistan has contributed very little amount of fish production (produced 1.9kg in 2013) as compared to production worldwide (produced 19.8kg fishes in 2013) due to inappropriate practices and awareness. This is the reason that compels the government to import the fishes from China, Myanmar, Viet Nam, Singapore, Thailand, and Burma which are major contributors in the fisheries worldwide. As fisheries contributes to provide food, income, and employment, it is considered as important part of Pakistan national economy. The aquaculture and fisheries sectors are in infancy and vulnerable to climate change or natural disasters. Therefore, preparedness to overcome the losses and recover damages to the sector need immediate attention **(FAO fisheries division, 2019).**

Challenges in aquaculture

As aquaculture is fastest growing sector for food production system worldwide, there are limitations in the expansion of this sector. The practice for aquaculture requires high density cultivation which cause noteworthy damage to the environment. The damage includes organic pollution, chemical pollution, fish for fish feed and diseases. Organic pollution is nutrient pollution cause by uneaten food and fish waste discharge in aquaculture. Continuous discharge of nutrients results in concentrated amount of nitrogen, phosphorous which cause negative ecological impacts like eutrophication, oxygen depletion or algal blooms **(David *et al.*, 2009).**

Chemical pollution is caused due to usage of prophylactic and therapeutic products. This is common issue as pathogenic community removal is necessary in aquaculture. These chemical additives are used to reduce the risk of diseases but it lead to antibiotic resistant strains of pathogen which can consequently risk the health of human beings **(WHO, 2012).** Moreover, these antibiotics residues are accumulated in the flesh of the organism and kill the Gastrointestinal tract (GIT) flora of the organism. Low value of fish as feed ingredients are used in some aquacultures. This negligence can indirectly affect aquatic ecosystems thousand miles from fish farm if it is in marine environment. The usage of low value fish causes the discharge of high concentration of phosphorous, nitrogen and contributes in chemical and organic pollution **(Tacon *et al.*, 1995).**

Aquaculture is intense cultivation practice, where diseases are expected problem that go hand in hand. This problem can impede economic development of many countries as diseases cause epidemics, high mortality, difficult to control, and they harm the aquatic organism in growth stages including overwintering, breeding, and maturation **(Bondad *et al.*, 2005).** To avoid these situations, probiotics are recognized as one of the solutions in aquaculture (Table 1).

Table 1 Marine bacteria as probiotics in aquaculture

Bacteria	***Source***	***Aquaculture host***	***Function***	***References***
Paenibacillus spp.	Marine sediments	Shrimp	Antibacterial	Ravi *et al.*, 2007
Paenibacillus polymyxa	Marine sediments	Shrimp	Antibacterial	Ravi *et al.*, 2007
Bacillus cereus	Marine sediments	Shrimp	Antibacterial	Ravi *et al.*, 2007
Vibrio alginolyticus	Turbot	Fish larvae	Improve survival rate	Gatesoupe, 1990
Lactobacillus plantarum	Rotifers	Fish larvae	Antibacterial	Gatesoupe, 1994
Streptomyces strain	Marine sediment	Shrimp	Antibacterial, improve survival rate	Das *et al.*, 2010
Vibrio alginolyticus	Pacific Ocean seawater	Shrimp	Increase of survival and weight shrimp's post-larvae; antibacterial	Garriques, 1990
Alteromonas sp	Coastal seawater	Shrimp	Increase of survival shrimp larvae; antibacterial	Haryanti *et al.*, 2017
Lactobacillus plantarum AH 78	Sediments, algae, sponge and corals, coastal water	Fish	Improved survival; antibacterial	Hamdan *et al.*, 2016

SELECTION CRITERIA OF PROBIOTICS

Commercialization of probiotics is a rigorous process and involves series of steps to ensure its quality and efficacy. Food and Agriculture Organization (FAO) has capitulated selection criteria of the probiotics having special properties to be selected as potential probiotics **(FAO, 2002).** The criterion of selection is given below:

1. The probiotics should have beneficial effect on host and provide protection against various pathogens.
2. It should not harm the host in any way (non-toxic, nonpathogenic or any unfavorable side effects).
3. It should survive in host gastrointestinal tract (Gastro-Intestinal Tract; inside and outside).
4. The product should have adequate amount of probiotic to confer health benefit to the host.
5. The quality should not be compromised and should be compatible if the probiotics are processed and stored to sustain the required properties.
6. The probiotic should be aerobic or facultative anaerobic to ensure its survival in prolong exposure of oxygen environment
7. The probiotics should not be drug resistance, they must have the ability to keep up the hereditary traits.

The potential probiotic should be identified accurately through variety of in-vitro test to examine its functional properties because the probiotics are condition, dose and strain specific. The probiotic strain must be well defined to obtain the specific probiotics product **(Morelli, 2000).**

PROBIOTIC MODE OF ACTION

Probiotics isolated from different habitats of marine environment are providing benefits to the host through acting against pathogens of the host, also providing health benefits to the aquaculture community. When the microbe is improving the health of the organism, it competes for colonization and chemicals or energy, produce antimicrobial compounds. Furthermore, if it acts against the pathogen of the aquaculture host, it improves water quality, stress conditions and immunity. It also contributes in nutrient, enzymes and increase the length of the intestinal villi.

Activity against host pathogen

Probiotics have various mechanism to confer benefits to the host such as competition for site, competition for chemical or energy source, production of inhibitory substances, antifungal activity of probiotic bacteria in aquaculture, and antiviral activity of probiotic bacteria in aquaculture (Figure 3). These modes of actions are used against the pathogen of the aquaculture host to indirectly help the aquatic organism to grow.

Competition for site

Interference of probiotics with the attachment of pathogen is a desirable quality for the probiotic selection. Competition of site is also known as competitive exclusion in which probiotic colonize the Gastro-Intestinal Tract and attach to the surface of epithelium and prevent the pathogen to harm the host **(Balcázar *et al.*, 2006; Lazado *et al.*, 2011)**. For example, *Enterococcus faecium* and *Lactobacillus pentosus* are reported to attach to intestinal mucus layer of shrimp and compete against host pathogen such as *Vibrio spp.* **(Sha *et al.*, 2016).**

Competition for energy source

Microbial population require available energy and chemicals in the environment in order to survive. These nutrients are also needed by other microbial communities present in the same environment and this make the microbes to compete for the chemical and energy source. This characteristic can be used against the pathogen of host in order to prevent them to cause disease. For example, bacteria producing siderophore (iron chelating agent dissolve complex iron and make it in available form of iron for bacteria) can be used as probiotics because they can consume the iron and prevent pathogenic growth **(Tinh *et al.*, 2008).**
P. fluorescens is reported to be competitive in inhibiting the growth of *A. samonicida* by using iron present in the same environment as the pathogen **(Gram *et al.*, 1999).** It was also reported that GP12 (*Psychrobacter* sp.) and GP21 (*Pseudomonas* sp.) are the probiotic candidate as they are capable of utilizing iron using siderophores **(Lazado *et al.*, 2011).**

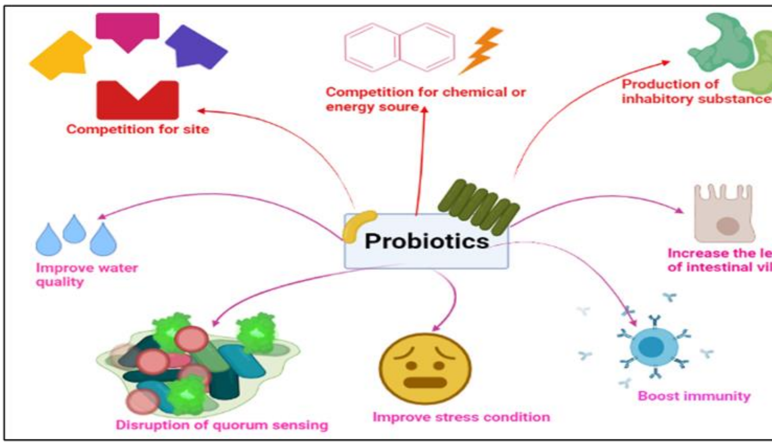

Figure 3 Probiotics mode of action. Probiotics actively works against pathogens by competing for site, chemical or energy source, producing inhibitory substances and disrupts quorum sensing. It also benefits the host by improving water quality, removing stress and boosting immunity.

Production of inhibitory substances

Probiotics can produce compounds and substances that show antifungal, antiviral or antibacterial activity on other microorganisms. The substances include siderophores, lysozymes, protease, bacteriocins, hydrogen peroxide among many others and the compounds includes volatile fatty acids (e.g., butyric, propionic, lactic, and acetic acid) and organic acids that can help in reduction of pH of GIT lumen to prevent opportunistic pathogenic growth **(Tinh *et al.*, 2008).** For example, Indole (2,3-benzopyrrole) is reported to have inhibitory effect against various pathogens of aquatic organisms i.e., *Aeromonas salmonicida*, *Edwaedsiella trada*, *Yersinia ruckeri* and *Vibrio anguillarum* **(Lategan *et al.*, 2006).**

Antifungal activity

Fungal diseases are also concerning issue in aquaculture. One of the major diseases, saprolegniosis cause serious economical damage to fish farms. Many antifungal drugs have been applied but biocontrol is considered the best solution for the disease. *Aeromonas media* A199 has shown inhibitory effect on *Saprolegnia parasitica* which cause serious fungal infection in fish (eel, silver perch). In other studies, the probiotic is added to the water column which consequently released the hyphal matter from the skin of the fish into the water **(Lategan *et al.*, 2004)** (Figure 4).

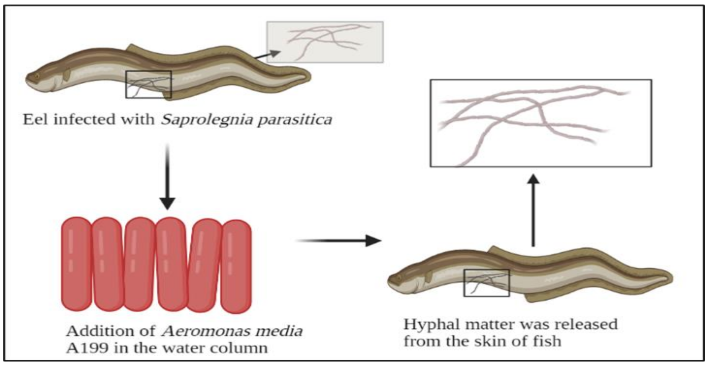

Figure 4 Antifungal activity of probiotic. *Saprolegnia parasitica* causes serious fungal infection in eel, and silver perch and after addition of *Aeromonas media* A199 in water column released the hyphal matter from the skin of the fish into the water (Lategan *et al.*, 2004).

Antiviral activity

Antiviral activity of probiotics got attention in past decade **(Lakshmi *et al.*, 2013)** as it is one of the most common diseases in aquatic environment causing serious damage to aquaculture organisms worldwide. Common viral diseases that affect aquaculture organisms include Hirame Rhabdovirus (HIRRV), Yellow Ascites Virus (YAV), Striped Jack Nervous Necrosis Virus (SJNNV), Ranavirus (EHN), Orthomyxovirus (ISA), White Spot Syndrome Virus (WSSV), Infection Pancreatic Necrosis Virus (IPNV), Novirhabdovirus (VHSV), Rhabdovirus (IHNV), Nodavirus (VVN), and many others.
For example, *Pseudomonas*, *Aeromonas* spp. and *Coryneforms* were reported to have antiviral activity against Hematopoietic Necrosis Virus (IHNV) **(Kamei *et al.*, 1988).** *Bacillus megaterium* strain was also reported to increase resistance of the shrimp against WSSV **(Li *et al.*, 2009).** *Bacillus* and *Vibrio* sp. have positive

effect on the shrimp against WSSV**.** When Lactobacillus bacteria were applied to olive flounder through food, it improved the resistance of the host against Lymphatic Disease Virus (LCDV) **(Harikrishnan *et al.*, 2010).** *Vibrio harveyi* strain 820514 was reported to have antiviral activity against WSSV when feed to black tiger shrimp **(Lakshmi *et al.*, 2013)** (Table 2).

Table 2 Reported probiotic bacteria for antiviral activity

Affecting virus	*Probiotic bacteria*	*Applied host*	*References*
Infectious Hematopoietic Necrosis Virus (IHNV)	*Corynrforms, Pseudomonas spp., Vibrio spp.* and *Aeromonas spp.*	Salmon	(Kamei *et al.*, 1988)
White Spot Syndrome (WSSV)	*Bacillus megaterium* strain	Shrimp	(Li *et al.*, 2009)
White Spot Syndrome (WSSV)	*Vibrio* and *Bacillus sp.*	Shrimp	(Balcazar, 2007)
Lymphatic Disease Virus (LCDV)	*Lactobacillus*	Olive flounder fish	(Harikrishnan *et al.*, 2010)
White Spot Syndrome (WSSV)	*Vibrio harveyi* strain 820514	black tiger shrimp	(Lakshmi *et al.*, 2013)

Antibacterial activity

Many probiotic bacteria have been reported to have antibacterial activity against known pathogens. For example, *Lactobacillus lactis* RQ516 was reported to have antibacterial activity against *Aeromonas hydrophila* in *Tilapia* **(Zhou *et al.*, 2010).** *Lactobacillus lactis* possessed antibacterial activity against *Yersinia rukeri, and Aeromonas salmonicida* in Rainbow trout **(Balcázar *et al.*, 2007).** *Leuconostoc mesenteroides* was able to prevent the M*ycobacterium marinum* T217, *Pseudomonas aeruginosa* T3, *P. putida* T4, *Vibrio harveyi* T34 growth in Nile tilapia **(Zapata *et al.*, 2013).** It was reported that *Bacillus subtilis* significantly decrease the motile Coliforms, *Aeromonads, Pseudomonads* in Ornamental fishes **(Ghosh et al., 2008).** *L. acidophilus, L. buchneri, L. fermentum, Lactococcus lactis,* and *Streptococcus salivarius* were found to prevent the growth of *Listeria innocua.* These probiotics were isolated from Spanish mackerel **(Moosavi *et al.*, 2014).** *Lactobacilli* spp. were isolated from the intestine of Rohu fish, Hari fish, Catfish, Gendi fish and Jillabe fish and these probiotics showed antibacterial activity against *Aeromonas* and *Vibrio* sp. While, *L. plantarum, L. lactis* subsp. *lactis* and *Staphylococcus arlettae* were isolated from native fish sauce of Malaysia reported to have significant inhibitory effect against *Staphylococcus aureus* and *Listeria monocytogenes* in fish **(Dhanasekaran *et al.*, 2008)** (Table 3).

The probiotics not only help the organism directly but indirectly as well. They can colonize in the aquatic organism and perform different mode of action such as ameliorate the water quality, disruption the quorum sensing, improve stress conditions, contribute to nutrient and enzyme production, increase the length of intestinal villi of fish, and boost immunity.

Table 3 Reported probiotic bacteria for antibacterial activity

Pathogenic bacteria	*Probiotics bacteria*	*Applied host*	*References*
Aeromonas hydrophila	*Lactobacillus lactis* RQ516	Tilapia	(Zhou *et al.*, 2010)
Aeromonas salmonicida, Yersinia rukeri	*Lactobacillus lactis*	Rainbow trout	(Balcázar *et al.*, 2007)
Mycobacterium marinum T217, Pseudomonas aeruginosa T3, P. putida T4, Vibrio harveyi T34	*Leuconostoc mesenteroides*	Nile tilapia	(Zapata *et al.*, 2013)
Coliforms, Aeromonads, Pseudomonads	*Bacillus subtilis*	Ornamental fishes	(Ghosh et al., 2008; Newaj-Fyzul & Austin., 2015)
Listeria innocua	*L. acidophilus, L. buchneri, L. fermentum, Lactococcus lactis,* and *Streptococcus salivarius*	Spanish mackerel	(Moosavi *et al.*, 2014)
Aeromonas and Vibrio sp.	*Lactobacilli*	Rohu fish, Hari fish, Catfish, Gendi fish and Jillabe fish	(Dhanasekaran *et al.*, 2008)
Staphylococcus aureus and Listeria monocytogenes	*L. plantarum, L. lactis* subsp. *lactis* and *Staphylococcus arlettae*	Fish	(Dhanasekaran *et al.*, 2008)

Ameliorate the water quality

As organic pollution accumulates the concentrated nitrogen, phosphorous and other organic compounds, it causes eutrophication, oxygen depletion and algal blooms which gradually leads to mortality of organism **(David *et al.*, 2009).** Application of probiotics improve the quality of water system thus benefiting the aquatic organism health.

For example, Gram positive *Bacillus* spp. is reported in improving the water system quality by converted organic matter into CO_2. Nitrifying bacterial culture were introduced in the aquaculture and it reduced the nitrite and ammonia toxicity and converted organic matter into bacterial slime or mass **(Balcázar *et al.*, 2006; Mohapatra *et al.*, 2013).** *Flavobacterium* sp. showed algicidal affect in aquaculture on some microalgae species **(Fukami *et al.*, 1997).** Addition of *Bacillus pumilus* in the water system enhanced shrimp's post larval growth and survival. It also Improved the pH, temperature, NH_3, H_2S and dissolved oxygen in rearing water (**Aguirre-Guzman *et al* 2012; Banerjee *et al.*, 2010)** (Table 4).

Table 4 Properties of probiotics in terms of improving water quality

Probiotic bacteria	*Property of probiotic*	*References*
Bacillus spp.	Converted organic matter into CO_2	(Balcázar et al., 2006; Mohapatra *et al.*, 2013)
Nitrifying bacteria	Converted organic matter into bacterial slime or mass	(Balcázar *et al.*, 2006; Mohapatra *et al.*, 2013)
Flavobacterium sp.	Algicidal affect	(Fukami *et al.*, 1997)
Nitrifying bacteria	Eliminated NH_3 and NO^-_2 toxicity	(Mohapatra *et al.*, 2013)
Bacillus pumilus	Enhanced shrimp's post larval growth and survival	(Banerjee *et al.*, 2010)
Bacillus pumilus	Improved the pH, temperature, NH_3, H_2S and dissolved oxygen in rearing water	(Aguirre-Guzman *et al* 2012; Banerjee *et al.*, 2010)

Disrupts Quorum Sensing (QS)

Quorum sensing is a process in which bacteria communicate cell to cell by chemicals or signaling molecules (autoinducers) that alters the response in population density and specie composition **(Mukherjee *et al.*, 2019).** QS disruption is potentially considerable strategy to prevent infection in aquaculture. *Bacillus cereus*, *Lactobacillus* and *Bifidobacterium* strains are reported to degrade the signaling molecule of pathogenic bacteria by producing different substances such as enzymes or antagonistic autoinducers**.** It was also reported that *Lactobacillus acidophilus* can inhibit the bacterial transcription and QS of *E. coli* O157 when these two specie grew together in same media **(Medellin-Peña *et al.*, 2007).**

Improve stressful conditions

Stress can be defined as any chemical or physical agent that cause reaction that leads to any disease or death of the organism. There are different stresses that affect the physiological and behavioral aspect of aquatic organism in aquaculture. The stresses can be abiotic such as nutritional, high density **(Lupatsh *et al.*, 2010),** thermal **(Logan & Somero, 2011),** chemical, toxins, anoxia, and hypoxia. Water, air, soil or even the aquatic organism's own body can be harmful for itself where toxins are accumulated in the host body and cause harm **(Smith *et al*., 2012).**

Table 5 Mode of action of probiotic bacteria in terms of improving stress conditions

Probiotic bacteria	Effect of probiotics	Aquatic host	References
Bacillus subtilis, Lactbacillus acidophilus, clostridium butyricum	Improved heat tolerance	Flounder	(Taoka *et al.*, 2006)
L. delbrueckii spp. delbruekii	Improved temperature stress tolerance (Decreasing the cortisol level)	European sea bass	(Carnevali *et al.*, 2006)
Bacillus spp.	Reduced handling stress (influence cortisol level)	Flounder	(Taoka *et al.*, 2006)
Pdp11	Improved high stocking density stress	Gilt-head bream	(Varela *et al.*, 2010)
Pediococcus acidilactcion MA 18/5	Improved antioxidant effect by modulation of antioxidant enzymes	Shrimp	(Castex *et al.*, 2009)
L. plantarum	Enhance the antioxidant state	Shrimp	(Chiu *et al.*, 2007)

As aquaculture requires intensive density of cultivation, there is a high possibility of disease outbreak that makes the aquatic organism more susceptible than the aquatic animal in wild. Probiotic bacteria can be provided to prevent these biotic stress conditions and boost their immunity to reduce the harmful effect of different stresses. Moreover, affect of probiotics can be assessed through heat shock tolerance of aquaculture organism **(Taoka *et al*., 2006).**
For example, *Bacillus subtilis, Lactbacillus acidophilus, Clostridium butyricum* improved heat tolerance in heat shock stress test for flounder. It was also reported that supplementing *Bacillus spp.* to flounder reduce handling stress by manipulating the cortisol level in host **(Taoka *et al*., 2006).** *L. delbrueckii spp. delbruekii* decreased the cortisol level in European sea bass when compared with the control group in temperature stress test **(Carnevali *et al*., 2006).** Probiotic Pdp11 was administered to gilt-head beam and it improved high stocking density stress **(Varela *et al*., 2010).** *Pediococcus acidilactcion* MA 18/5 was evaluated for its antioxidant effect on shrimps and it showed the activity of catalase and superoxide dismutase **(Castex *et al*., 2009).** *L. plantarum* was reported to enhance the antioxidant activity in shrimp **(Chiu *et al*., 2007)** (Table 5).

Improves digestion

Probiotics have a positive effect on the digestive process of aquatic organisms of aquaculture **(Balcázar *et al*., 2006).** These probiotics are known to produce different extracellular enzyme such as lipase, protease, and growth promoters **(Xianghong *et al*., 2000).** *Bacteroides* and *Clostridium* sp., are reported to have potential of providing fatty acid, essential amino acid, and vitamins to the aquatic host **(Balcázar et al., 2006; Tinh et al., 2008).** When probiotic bacterial strain CA2, a bacterial strain which is defined as protein capsule in seawater of Whiskey Creek Hatchery, Oregon **(Douillet & Langdon, 1994).** It was named as Stappia sp. Strain FG-4 (Wang *et al*., 2022), was supplied with axenic algae and fed to gnotobiotic oyster larvae, it showed efficient utilization of nutrient as well as improved growth performance **(Douillet & Langdon, 1994).**

Increases length of intestinal villi in fishes

Although there is limited research on the effect of probiotics on the morphology of intestine. However, previous research showed potential of probiotic bacteria to increase the villi surface area of fish **(Daniels *et al*., 2010).** Probiotics can improve the intestinal wall thickness, villus density and villus height in fishes **(Asaduzzaman *et al*., 2018)** (Figure 5). For example: *Pediococcus acidilactici* was reported to heighten the enterocyte microvilli in the proximal intestine of rainbow trout. Also, *Bacillus spp.* was reported to increase microvilli density and length of larvae and post larvae in European lobster after the probiotic supplement. Though, increase in absorptive characteristics were not observed (**Daniels *et al*., 2010).** *Lactobacillus rhamnosus GG got its name from the humans named Sherwood Gorbach and Barry Goldwin who gave their fecal samples and this bacteria was isolated from it **(Doron et al., 2005).***

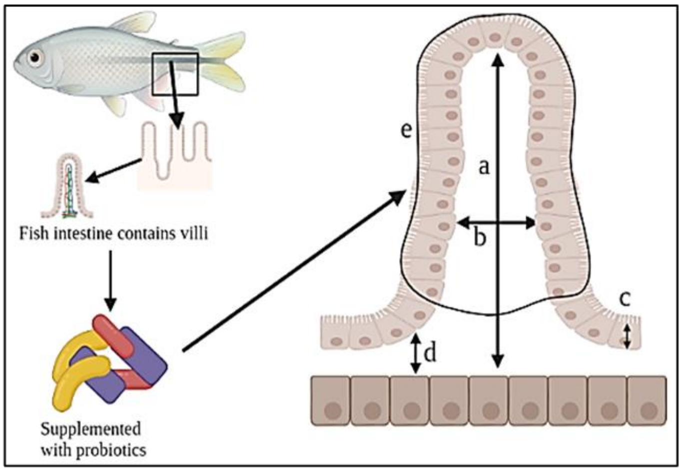

Figure 5 Probiotics increases intestinal villi size in terms of a) length b) width c) crypt depth d) intestinal wall thickness and e) area (Asad-uzzaman et al., 2018)

Boost immunity

Immune system is essential part of any organism. As aquaculture host lives in the environment where it has the susceptibility to have outbreak, probiotics can improve their immune system to boost the aquatic organism performance against the pathogen. Probiotics stimulate immune system of host by stimulating the cytokines activity of immune cells, rising the antibodies level, lysozymes, acid phosphatase, and complement **(Balcazar *et al*., 2007),** boosting the phagocytic activity of tumor necrosis factor alpha, cytokines (interleukins) **(Nayak, 2010),** antimicrobial peptides **(Mohapatra *et al*., 2012)**. These components improve immune system, resistance to pathogen and growth performance **(Lakshmi *et al*., 2013).**
Some probiotics can stimulate cellular immunity rather than activating the humoral immunity. Feeding probiotics with diet can improve survival rate. Some probiotics can improve innate immunity in various shrimp species. Previous research reported that probiotics can increase the production of agglutinins, phenol oxidase, anticoagulants, and processes like phagocytosis, encapsulation, and formation of nodules **(Lakshmi *et al*., 2013),** also, antiapoptotic proteins, free radicals, antimicrobial peptides, bacteriocins, siderophores, lysozymes, protease, hydrogen peroxide, polmyxin, and organic acids **(Balcazar *et al*., 2007).** Probiotics are reported to increase T- cells and agglutinins in mucosal intestine of larval sea bass **(Picchietti *et al*., 2009).** When rainbow trout was fed with probiotics, it enhanced the phagocytosis of mucosal leucocyte in rainbow trout group **(Balcazar *et al*., 2006).**

SCREENING STEPS TO IDENTIFY MARINE BACTERIA AS PROBIOTICS IN AQUACULTURE

There are three steps to identify marine bacteria as potential probiotics (Figure 6). First is pre-experimental screening in which the marine bacteria is isolated, cultured and tested for its activities in favor of host health or against the pathogen.

Second is experimental screening in which the microbes are delivered to the aquaculture host and the results are observed. Finally, in the post-experimental screening, the marine bacteria are identified to the strain level and its fate is assessed—whether it has the potential to be used as probiotics and have the ability to be produced in mass culture **(Rashad *et al.*, 2015).**

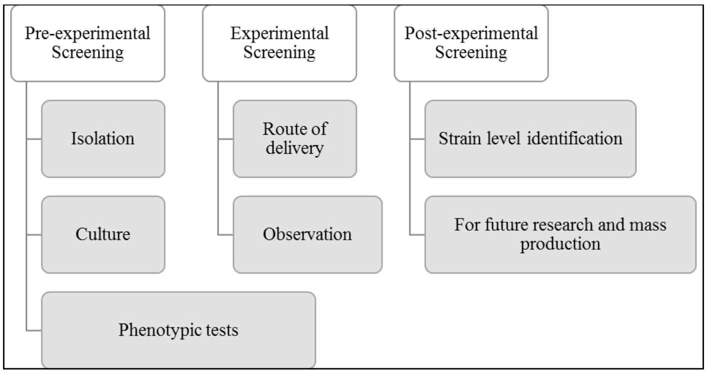

Figure 6 Screening steps to identify marine bacteria as probiotics. Pre-experimental screening consists of isolation, culturing, and phenotypic test of the potential marine bacteria. Experimental screening consists of delivering the probiotics in the aquaculture host and observe the results. In post-experimental screening, the marine probiotic is identified to the strain level and its fate is assessed- whether it has the potential to be used as probiotics and have the ability to be produced in mass culture.

PRE-EXPERIMENTAL SCREENING

Marine environment provides vast area to scavenge for the desirable probiotic bacteria. The pre-experimental screening process begins with the isolation. Marine bacteria can be isolated from three sources, sea water **(Ren *et al.*, 2020),** sediments **(Zhou *et al.*, 2018)** and marine organisms e.g., fish **(Alonso *et al.*, 2019)** and shrimps **(Wang *et al.*, 2018).** After the isolation, it is culture and tested for phenotypic traits (Figure 7).

Treatment to isolate bacteria from sediments

Isolation of sample from sediment sample can be treated differently before culturing. All these samples are serially diluted and then cultured. These treatments are

1. Simple serial dilution using sterile synthetic seawater and cultured using pour plating technique
2. Samples are heated by incubating at standard temperature to isolate the desired microbe, mixed in the sterile synthetic seawater and serially diluted **(Rashad *et al.*, 2015).**
3. Samples can be air dried using Lamina Air Flow (LAF) at room temperature overnight, mixed with sterile synthetic seawater and serially diluted **(Bredholt *et al.*, 2008).**
4. Centrifugation of samples in synthetic seawater at standard rpm and time and at room temperature then serially diluted **(Rashad et al., 2015)**.
5. Sediment sample can be shaken by rotary shaker in synthetic sea water at room temperature and standard rpm. As material in the sample is settled down and the suspension are serially diluted **(Poosalra & Krishna, 2013).**
6. Dual stage method in which air-dried samples (in synthetic seawater) are shaken in rotary shaker and the suspension are serially diluted **(Rashad *et al.*, 2015).**
7.

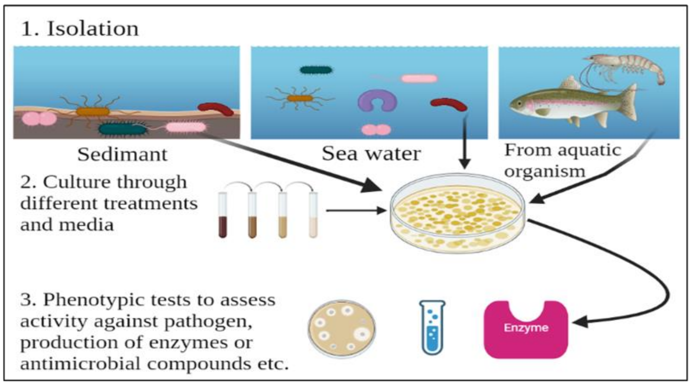

Figure 73 Pre-experimental stage of screening the marine bacteria as potential probiotics. The pre-experimental screening process begins with the isolation from different sources and treated through different methods. It is culture and tested for phenotypic traits. After this step, the probiotic screening enters experimental screening.

Treatment to isolate bacteria from seawater

The seawater sample will be transferred to selective media broth or in synthetic seawater and serially diluted then cultured in selective media **(Rashad *et al.*, 2015).**

Treatment to isolate sample from organism

The microbe is usually isolated from its host. The organism's mid gut is usually collected. The intestines are homogenized and enriched with media broth or serially diluted in sterile synthetic sea water and then cultured in selective media **(Rashad *et al.*, 2015**). The culturing media depends on the isolated micro-organism as the media is selective and suitable for the desired microbe. E.g., International *Streptomyces* Project (ISP) is specifically used for *Streptomyces* strains. Fish peptones are also reported to culture *Phaeobacter* sp. and *Pseudomonas fluorescens* **(Vazquez *et al.*, 2020).** De Man, Rogosa and Sharpe agar (MRS) media can be used to culture *Lactobacillus* species **(Alonso *et al.*, 2019).** After culturing, the bacteria are investigated further for phenotypic test. These are invitro test in which the microbe is tested to produce extracellular enzyme **(Xianghong *et al.*, 2000),** antimicrobial compounds **(Tinh *et al.*, 2008)** or determine its activity against host pathogen **(Lategan *et al.*, 2004).** After these tests, the marine bacteria are entered in the experimentally screened.

EXPERIMENTAL SCREENING

In experimental screening, the microbe are delivered to the aquaculture host. There are five methods to deliver the probiotics to host: Deliver through injection, bathing, live food, addition to artificial diet, addition to culture water. After the delivery of the probiotics, the results are observed, and the experimental stage enter the post-experimental screening (Figure 8).

Injection

Probiotics can be injected in the aquaculture organism. The probiont is isolated and mixed in saline solution and added to the aquaculture host. It is not recommended because this method is not applicable for the larval stage of the aquaculture organism and for large number of hosts as it is time consuming and need expert for handling fish **(Jahangiri & Esteban, 2018; Camara-Ruiz *et al.*, 2020).**

Bathing

The probiont is cultured in broth media and then diluted with sterile water. The host is bath in this solution for desired time and returned into its holding tanks or ponds **(Gram *et al.*, 1999).** The host can be introduced into the tank already containing the probiotic. After the exposure, the organism is transferred to its original pond or tank **(Klakegg *et al.*, 2020b).**

SCREENING PROBIOTIC ACTIVITY

Artificial diet

Artificial diet is prepared, and probiotic is added in it. The diet contains the standard nutrient requirement for the host. The probiotic can be in the form of powder or capsule that will be mixed in the diet and supply to the host **(Pramanick *et al.*, 2019).** It is not applicable for the larval stage of fish as the digestive system is not developed to digest the feed **(Hamre *et al.*, 2013).**

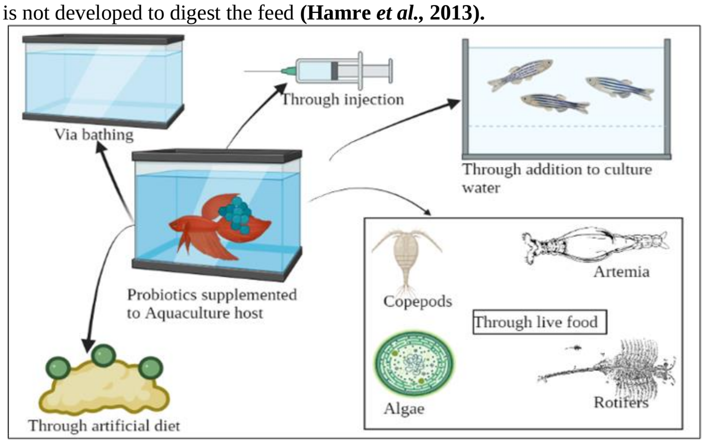

Figure 8 Experimental stage of screening the marine bacteria as potential probiotics. The experimental screening process has two stages. It will be delivered through five source and observed for the results and then the screening step enters the post-experimental step.

Addition to culture water

Probiotics can enhance the quality of water of aquaculture by bioremediation and biocontrol of pathogen **(Baerjee & Ray, 2017).** The probiotics is directly added to the culturing water. It is highly recommended administration method with live feed as it is available for all stages of fishes. This method is not applicable for fishes which are in open sea cages **(Camara-Ruiz *et al.*, 2020).**

Live food

Live food supplemented with probiotics is strongly recommended administration method. There are four organism groups that are used as live food for aquaculture: artemia **(Hamsah *et al.*, 2019),** rotifers **(Najmi *et al.*, 2018),** copepods **(Rasmussen *et al.*, 2018)** and microalgae **(Cheng *et al.*, 2020).** These food sources are small in size and fit to deliver probiotics to the host **(Dhont *et al.*, 2013).** Probiotic not only enhance the nutritional value of the aquaculture host but also the live food organisms **(Contreras-Tapia *et al.*, 2020).** They provide essential compounds such as vitamins or organic nutrients that are lacking in the diet. Thereby, increasing the population of these live feed and inhibit the pathogen growth. However, every group which is used as live food has its own advantages and disadvantages **(Dhont *et al.*, 2013).** It is necessary to evaluate them according to their characteristics and then utilize them.

OBSERVATIONS

The results of experimental steps are analyzed in the observation step, the dosage of the probiotics will be decided to ensure its attachment to the external surface or gut region of the organism. Moreover, the other mode of action of the probiotics are also observed via in vivo tests. For example, activity against the pathogen in which the activity or growth of the pathogen is complete prevented or simply postponed and immune responses of the organism are also evaluated (**Das *et al.*,2008).**

POST-EXPERIMENTAL SCREENING

The screening process complete with the strain level identification and suggestion for further research or parameters for mass scale production **(Das *et al.*,2008)** (Figure 9)

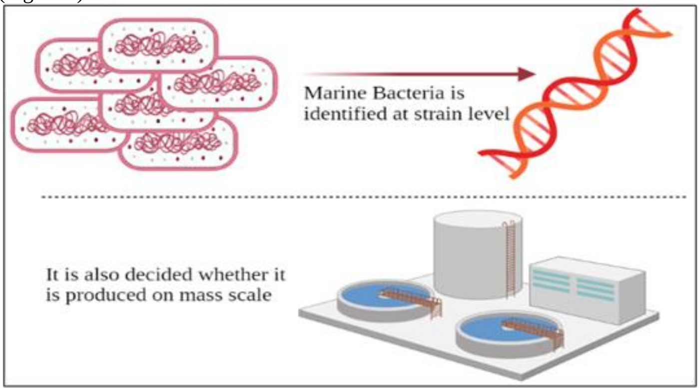

Figure 94 Post-experimental stage of screening the marine bacteria as potential probiotics. The probiotic is identified to strain level and assessed for parameters for mass scale production

CONCLUSION AND RECOMMENDATIONS

Aquaculture is highly nutritious food source for 7.6 billion population. Its limitations can be overcome through different methods including usage of probiotics. Terrestrial probiotics has been studied for a long time and the marine origin probiotics need attention as 91 percent of ocean is still unknown. Ocean has diversity of microorganism that is providing different products for human welfare, still the unknown part of the ocean is understudied to pour out the facilities for mankind. Marine bacteria are also contributing to the industrial revolution of different industries such as food, pharmaceuticals, nutraceuticals etc. As marine bacteria are potential probiotics, research is essential to identify more bacteria to exploit them for our advantage and use it in the aquaculture to produce healthy and safe food. Pakistan is one of the developing countries and we are depended on the export of food and other goods. Aquaculture has the potential to boost the economic revenues in our country. It is necessary to find solution to progress in food and health sector to provide healthy lifestyle where aquaculture has promising solutions of quality food for us. It is crucial to aware our farmers about the economic importance of the aquaculture and educate them about the farming of marine organisms. Government should collaborate with scientists related with the field of aquaculture in order to provide sustainable and stable economic plan for aquaculture. The trainings regarding fish farming practices should be offered to motivate the farmers for aquaculture.

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