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Review

Microbial biofilms as oral vaccine and for ameliorating aquatic environment: a future potential for aquafarming

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Abstract
Microbial biofilms have traditionally been viewed as scourges associated with colonization on medical devices and implants leading to drug resistance and persistent infections. In recent few years, antibiotics have been rampantly used to curb the disease outbreaks in animals, leading not only to escalates antibiotic resistant bacteria and genes but also negative impacts on fish and the environment health. Therefore, vaccines are getting prime importance in aquaculture as an alternative to the antibiotics. Biofilm oral vaccine is another exciting avenue of biofilm utilization for improving fish health. Oral deliveries of vaccine preparations have traditionally failed to induce protective immunity in fish, whereas biofilms of pathogenic bacteria grown on a defined substrate were effective in inducing higher antibody titers and protective immunity. Our studies on oral biofilm vaccines in catfish and major carps models have proved an enhanced protective immunity against the pathogenic bacteria. By virtue of the distinct antigenic makeup and adjuvancy of biofilms assemblage, novelty of biofilm oral vaccine has largely been its safe and effective performances in fish species. Here, we review on the state-of-art knowledge of microbial biofilms as fish vaccine and as amelioration of aquatic environment perspective towards enhanced fish production.

Keywords Biofilm · Fish vaccine · Aquatic environment · Fish production

1 Introduction

Fish, the cheapest source of animal protein could be "nutrition-sensitive" addressing health inequities and global harmonized trade environment, toward social equity and sustainable environmental [1]. Small-scale commercial fish farming plays a significant role in rural development and poverty reduction [2]. Access to equality and affordable fish food is considered a critical factor for commercial success of fish production [3]. In developing countries, where poverty is really extreme, the conventional type of farming with artificial feeds become practically uneconomic and hence, autonomous operation of production without or with limited use of external means to improve natural pond productivity plays a significant role. In this direction, concept on use of artificial substrates as peri-phyton/biofilms

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for increasing fish production form potential as a low cost technology in the developing countries. Substrates are known to promote colonies of microbes, referred to as microbial biofilms that provides food for fish and shellfish [4]. Microbial biofilms have been extensively evaluated for immune prophylaxis (vaccines in fin fish and immunostimulants in shellfish), fish growth enhancement and improved water quality [5–8]. Microbial biofilms has better gastrointestinal stability and protective responses against the bacterial fish pathogens in comparison with free cells conventional vaccines[6, 7]. Fish perform best for oral vaccines targeting gut mucosal immunity as compared with injection vaccines [9]. Bacterial biofilms could be used as novel management strategies towards vaccine development against fish pathogens [10].

The biofilms colonies growing in aquatic environment may include combinations of algae, protozoans and fungi embedded with extracellular polymeric substance matrix secreted by bacteria [11]. Studies have also recorded that biofilms density on substrates can be as high as 100–1000 fold greater per unit weight/ volume as compared to that of free-floating natural food availability for the fish [12, 13]. Recent report confirms that simple changes in fish farming technology and management practices could help in the global transition of intensive forms of aquaculture with more sustainable [14]. In this context, microbial biofilms based fish research could be one of the strategies for fish production enhancement keeping the view on improved fish health and better aquatic environment for fish particularly in the South Asia and African countries. With this backdrop, the prime objective is to document the recent developments as well as the findings reported from our earlier studies those claim as the first scientific studies on microbial biofilm as fish vaccine and for ameliorating environment health in aquaculture [15–18]. This technology could be widely beneficial for the aquaculture system practised in South Asia and South East Asian countries, spreading more trials on different substrates and aquatic bacteria as potential biofilm vaccines in both fin fish and shellfish farming.

1.1 Biological basis of microbial biofilms on substrates

1.1.1 Development of microbial biofilms as fish food

Biofilm is a consortium of microorganisms that exhibit syn-trophy between the immobilized cells that assemble, adhere and multiply on solid surfaces while enmeshed in extracellular polymers that provide shelter for the assemblage of microorganisms [15, 16]. Biofilms are essentially organized communities that grow spatially differentiated on biotic and abiotic surfaces (Fig. 1. adopted from [19] AMS, L. No. 4775351297524). Figure 2. depicts the stages of microbial biofilm development that begins with adherence of planktonic forms progressing through colonization of solid surface, maturation and finally dispersion of free cells. The microbial constituents are known to interact among themselves by releasing, sensing and responding to diffusible signal molecules referred to as quorum sensing that determines the social life within the biofilm community [17, 18]. The biofilm community, per se, represents a physiologically unique ecosystem compared to the constituents' planktonic counterparts and as such due to assemblage nature of growth; biofilms present a highly dense biomass of cells.

1.1.2 Cell composition of Substrate based microbial biofilms

Wide ranges of microorganisms are found within biofilms [16]. However, bacteria act as colonizing organisms at solid–liquid interface and normally constitute the predominant organisms in most biofilms. Heterotrophic bacteria are the most important constituents of biofilm suggesting that organisms are able to derive sufficient nutrient from the water [19]. The ability of both Gram-negative and Gram-positive bacteria to colonize on solid surfaces, their rapid growth rate, adaptability to changing environment and capability to produce extracellular polymeric substances (EPS), are important in the adsorption and aggregation of cells [18]. Aquatic fungi are also important in the colonization and deterioration of submerged wooden structures whereas algae are important component of biofilms exposed to sun light [20]. Likewise, predatory protozoans are generally found in mature biofilms that reinvigorate the community by feeding on ageing cells [21].

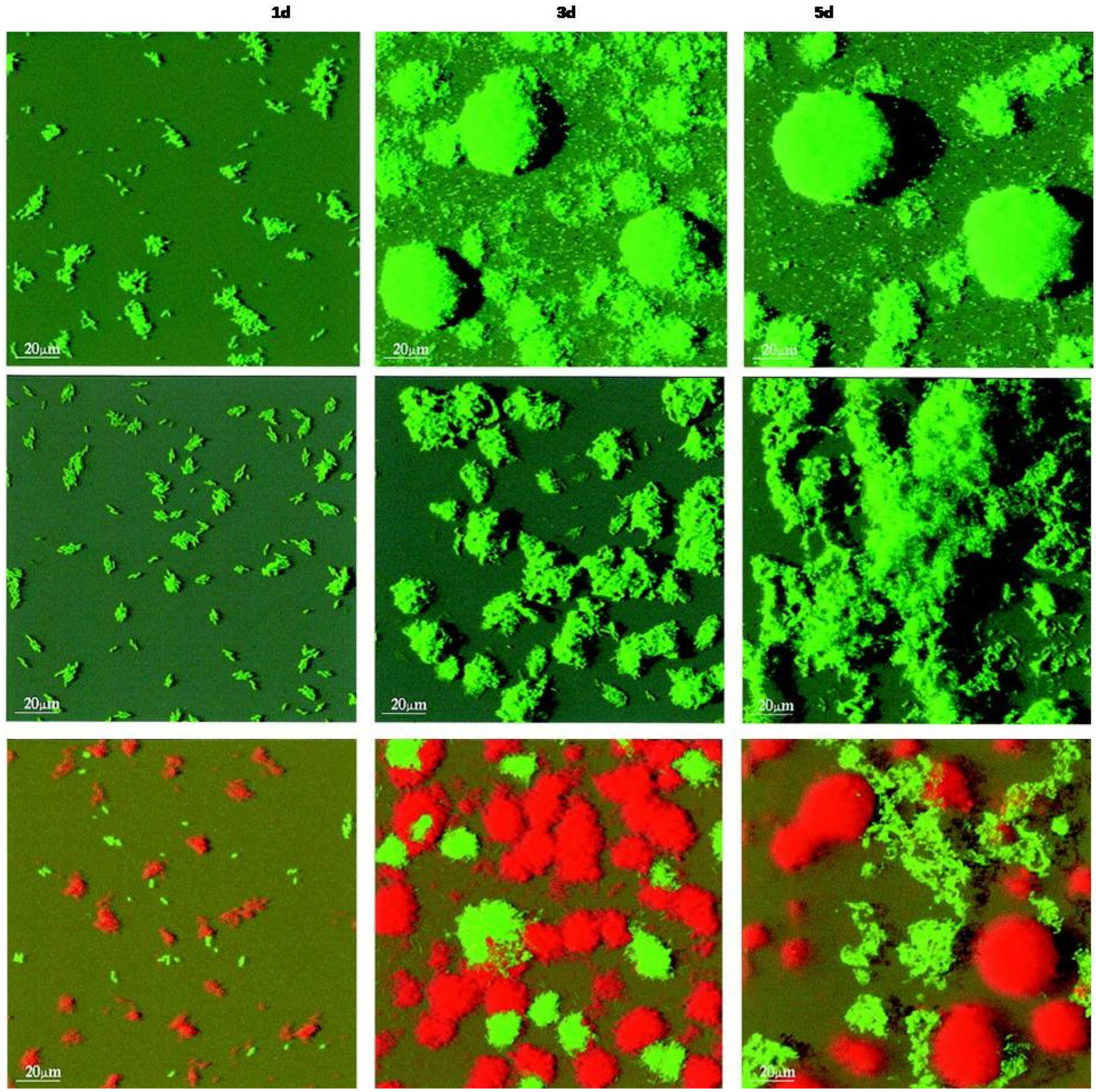

Fig. 1 Confocal scanning laser micrograph of 1, 3 & 5d old *Pseudomonas* biofilms growing in flow chambers showing spatial organisation. Top row green fluorescent protein (GFP) tagged *P.* sp.; Middle row GFP tagged *P. putida*; Bottom row, GFP tagged *P.* sp. and Red fluorescent protein (DsRed) tagged *P. putida* (Adopted from Toller-Nielsen et al. 2000; J. Bacteriol. 182:6482–6489. L No. 4775351297524)

1.1.3 Properties of substrate based microbial biofilms

The characteristic of a biofilm is directly influenced by the composition of microbial cells and the chemical properties of extracellular polymeric substances (EPS) matrix that primarily constitutes polysaccharides, proteins and nucleic acids [15]. The EPS accounts for 50 to 90% of the total organic carbon in biofilm and is the single most important criterion for the initiation, recruitment, growth and survival of the biofilm community [22]. Considerable research has focused on understanding the biofilm architecture in order to gain insights into their physiologic states, nutrient channels, efflux pumps etc. to fully appreciate the basis of drug resistance and dissemination that have been the greatest challenges in public health in combating infestation on medical devices and implants [23–25]. Most bacteria produce polymeric substances as a protective pericellular cover while growing in suspension or in biofilm mode. The polymer appears as a

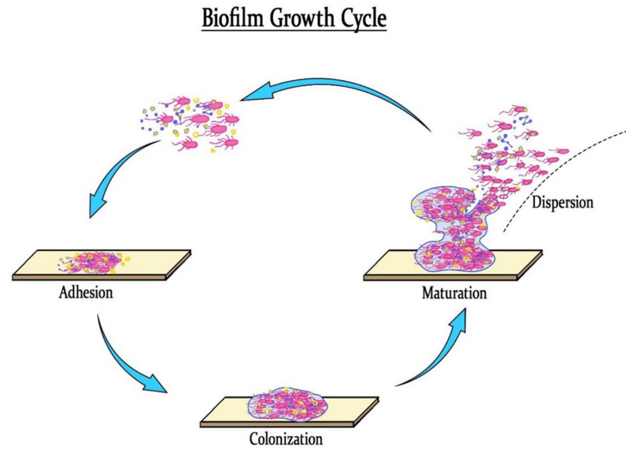

Fig. 2 Schematic diagram of microbial biofilm growth cycle that includes adhesion followed by colonization, maturation and finally dispersion

highly hydrated capsule attached to the cell or secreted as a viscous slime [22]. While most bacteria are able to produce EPS, their composition remains highly species dependent and environment specific [22]. Microbial extracellular and cell surface polymers are important in biofilm mode of growth for at least two reasons: (1) to facilitate interaction between a bacterial cell and substrate that is largely determined by physical properties of the macromolecules at the cell surface, and (2) be responsible for the integrity of the microbial biofilm.

1.2 Microbial biofilms as oral vaccines for improving fish health

Havocs from persisting bacterial infections in aquaculture systems are frequent that often trigger massive economic losses worldwide. High prevalence of bacterial diseases in aquaculture has resulted frequent use of antibiotics, leading to the spread of antimicrobial resistance (AMR) bacteria [26]. Recent study showed the evidence for the presence of *bla*CTX-M-55, QnrVC5 antimicrobial resistance genes, in *Vibrio vulnificus* isolates in Asian Seabass [27]. Multi-drug resistant isolates of *Edwardsiella tarda* in Nile tilapia and catfishes have been a threat to fish farming sector in Egypt [28]. Algammal et al. [29] reported that existence of MDR *Bacillus cereus* in *Mugil seheli* that reflects a threat to the public health and the aquaculture sector. Efficacy of antibiotics in the preventive and therapeutic management of aquatic health has always been debatable due to low efficacy as well as growing concern of residual antibiotics in fish marked for human consumption [30]. Therefore, vaccines have been the best alternative against use of antibiotics. Oral vaccination is probably the most ideal intervention and a fully integrated option to protect fish from infectious diseases in commercial aquaculture. However conventional oral vaccines have yielded poor and inconsistent results in fish [31, 32]. Therefore, there is an obvious need for an effective oral vaccine that might be (a) stable for antigen delivery into the immunologic sites in hind gut to build pathogen specific immunity, and (b) scalable to other disease situations and culture practices for adaptations. It is largely believed that orally delivered antigens suffer from gastric destruction in the foregut and stomach, and an effective oral vaccine would at least need protective encapsulation during its journey through the hostile acidic gut environment [33] Incidentally, it had been observed that bacterial cells in biofilm masses have high resistance to heat, biocides and antibiotics. Therefore, it seemed logical to screen bacterial biofilms as a candidate oral vaccine (Fig. 3).

Motile Aeromonad Septicemia (MAS) primary caused by *Aeromonas hydrophila*, a component of the aquatic microflora, is a significant bottleneck in warm water aquaculture [33]. In this background designing an effective MAS vaccine and employing biofilms for an oral delivery appeared a novel combination. Biofilm of *A. hydrophila* was developed on chitin and/or sugarcane bagasse substrates and were systematically tested in fishes with herbivore, carnivore and omnivore feeding habits representing full spectrum of gastro-intestinal environments [5, 13]. Higher levels of systemic antibody perticularly of IgM and protection from bacterial challenge were observed in *A. hydrophila* biofilm vaccinated carps compared to those vaccinated with *A. hydrophila* free cells [33, 34]. Interestingly, promoting biofilms in fishponds also had a positive impact on fish health. Promoting biofilm growth in pond environmental supports fish species for grazing on the substrate bound biofilm floccules and eventually the fish naturally get enhanced immunity. Carp raised in biofilm promoted pond registered a higher antibody titer and protection from *A. hydrophila* [29]. Mean antibody titer from biofilms promoted pond was significantly higher than that from the untreated control. Percentage survival of carps from treatment was 75% compared to 31.25% in the control, with a relative percent survival of 63.64%. Thus, the study indicated that biofilm, besides serving as fish food, can also serve as an effective delivery vehicle for oral vaccine. Series

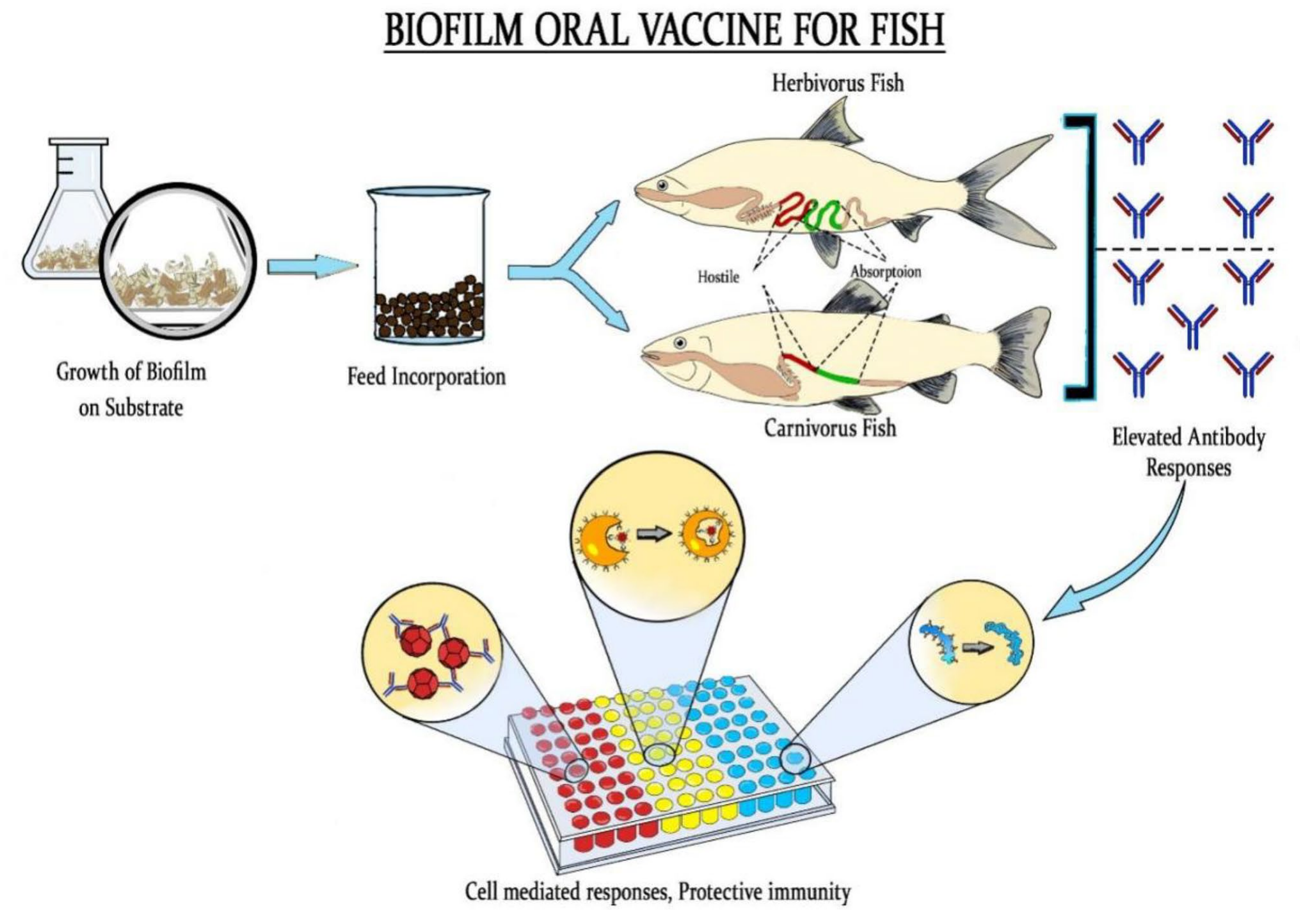

Fig. 3 Schematic diagram of Biofilm oral vaccine for improved fish health through enhanced non-specific (CMI) and specific immune systems (Antibody)

of subsequent studies attempted to optimize the vaccine dose while establishing a therapeutic window for optimal vaccine safety and efficacy performances in carp, catfish and shrimp [6, 35, 36].

Microbial biofilm of *A. hydrophila* was evaluated as oral vaccination in *Clarias batrachus,* an omnivore species possessing a highly acidic foregut and stomach with relatively short intestine [36]. Biofilm (BF) and free cell (FC) were incorporated as fish paste and fed for 20 days and antibody titer was monitored up to 60 d post vaccination. Antibody titer and relative percentage of survival (RPS) following challenge with *A. hydrophilla* were significant higher in BF fed catfish compared to FC indicating better performance of biofilm vaccine. On further analysis, the BF cells of *A. hydrophilla* were chemically distinct from their FC counterparts where BF cells expressed three new proteins, while repressing 15 preexisting proteins from FC. In addition, the S-layer proteins were lost in BF cells, while the lipopolysaccharides (LPS) showed an additional high molecular moiety [37]. It was imperative to notice a possible correlation between the altered chemistry and improved immunogenicity found in BF vaccine.

The effect of biofilm developed on artificial substrates in carp ponds and their resistance to *A. hydrophila* infection was evaluated in rohu, *Labeo rohita* [5]. Fingerlings of rohu were reared with sugarcane bagasse substrate for 98 days developed significantly higher antibody titers and protection from challenge against *A. hydrophila* compared with those without substrate. Similarly, *A. hydrophila* biofilm oral vaccine was evaluated in snakehead, *Channa striatus*, a carnivorous model [7]. The fish were fed with BF and FC of *A. hydrophilla* for 20 days and a monoclonal antibody-based ELISA measured appearance of anti- *A. hydrophilla* titer. BF vaccinated snakeheads mounted significantly high antibodies as well as greater relative percent of survival (88%) compared to that of FC [7].

Biofilm of pathogenic bacteria was initially hypothesized to be a good vaccine candidate for oral delivery in fish [28, 31]. Systemic antibody titers as well as protective immunity to homologous challenge were higher in orally immunized fishes than those immunized with free cells of same bacterium or non-immune controls [37]. In spite of low antibody titers, the complete protection to a virulent challenge established involvement of a vaccine-associated cell mediated immunity. In view of antigenicity of biofilm, where the biofilm phagosomal signalling is largely unknown, biofilm mass was seen to be taken up by macrophages from gut sites and entering pronephrous and spleen [34]. High antigenic mass because of the assembled micro colonies growing in biofilm mode does not seem to create a situation of either antigen excess or oral tolerance, may be meeting the higher priming threshold for oral antigens. Even though antigen presentation and T cell response are not well studied in fishes at epitope level, observations of immune induction ability of biofilm oral vaccine tenders a wide potentiality for further study and therapeutic usage, underlining as a promising tool for fish health researchers.

1.3 Microbial biofilms for improving water quality for aquafarming

Microbial biofilm growing on different substrates has a significant impact on water quality parameters particularly on ammonia, dissolve oxygen (DO) and nutrient levels both in closed and open waters [12]. For aquaculture, DO, alkalinity, pH, and total nitrogen play critical role for growth and survival of fish. Often, fish species in both closed and open waters experience mass mortality due to decreased DO levels and increased ammonia concentrations [38]. Though there are large numbers of literature on biofilm in natural ecosystems and its relevance to pollution, there has been limited information on role of microbial biofilms on water quality environment and fish health.

1.3.1 Role of microbial biofilms on *Ammonia*

Microbial biofilms in aquaculture system acts as an *in-situ* bio-filter by reducing toxic ammonia level in the system. The impact of bacterial biofilms on intensive culture of *Daphnia*, observed a significant difference in ammonia levels, which was attributed to presence of nitrifying bacteria in the biofilm assemblage [39]. Similar observations were also made with sugarcane bagasse as substrate [40]. The decrease in ammonia with plant based substrates was directly related to biofilm population density on the substrate. A 60-d study on ammonia dynamics in carp nursery ponds supplied with sugarcane bagasse showed significantly low ammonia concentrations than nursery ponds without substrate [35].

A positive effect of biofilm on water quality in the culture of *Farafantapenaeus paulensis* indicated lower level of nitrogen and phosphorus in a biofilm -treated group than the control group [41]. Another study found that ponds with substrate had 8–10 times higher counts of nitrifying bacteria compared to control ponds. It concluded that the lower ammonia levels in substrate-based ponds could be attributed to the conversion of ammonia to nitrite and nitrate by the nitrifying bacteria. [5].

1.3.2 Role of microbial biofilms on dissolved oxygen (DO)

Microbial biofilms improves the oxygen balance and nitrogen-related processes in water column through providing more autotrophic bacteria, larger surface and greater grazing space for fish [42]. Biofilm density on the substrate determines DO regime in pond [40]. Oxygen concentration decreased drastically to 1–2 ppm during the first week of fertilization with biodegradable substrates at 2000 kg/ha (dry wt) [43]. Therefore, substrate addition depending on the nature of the substrate has influence on the oxygen regime in ponds. Less biodegradable substrates such as bamboo will harbour less biofilm compared to more biodegradable substrates such as sugarcane bagasse and paddy straw [43]. DO gradually improved with time following algal colonization and hence stocking fish week post fertilization is advised. Hence, fish farmers can improve their yield from biofilms-based aquaculture by taking advantage of biodegradable substrates and choosing mixed fish species from different feeding niches in order to minimize farming cost due to artificial feeding and pond fertilization.

1.4 Cases of successful microbial biofilms based fish production

Microbial biofilms based aquaculture has been reported as the beneficial for many commercial fish species including carp, tilapia, shrimp and freshwater prawn cultures [44, 46, 47]. Different natural and artificial substrates have been observed as the house for microbial biofilms formation. These biofilms are subsequently utilized as fish food, maintaining optimal water quality for fish growth and enhancing fish immunity, leading to better survival and disease resistance. The fundamental concept of this technology is that substrates promote the formation of microbial biofilms, which provide food, maintain water quality, and improve fish immunity [36, 37].

1.4.1 Microbial biofilms based carp culture

Microbial biofilms based fish farming was found to be ideal for growth and enhanced production for catla (*Catla catla*)—rohu (*Labeo rohita*) with stocking at 40:60 [44]. Studies on different type of substrates that support the microbial biofilms growth with increased fish production particularly carps showed that bamboo provides better growth than PVC [45]. In addition to bamboo, paddy straw and sugarcane bagasse have been used for the microbial biofilms growth towards carp production. Hence, substrate type and composition have a strong effect on both microbial biofilms growth and fish productivity.

1.4.2 Microbial biofilms based *tilapia* culture

Tilapia (*Orechromis niloticus*), is an ideal fish species for substrate based aquaculture. Study showed that the utilization of microbial biofilms as partial replacement of commercial food in organic tilapia (hybrid of *Oreochromis aureus* × *O. niloticus*) has a reduced commercial food input by 30–40% without hampering fish growth [46]. The combined effects of periphytic surface area and stocking density of tilapia on growth performances and immunological parameters and recommended that 70 fish/m^3 and 2 ps units (surface area of 1.4 m^2) is ideal for best growth and health status, for microbial biofilms based cage culture [13].

1.4.3 Microbial biofilms based shrimp culture

The biofilms provides a suitable habitat for biotic communities that can serve as an excellent food for shrimp and also, provide a sheltered habitat for maturing individuals during moulting. The synergistic effects of biofilms and supplementary feeding resulted in better growth of the pink shrimp cultured in captive condition (tanks and net cages) that has been validated deploying stable isotopes ($\delta^{13}C$ & $\delta^{15}N$) and the analysis showed that biofilms could spare 49% and 70%, carbon and nitrogen, respectively for shrimp growth [47]. The effects of artificial substrates (polypropylene fabrics) in culture system of white-leg shrimp, *Litopenaeus vannamei,* showed that inclusion of artificial substrate could enhance growth and survival of the shrimp, and also provide positively affected the bottom distribution of shrimp that can be used as strategy for marinating high-stocking density for more production without compromising the health of the animals [48]. Substrates provide vertical and horizontal expansion of microbial biofilms in a culture system, thus, reduce stocking stress and also maintain the higher shrimp production [48, 49]. Importance of substrates in culture of the freshwater prawn has been realized in early 90's. Studies conducted in Israel indicated that adding substrate to ponds allowed for an increase in prawn production by 14% while average size increased by 13% [50, 51]. Tidwell et al. [52], evaluated added substrate under temperate conditions and reported that in prawns stocked at relatively low density (59,280/ha), production and average size were increased by 20 and 23% respectively.

The microbial biofilms based fish/shrimp culture has taken center stage due to its practical feasibility and benefits, however, research on interactive and synergetic effect both in combination and varying stocking densities, is need of the hour. New approaches, such as integrated multi trophic aquaculture (IMTA) has already been in place with live substrates such as seaweeds, integration with fish and molluscans [53]. The effect of red seaweed (*Gracilaria tenuistipitata*) culture with Nile tilapia (*Oreochromis niloticus*) applying diverse feeding regimes revealed that symbiotic culture of Nile tilapia and red seaweed fed with 80% feed allowance, led to reduce the feed cost of the total operation by 28.9% as compared to control group without compromising the growth-performances and marinating the water quality [53]. Different substrates including bambo poles [54], sugar cane bagasse [55], rice straw and kanchi [56], eichhornia [43], mosquito screen [57] at pond level and chitin flakes [7, 33, 34] at laboratory leve lhave been used for the microbial biofilms development towards fish production through improved fish and pond health as oral vaccine and ameliorating the water quality parameters.

1.5 The way forward

Microbial biofilms is emerging as a low-cost technology for fish production in developing countries through improved water qualities and fish health as oral vaccine as well. One of the key components for successful fish farming is judicious selection and deployment of biodegradable substrates that have proven success in raising fish and shellfish from all feeding niches. Therefore, more research attention should be focused towards the substrates based aquaculture. Besides food generation, microbial biofilms also plays a role in improving water quality and fish health particularly by acting as a biofilter to eliminate toxic metabolites as well as serving as an oral vaccine to induce pathogen specific immunity. At this juncture, there is a greater need for deeper understanding on the fish and shellfish immune system functions through advanced gut microbiome and detailed immunological profiling for development of effective vaccine. Furthermore, deeper understanding on the microbes and their association in the ameliorating water quality is needed for development of the microbial consortium for aqua-farming. Though the technology is not well propagated in the commercialized aquaculture, there is a need to develop different farming practices with diverse fish species.

2 Conclusions

The current review highlighted the mechanisms of microbial biofilms formation in different substrates-and their role in the water quality improvement and most importantly development of oral vaccine against the pathogenic fish bacteria. The microbial biofilm has shown to be much effective as oral vaccine by improving the cell mediated immunity and antibodies particularly IgM level in fish. While, the microbial biofilms ameliorate the water quality particularly on DO, ammonia, pH, therefore sustain the pond and fish health for better production. The review furthermore covers the cases of fish species including carps, tilapia, shrimp, and freshwater prawn cultured, in the biofilms/substrate based pond practices received higher production and underlined the need for better field trials and propagation of this low cost technology in the commercialized aquaculture practices particularly in South Asia and South East Asian countries.

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Data availability No datasets were generated or analysed during the current study.

Declarations

Ethics approval and Consent to participate This is a review based on our team research already published and the work carried out by other authors elsewhere. Hence, the ethical statement of animal use is not essential.Informed consent was obtained from all individual participants included in the study.

Consent for publication All authors are hereby provide their consent to publish the article.

Competing interests The authors declare no competing interests.

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