ELSEVIER

Aquaculture 200 (2001) 223–247

Aquaculture

www.elsevier.com/locate/aqua-online

Interactions between fish larvae and bacteria in marine aquaculture

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Abstract

Modern aquaculture provides effective means for intensive seafood production under "controllable" conditions. This rapidly growing industry, however, has experienced relatively severe disease problems owing to lack of control of the microbiota in rearing systems. Disease control is an inherent part of any intensive animal production system; however, in the aquatic environment, the intimate relationship between bacteria and their host and the frequent use of open production systems adds to this challenge. The use of antibiotics in aquatic ecosystems is presently kept to a minimum, and fortunately, vaccines and other health control means have so far kept most diseases under relative control. Various organisms, however, may not respond to vaccines, and new diseases or variants are a constant challenge to the industry. In aquaculture, eggs are kept in incubators with a microflora that differs considerably from that in the sea, and become heavily overgrown with bacteria within hours after fertilisation. Fish larvae ingest bacteria by drinking and are, thus, primed with antigens before active feeding commences. This may result in the formation of an indigenous larval microflora; however, at present, we know little about this process. The microflora of marine invertebrates may harbour bacteria that are pathogenic to other organisms and, thus, invertebrate co-inhabitants or food organisms in aquaculture may serve as vectors for transfection of fish pathogens. In intensive egg production and larviculture, the numbers of bacteria are kept low by various forms of water treatment and disinfection. These approaches, however, may disturb the balance between microbial communities, or favour proliferation of opportunistic bacteria or unpredictable development of bacterial communities. Thus, there is a need for better microbial control during intensive larval production. The use of probiotics has proven advantageous in domestic animal production, and microbial management may also have a potential in aquaculture. Better control of host–microbe interactions is a prerequisite for stable production of marine larvae in intensive systems.

Keywords: Fish larvae; Fish eggs; Bacteria; Microflora; Normal flora; Host interactions

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0044-8486/01/$ - see front matter
PII: S0044-8486(01)00702-5

1. Introduction

During the intensive hatching of eggs and rearing of marine larvae, various forms of interactions between bacteria and the biological surfaces may occur. This may result in the formation of an indigenous microflora or be the first step of infection. In the aquatic environment, bacteria travel easily between habitats and hosts, and a better understanding of host–microbe interactions and natural defenses is imperative for the successful mass production of larvae. The continued development of aquaculture relies on improved microbial control to prevent the proliferation and spreading of pathogens.

Fish eggs are kept at high densities in incubators with a microflora that differ in numbers and characteristics from that in the sea, and the eggs become heavily overgrown with bacteria within hours after fertilisation. The diverse flora that eventually develops on the egg surface reflects the bacterial composition of the water; however, species-specific adhesion to surface receptors may also affect the composition of the epiflora. Members of the adherent microflora may damage developing eggs; however, we do not yet know whether a natural epiflora may bestow some protection in the sense that a heterogenous epiflora may prevent microcolony formation or domination by

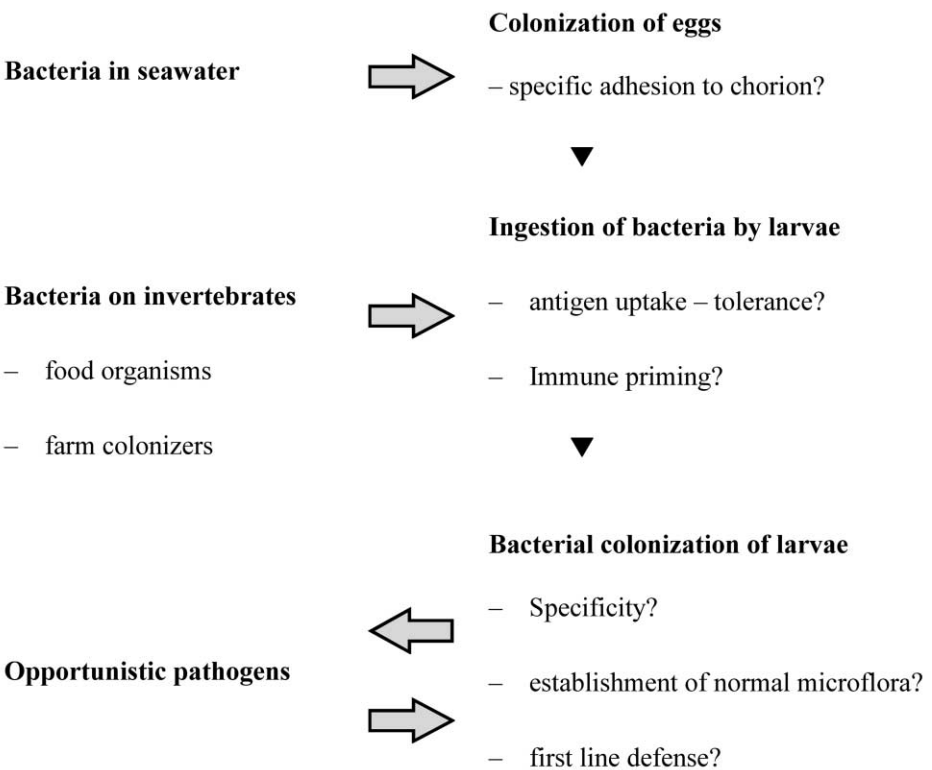

Fig. 1. Steps and interactions in bacterial colonization of fish eggs and larvae.

potentially harmful bacteria. Factors that may protect eggs from bacterial invasion or infection are still poorly understood.

Newly hatched larvae live in close contact with eggs and debris. Fish larvae ingest bacteria by drinking and are, thus, primed with antigens before active feeding commences. This may result in the formation of an indigenous larval microflora; however, at present, we know little about the establishment and role of the normal microflora of fish larvae. Sequestering and cellular uptake of intact bacterial antigens by newly hatched larvae may affect their immune development. Host colonization or adhesion factors of pathogens in the aquatic environment, however, are still poorly described. For a recent review of bacterial colonisation of fish larvae, see Hansen and Olafsen (1999).

The microflora of marine invertebrates and plankton may be dominated by opportunistic or potentially pathogenic vibrios at certain times of the year, and marine invertebrates may harbour bacteria that are pathogenic to other organisms. The defense factors of invertebrates are not fully understood; however, bacteria may persist in their tissues and body fluids and, thus, marine invertebrates or food organisms may serve as vectors for transfection of fish pathogens. A better perception of these factors is essential to understand epidemiology in aquatic environments. For a recent review of defense factors and disease prevention in invertebrates, see Roch (1999).

Successful aquaculture will rely on better insight into the complex interactions between the cultured organisms and the bacterial communities that develop in the rearing systems (Fig. 1). The use of probiotics or microflora control may also have a potential in aquaculture. For a recent review of probiotics in aquaculture, see Gatesoupe (1999).

2. Host–microbe interactions in marine microbial ecology

Intensive aquaculture implies that food is added at high concentration to water, which results in an excellent medium for growth of heterotrophic or opportunistic bacteria, and this will affect selection and growth of microorganisms in the nets or cages. Many fish diseases are caused by opportunistic pathogens. Thus, feed composition affects the commensal microflora and results in the proliferation of bacteria that normally have restricted opportunities to grow. Stress caused by handling or transport of fish reduces the resistance against infection. The use of antibiotics may dramatically change the intestinal microflora of the fish and, thus, impair its first-line defenses (Austin and Al-Zahrani, 1988; Strøm and Ringø, 1993). Thus, it is imperative to understand the factors that affect the proliferation of microorganisms in the environment and the microbial ecology of the farm. Even though such intimate relationships exist between fish and bacteria, little is known about early immunity or tolerance in fish larvae, or about the establishment of a “normal microflora” in larvae.

In principle, microorganisms in the aquatic ecosystem inhabit a continuous medium with easy access to host surfaces. All aquatic organisms are exposed to a varied microflora that harbours multiple pathogens. Many of these organisms are filter feeders that live in areas with a dense microflora. They accumulate bacteria from the environment and may carry bacteria on their surfaces and in their tissues and body fluids

(Olafsen et al., 1993). Complex and fine-tuned interrelations have evolved between aquatic organisms and their indigenous microflora—including pathogens. Yet, there are still gaps in our perception of how host bacteria may be pleomorphic and shift between free-living microforms and more metabolically active and bulky adherent forms (Morita, 1985; Kjelleberg et al., 1987). Plankton blooms may cause dramatic changes in biomass, and microorganisms may respond by chemotaxis towards the released nutrients. Many aquatic microorganisms adhere well to favourable surfaces to ensure a stable supply of nutriments. Settlement and colonisation by invertebrate larvae may be induced on surfaces covered with bacteria (Fitt et al., 1989). In a fish farm, this will lead to the proliferation of invaders or fouling organisms with their own microflora, thus accounting for a varied microbial community. We still have relatively scarce knowledge about the free-living and adherent microflora of the aquatic environment. One reason for this is that many typical marine microorganisms do not grow well on laboratory media (Morita, 1985; McKay, 1992). Culturable bacteria may constitute as little as only 0.01–10% of the viable bacterial population from the marine environment and, thus, a multitude of hitherto unknown bacteria may invade fish farms.

The number of marine vibrios have been observed to coincide with variations in planktonic particles in the sea, and the microflora of marine invertebrates, seaweed, and plankton may be dominated by vibrios at certain times of the year (Simidu et al., 1971; Kaneko and Colwell, 1978; Huq et al., 1983; Pedrós-Alió and Brock, 1983). Some fish pathogens may survive for months in seawater (Hoff, 1989b; Effendi and Austin, 1991; Thorsen et al., 1992), and even longer in sediments (Enger et al., 1989). Prolonged survival of *Aeromonas salmonicida* in water, sediments, seaweeds, and various invertebrates has been demonstrated (Effendi and Austin, 1994). *Vibrio anguillarum* survived better in seawater if particles were present (Olafsen et al., 1981), and also survived in eels kept in freshwater (Rødsæther et al., 1977), where the pathogen would not normally survive.

3. Host–microbe interactions of invertebrate co-inhabitants and food organisms

Aquatic invertebrates are natural food sources for fish larvae and are also co-inhabitants of larval ecosystems. This relationship will imply that the establishment of a larval microflora will also be influenced by the indigenous microflora of invertebrates, whether they are food organisms or co-inhabitants of larval ecosystems or rearing facilities. Aquatic invertebrates live in areas with an abundant microflora that may provide food particles for filter feeders, but also act as a potential source of infection. They are, thus, continually confronted with infectious microorganisms. The complex relationship between marine invertebrates and their microflora is not fully understood, since invertebrates lack immunoglobulins (Ig), T cell receptors (TCR), Major histocompatibility complex (Mhc), and do not exhibit immune memory in a strict sense. For reviews, see Olafsen (1996), Vasta and Ahmed (1996), and Roch (1999). Defense reactions and disease control in crustanceans have been, because of their economic importance, comprehensively reviewed (Söderhäll and Cerenius, 1992; Bachère, 2000; Bachère et al., 1995, 2000; Sritunyalucksana and Söderhäll, 2000).

Invertebrates exhibit many functional traits of the immune system similar to those found in mammals, such as cytokine-like molecules, components similar to acute phase reactants (Olafsen, 1996; Vasta, 1996) or complement activators (Vasta et al., 1999). Humoral factors in invertebrates were believed to be innate and non-inducible although activities, such as lysozyme, lysosomal enzymes, lectins, or antibacterial components, respond to challenge (Chu, 1988; Lassegués et al., 1989; Olafsen et al., 1992). In recent years, the existence of an adaptive immune system in invertebrates has emerged (Arala-Chaves and Sequeira, 2000). Thus, it now seems reasonable to assume that a peculiar form of adaptive immune response, quantitatively and qualitatively different from that of vertebrates, probably exists in invertebrates.

In healthy mammals, and in fish, the concerted actions of the nonspecific defenses and the specific immune system prevent bacteria from entering and proliferating in tissues and body fluids. It appeared that the coelomic fluid of healthy echinoderms was aseptic (Wardlaw and Unkless, 1978; Kaneshiro and Karp, 1980); however, it has also been observed that healthy marine invertebrates may contain bacteria in their digestive system, gill, mantle fluid, body fluid (hemolymph) and other soft tissues (Farley, 1977; Kueh and Chan, 1985; Stein et al., 1987; Olafsen et al., 1993), and eggs (Zachmann and Molina, 1993). These filter feeders ingest bacteria from the environment (ZoBell and Feltham, 1938), and bacteria colonize their integument and gut (McHenery and Birkbeck, 1985). Marine shellfish may also act as specific vectors for the spreading of viruses (Atmar et al., 1993).

Bottom-dwelling marine bivalves accumulate large numbers of microorganisms from the seawater by filter feeding and, thus, harbour an exceptionally rich microflora with *Vibrio* species predominating. The *Vibrio* group comprises microorganisms with a dynamic role in the marine and brackish environments. They are isolated from seawater, sediment, plankton, invertebrates, and fish, and are abundant members of the microflora of aquatic organisms (Colwell, 1984). We know relatively little about how vibrios interact with aquatic organisms; however, apparently, they are the most commonly isolated members of the "normal" microflora. Marine vibrios are frequently found in association with invertebrate surfaces and also in hemolymph and internal organs of healthy bivalves (Vasta, 1990; Olafsen et al., 1993). *Pseudomonas* and *Vibrio* are natural constituents of the bacterial flora of the molluscan digestive tract.

A specific relationship exists between some marine vibrios and their invertebrate hosts. This interaction may be specific, since bivalves may have lectins that bind vibrios (Olafsen et al., 1992), including *V. cholerae* and *V. vulnificus* that are pathogenic to man (Tamplin and Fisher, 1989; Fisher, 1992; Tamplin and Capers, 1992). Invertebrates apparently possess effective defenses to regulate the internal proliferation of bacteria (Olafsen, 1988), but may nevertheless act as vectors for bacteria that are pathogenic to other organisms. The tendency to harbour indigenous *Vibrio* species is also evident from the fact that vibrios persisted in bivalve hemolymph and soft tissues after depuration in UV-treated seawater (Greenberg et al., 1982; Eyles and Davery, 1984; Tamplin and Capers, 1992). Thus, filter feeders, such as marine bivalves, may harbour viable human pathogens, *V. cholerae*, *V. damsela*, *V. fluvialis*, and *V. vulnificus* (Fisher and DiNuzzo, 1991), and may act as vectors for transmittance of diseases (Blake et al., 1980; Janda et al., 1988; Tamplin and Fisher, 1989; Tamplin and Capers, 1992). We found that

hemolymph and soft tissues of Japanese oysters (*Crassostrea gigas*) and horse mussels (*Modiolus modiolus*) kept in seawater at temperatures below 8 °C contained bacteria (Olafsen et al., 1993). Dominating bacterial groups were *Pseudomonas*, *Vibrio* and *Aeromonas*. Following addition to the seawater of psychrotrophic fish pathogens (*V. anguillarum* and *V. salmonicida*), it was observed that these bacteria massively invaded the hemolymph and soft tissues even at low temperatures. Thus, bivalves may also serve as vectors for fish pathogenic psychrotrophic vibrios (Olafsen et al., 1993).

Lectins are receptor-specific recognition factors that bind carbohydrates or glycoproteins and agglutinate cells or precipitate glycoconjugates (Boyd and Shapleigh, 1954). Lectins have been identified in all taxa, from viruses and bacteria to vertebrates. Microbial lectins facilitate the adhesion of bacteria to host tissues, and host lectins may act as opsonins to potentiate the uptake of microorganisms by phagocytic cells. Invertebrate lectins are generally believed to take part in humoral defense reactions against bacteria by reacting with nonself ligands and augmenting the phagocytic response (Olafsen, 1986; Renwrantz, 1986; Vasta and Marchalonis, 1987; Olafsen, 1988; Vasta, 1991). Humoral lectins are found in the body fluids of most invertebrates; however, their biological role(s) are not yet firmly established. By acting as opsonins, invertebrate lectins may take part in the recognition and clearance of bacteria by phagocytic cells (Hardy et al., 1977; Vasta et al., 1982; Renwrantz and Stahmer, 1983; Olafsen et al., 1992). For recent reviews of the function of lectins in mulluscs and crustaceans, see Olafsen (1996), Vasta (1996), Vasta et al. (1999), and Marques and Barracco (2000).

Invertebrate lectins react with a variety of cells and ligands and also with natural antigens, such as bacteria. Natural agglutinins for bacteria have been described in a variety of marine invertebrates, reviewed in Olafsen (1996). The retention of pathogenic vibrios in oysters may partly be explained by the existence of lectins that agglutinate them (Tamplin and Fisher, 1989; Fisher, 1992; Fisher and DiNuzzo, 1991). The lectins agglutinated a wide variety of bacteria, but had specific recognition capabilities for some bacteria (Tamplin and Fisher, 1989; Fisher and DiNuzzo, 1991), including all serovars and biovars of *V. cholerae* (Tamplin and Fisher, 1989). Thus, it appears that bivalves have natural agglutinins for marine vibrios, and the reaction with some of these bacteria is specific, since lectins react with purified LPS from marine pathogens, including *V. anguillarum* and *V. salmonicida* (Tunkijjanukij et al., 1997). A number of LPS-binding lectins have been described from invertebrates (Azumi et al., 1991; Kawasaki et al., 1993; Vargas-Albores et al., 1993; Murali et al., 1994; Hypsa and Grubhoffer, 1995); however, they reacted mainly with LPS from nonmarine bacteria. Purified lectins from the horse mussel also demonstrated antibacterial activity against a range of fish pathogenic bacteria, including *V. salmonicida*, *V. anguillarum*, *V. viscosus*, and *V. wodanis* (Tunkijjanukij and Olafsen, 1998), thus suggesting that the lectins may also be directly involved in bacterial killing.

Bacterial resistance to the bivalve defense mechanisms (i.e., phagocytic hemocytes) may partially explain their prevalence in bivalve tissues (Harris-Young et al., 1993). It is also possible that bacteria that survive bivalve humoral defenses may later establish more stable relationships within their tissues. Nevertheless, the ability for some bacteria to specifically colonize invertebrate hosts implies that they will readily be transfected to

fish larvae when invertebrates are used as food organisms or coinhabit the rearing facilities.

Because of their economic importance, disease management of aquatic invertebrates, particularly shrimp, has been well described (Roch, 1999; Bachère, 2000; Rodríguez and Le Moullac, 2000). Even though invertebrates are not capable of anticipatory immune responses in a strict sense, induction of antibacterial activity has been observed following vaccination of shrimp, *Penaeus vannamei* (Alabi et al., 2000). Different approaches to improve health of farmed invertebrates have been successfully employed, including ecosystem management (Kautsky et al., 2000), and microbial control through the use of bacterial or yeast probionts (Scholz et al., 1999; Rengpipat et al., 2000; Riquelme et al., 2001; Sung et al., 2001). This research has also formed a platform for an "ecosystem approach" in microbial management of rearing systems for fish larvae (see below).

4. The egg microflora

Poor egg quality and resulting mass mortalities have been serious problems in larval production systems. Intensive incubation techniques, often resulting in eggs overgrown by bacteria, may affect the commensal relationship between the indigenous microflora and opportunists, hamper egg development and subsequently affect hatching, larval health and ongrowth. Thus, the microflora on eggs and in larval incubators may also affect the short- and long-term health of farmed fish.

Use of antibiotics may result in alterations in the microflora that could be unfavourable (Austin and Al-Zahrani, 1988; Hansen et al., 1992b) and, thus, there is an urgent need to control the microflora in incubators by other means. In aquaculture, eggs may be sterilised by various techniques to remove the adherent microflora and to prevent the transmission of bacterial pathogens during transport. Such methods (UV-irradiation, ozonisation, membrane filtration, and antibiotics) are used in intensive larviculture; however, these approaches disturb the balance of microbial communities and favour exponential growth of opportunistic bacteria (Prieur, 1982; Li and Dickie, 1985; Jeanthon et al., 1988; Baticados and Pitogo, 1990; Salvesen et al., 2000a,b). After disinfection, there is low competition for nutrients and opportunistic bacteria with high growth rates may proliferate (Andrew and Harris, 1986). This uncontrollable rapid recolonisation of the disinfected water may result in the development of a bacterial community favourable for larval development; however, the process is unpredictable and may also result in an outbreak of pathogenic bacteria with a detrimental effect on larval rearing. Removal of the egg epiflora will reduce the microbial heterogeneity and, thus, make the eggs vulnerable to opportunistic colonisation by bacteria. Even though surface disinfection of eggs is the prevailing method today, we should, in the future, try to obtain better control of the microflora by other means. In a sense, this is a general phenomenon that has been observed in medicine following the use of antibiotics, transplantation, or depletion of the normal flora (Gristina, 1987; Rook and Stanford, 1998), and described as a race for the surface receptors.

Fish eggs are readily colonised by bacteria (Oppenheimer, 1955; Shelbourne, 1963) and within a couple of days after spawning eggs may be heavily overgrown (Hansen and

Olafsen, 1989). The epiflora of fish eggs have been described in a number of papers, reviewed by Hansen and Olafsen (1999). Stalking and budding bacteria (Austin, 1982) and bacteria with adhesion structures appear to be abundant in the egg epiflora (Hansen and Olafsen, 1989). *Leucothrix mucor* is frequently observed on eggs, and because of its filamentous growth may be misleadingly identified as a fungus infection in hatcheries (Hansen and Olafsen, 1989). When identified, it may be readily treated.

Bacterial colonisation may have adverse effects on eggs (Hansen et al., 1992a) and on the developing embryo (Bergh et al., 1992, 1997). A negative correlation has been demonstrated between bacterial colonisation and the physical strength of fish eggs (Kjørsvik et al., 1990). Some pathogens may dissolve the chorion and zona radiata of the egg shell, such as the psychrotrophic *Flexibacter ovolyticus* that constitutes a major part of halibut egg epiflora and results in high larval mortalities after hatching (Hansen et al., 1992a). Bacterial overgrowth may result in hypoxia in the developing embryo, or hatching delay (Helvik, 1991). Release of exoproteolytic enzymes from the adherent bacterial epiflora may damage the chorion (Hansen and Olafsen, 1989) or destroy the zona radiata (Bergh et al., 1992; Hansen et al., 1992a), and bacterial exotoxins or toxic metabolites may harm the developing embryo (Barker et al., 1989). Pathogens may also be transferred vertically by intra-ovum infections (Evelyn et al., 1984). However, it has been observed that nonpathogenic bacteria may also be present in salmon ova (Sauter et al., 1987). Bacteria have also been demonstrated on the surface of unfertilised cod eggs that were aseptically dissected from the ovaries (Hansen and Olafsen, 1989). The microbial community of the ambient seawater may also influence the composition of the bacterial egg epiflora. Receptors present on the egg surface, however, may selectively favour colonisation of specific bacteria. This is, to some extent, reflected in the species-specific differences found between bacterial groups that colonize cod and halibut eggs (Hansen and Olafsen, 1989). The natural adherent microflora may protect eggs in a variety of ways, as previously discussed (Hansen and Olafsen, 1999). Cod and herring eggs are distributed in the surface and tidal zone, respectively, while halibut eggs are normally distributed at depths of about 200 m (Haug et al., 1984). Hence, conditions in incubators may not favour a "natural" halibut egg microflora. Exoenzymatic activity of the egg epiflora may also erode the chorion and, thus, affect secondary colonisation to exposed attachment sites (Hansen and Olafsen, 1989).

It is necessary to keep control on the microbial community composition, including pathogens and opportunists in incubators. Thus, a dense, nonpathogenic and diverse egg epiflora may be a barrier against colony formation by pathogens. To better control bacterial colonisation of eggs, we need to define community compositions that restrain the adhesion of harmful bacteria.

5. The larval microflora

In aquaculture, fish larvae are kept in incubators with hatching eggs and debris, resulting in a 1000-fold increase in bacterial counts of the ambient water through hatching (Hansen and Olafsen, 1989). Marine fish larvae start drinking before the yolk sac is consumed and bacteria, thus, enter the digestive tract before active feeding

commences. Older larvae may also ingest bacteria by grazing on suspended particles and egg debris (Olafsen, 1984; Olafsen and Hansen, 1992; Beveridge et al., 1991). Thus, the microflora of eggs and other organisms in the farm will affect the primary microflora of fish larvae. There is, however, little information on bacterial colonisation of mucosal surfaces of healthy fish larvae, apart from a few reports, reviewed in Hansen and Olafsen (1999).

A variety of bacterial genera and species have been isolated from slime and external surfaces of adult marine fish. For reviews, see Cahill (1990), Sakata (1990), and Hansen and Olafsen (1999). Some specificity has been observed in bacterial colonisation of external and internal mucosal surfaces of fish (Austin, 1982; Mudarris and Austin, 1988; Hansen and Olafsen, 1989; Cahill, 1990). The intestinal microfloras have been described for various fresh and seawater fish species and, although the majority of studies have concentrated on adult fish, some reports describe the microflora of larvae and juveniles, reviewed in Hansen and Olafsen (1999). The intestinal microflora of fish appears to be affected by the flora of the ambient water and in the diet, and a "normal" intestinal microflora appears to be established when the yolk sac is absorbed and the digestive tract activated. In tilapia (*Tilapia mossambica*), an "adult" intestinal microflora was established 20 to 60 days after hatching (Sugita et al., 1982). With the introduction of modern methodology, our understanding of the commensal flora is likely to change (Spanggaard et al., 2000). In halibut (*Hippoglossus hippoglossus*), the glycoproteins of the mucus shifted from predominantly neutral to a mixture of neutral and sulphated during development from a pelagic larvae to bottom dwelling flatfish (Ottesen and Olafsen, 1997), thus indicating a shift in adhesion sites for bacteria during larval development with implications for shifts in the microflora during larval ontogeny.

Several reports describe bacteria firmly attached to the intestinal mucosa (Yoshimizu et al., 1976; Onarheim and Raa, 1990; Sakata, 1990; Strøm and Olafsen, 1990; Onarheim et al., 1994), and it is accepted that fish contain a specific intestinal microflora consisting of aerobic, facultative anaerobic, and obligate anaerobic bacteria. The composition may change with age, nutritional status, and environmental conditions (Trust et al., 1979; Sakata et al., 1980; Sugita et al., 1985; Conway et al., 1986; MacFarlane et al., 1986; Sugita et al., 1991). Vibrios are common members of the indigenous microflora of healthy fish (Rødsæther et al., 1977; Colwell, 1984; Kanno et al., 1990; Olafsen, 1993). The term "gut group Vibrios" was introduced by Liston and Colwell (Liston, 1957) and indicated that members of the genus *Vibrio* dominated in the intestine of marine fish. Since then, it has been found that *Vibrio* and *Pseudomonas* prevail in the intestinal tract of a variety of species of marine fish (Sera and Ishida, 1972; Sakata et al., 1978; Onarheim et al., 1994). Marine bacteria adhere well to mucosal surfaces. Fish pathogenic bacteria, such as *V. salmonicida* and *V. anguillarum*, have been shown in vivo to adhere to the intestinal epithelium of fish larvae (Hansen et al., 1992b; Hansen and Olafsen, 1999; Olafsen and Hansen, 1992). Adhesive properties of bacteria from fish have also been studied in vitro using a single substrate, including mucus, for adhesion (reviewed in Hansen and Olafsen, 1999).

Adhesion to mucus is one of the first steps in the infection process. We tested the in situ adhesion of pathogenic and nonpathogenic bacteria isolated from fish for adhesion to cryosections from different mucosal surfaces of Atlantic salmon (*Salmo salar*) by

immunohistochemistry (Knudsen et al., 1999). The majority of the bacteria tested, *V. anguillarum* serotype O1, *V. salmonicida*, *V. viscosus*, *F. maritimus*, and "gut group Vibrios", i.e., *V. iliopiscarius* and apathogenic isolates of *V. salmonicida*, all adhered well to mucus on all salmon epithelial surfaces tested, including sections from foregut, hindgut, pyloric cecae, gills, and skin. In contrast, *V. anguillarum* serotype O2 did not adhere to mucus, but adhered to all other components of the tissues. Thus, adhesion to mucus appears to be a widespread trait of marine bacteria and not restricted to pathogens or virulent strains. It is feasible, however, that some pathogens, like *V. anguillarum* O2, have an invasion strategy that does not involve mucus adhesion.

Lactic acid bacteria have been isolated from the intestinal mucosa of cod (*Gadus morhua*), saithe (*Pollachius virens*), capelin (*Mallotus villosus*), herring (*Clupea harengus*), and Atlantic salmon (*S. salar*) in seawater (Schrøder et al., 1980; Stroband and Kroon, 1981). They all belonged to the genus *Lactobacillus*, resembling *L. plantarum*. In freshwater salmonid fry, lactic acid bacteria were the major part of the adherent intestinal microflora, while in marine species, they constituted only a minority. These lactic acid bacteria produced growth inhibiting factors that could inhibit various *Vibrio* spp., especially *V. anguillarum*. For a review of lactic acid bacteria in fish, see Ringø and Gatesoupe (1998).

Bacteria may play a role as food for marine invertebrates and fish (ZoBell and Feltham, 1938; Seki, 1969; Wavre and Brinkhurst, 1971; Olafsen, 1984; Bitterlich and Schaber, 1986; MacDonald et al., 1986) by furnishing cell substances or micronutrients, such as essential fatty acids (Ringø et al., 1992), vitamins (Kashiwada and Teshima, 1966; Sugita et al., 1991), minerals, or even enzymes (Goodrich and Morita, 1977; Lemos et al., 1985). The alimentary canal of teleostean fish larvae is histologically and functionally undifferentiated at the time of hatching, and the larval gut, thus, resembles that of stomachless or agastric fish, in which intracellular digestion predominates (Stroband and Dabrowski, 1979; Govoni et al., 1986). In fish larvae with a straight gut, ingested food accumulates in the posterior part of the gut (Iwai and Tanaka, 1968; Blaxter, 1988; Hansen et al., 1992b), and also bacteria accumulate and are endocytosed in the hindgut (Olafsen, 1984; Olafsen and Hansen, 1992). Ingestion of bacteria by drinking and subsequent endocytosis in the hindgut could, thus, sustain small, newly hatched larvae with essential nutrients before active feeding commences.

The functions of the indigenous intestinal microflora are well understood in warm-blooded animals. In fish, however, the roles or even existence of an indigenous intestinal microflora have been disputed (Wood, 1967; Seki, 1969), but apparently a primary transient microflora become established at the larval stage, developing into a persistent flora at the juvenile stage or after metamorphosis. Our understanding of this process may change as new molecular tools are being adapted to the study of the transient or indigenous microflora of fish (Spanggaard et al., 2000).

6. Larval defense factors

Fish are among the most primitive animals capable of mounting an anticipatory immune response, and we would expect its intimate relationship with bacteria to be effectively regulated. The epithelium with its mucus layer forms a barrier against

external environments that harbours a multitude of potentially harmful microorganisms. A continuous secretion and shedding of mucus produced by goblet cells in skin, gills, and mucosa of the gastrointestinal tract may prevent microbial colonisation. Natural defense factors in mucus, such as complement, lysozyme, antibacterial factors, and lectin-like agglutinins, may bestow protection and may explain observations that body surface mucus of healthy fish did not contain many bacteria (Crouse-Eisnor et al., 1985). Large numbers of *V. anguillarum*, however, were found in mucus during advanced infections (Kanno et al., 1990). Following stress or infection, mucus secretion is increased (Blackstock and Pickering, 1982), and increased numbers of bacteria in the seawater may also result in increased epidermal mucus production in halibut larvae (Ottesen and Olafsen, 1997). As most bacterial infections take hold on mucosal surfaces, however, increased mucus production may not be unequivocally beneficial.

In adult fish, evidence suggests that local mucosal and secretory immunity is important in protection against bacterial infections. Teleosts possess intraepithelial lymphoid tissue, although less organised than in mammals. Macrophages, lympoid cells, and secretory immunoglobulin-forming cells are infiltrated within the intestinal epithelium (Tomonaga et al., 1985; Rombout and van den Berg, 1989), and uptake of intact antigens by intestinal epithelial cells of adult fish has been extensively observed. Apparently, however, fish lack specialised intraepithelial cells for antigen uptake, and it appears that enterocytes may serve a similar purpose of antigen sampling (Rombout and van den Berg, 1989). Cells capable of sequestering intact antigens are found particularly in the hindgut of teleosts (Olafsen, 1984; Olafsen and Hansen, 1992). Induction of immune responses against particular antigens can possibly be obtained by the hindgut, and the absorptive cells may present antigens to the immune system (Rombout and van den Berg, 1989). Secretion of interleukin-like factors suggests that epithelial cells may also have an immunomodulatory function (Sigel et al., 1986).

Relatively little is known about ontogeny of immunity in fish. It has been inferred that the "immune capacity" of fish larvae is not fully developed until they are several weeks old (Chantanachookhin et al., 1991); however, this may vary among fish species. Until then, larvae probably rely on nonspecific defense reactions. Ig-positive cells are detected after a few weeks. Antibody response to bacteria has been observed 3 weeks posthatching (Mughal and Manning, 1985), while tolerance to commensal bacteria has not yet been described. Depending on the species, fish from 2–3 weeks may be vaccinated by injection, some protection may be obtained by immersion, while oral administration usually yields less protection. It was suggested that oral vaccination prevented colonisation of *V. anguillarum* resulting from anti-*Vibrio* agglutinins in skin mucus (Kawai et al., 1981); however, there is still a lack of information concerning immune regulation of the normal microflora in fish. In general, it is observed that lymphocytes appear later in the development of marine fishes than in freshwater species, see Schrøder et al. (1998) and references therein. While recent research has resulted in a better understanding of the ontogeny of the immune system in fish (Castillo et al., 1993; Takemura, 1993; Abelli et al., 1996; Padros and Crespo, 1996; Schrøder et al., 1998; Trede and Zon, 1998; Jones et al., 1999), there is still no complete understanding of the immunological factors that affects the establishment or regulation of a commensal microflora in fish.

Fish larvae ingest bacteria that are propulsed towards the posterior gut segment, which may be occluded with bacteria (Hansen et al., 1992b). Bacteria are endocytosed by epithelial cells in the hindgut of immature larvae (Hansen et al., 1992b; Olafsen and Hansen, 1992), probably by unspecific uptake. Very young cod larvae also take up intact bacterial antigens in columnar epithelial cells in the foregut, demonstrated by immunohistochemistry, and these antigens penetrate the gut epithelium (Olafsen and Hansen, 1992). The uptake demonstrated specificity for some of the tested bacteria, while latex beads were not ingested (Olafsen and Hansen, 1992). Production of specific antibodies, however, has not been observed in fish at this age, and it is believed that the immature larvae rely mostly on nonspecific defense mechanisms and phagocytosis (Fletcher, 1982).

In mammals, prefeeding protein antigens causes reduction in the subsequent systemic immune response. This is known as oral tolerance, most easily elicited with T-cell-dependent antigens and facilitated by T suppressor cells. Oral tolerance to various soluble antigens has been observed in fish (McLean and Ash, 1987; Rombout and van den Berg, 1989); however, it is not yet known whether sequestering of intact bacterial antigens in gut epithelial cells may result in immunity or tolerance to bacteria in fish.

The first reaction to infection in mammals is characterised by rapid increase of a variety of acute phase proteins, such as C-reactive protein (CRP) (Kolb-Bachofen, 1991; Köttgen et al., 1992) and serum amyloid component (SAP). These plasma proteins have been isolated in vertebrates from fish to man. An acute phase reaction takes place in humans, monkeys, dogs, and rabbits, while in other animals (cow, goat, rat, and fish), these proteins are present in varying concentrations in normal plasma (Kolb-Bachofen, 1991). CRP and SAP are members of the pentraxin family of serum proteins primarily synthesised in the liver. They are characterised by having five or ten identical subunits arranged in a planar pentameric ring structure. They exhibit calcium-dependent binding to a variety of ligands, reviewed by Tennent and Pepys (1994). CRP is defined by its binding to the phosphorylcholine moiety of the C-polysaccharide of the cell wall of *Streptococcus pneumoniae* in the presence of calcium ions (Tillett and Francis, 1930). CRP also binds, though more weakly, to carbohydrates, including agar and other galactans in a lectin-like manner. SAP recognizes agarose, zymosan, and phosphomannans (Tennent and Pepys, 1994). In mammals, CRP and SAP are major acute phase proteins, but still their activities are among the least well known of the acute phase proteins, reviewed by Steel and Whitehead (1994). The biological role of CRP is not yet fully understood, but it acts as an opsonin (Hokama et al., 1962), and once attached to a ligand may activate complement via the classical pathway and stimulate the action of phagocytic cells (Kilpatrick and Volanakis, 1985; Kolb-Bachofen, 1991). Various invertebrate lectins also exhibit CRP-like properties (Robey and Liu, 1981; Tunkijjanukij et al., 1997), and reviewed in Vasta (1990) and Olafsen (1995), but they are constitutive proteins. Also, the CRP of *Xenopus* is reported not to react as an acute phase protein (Lin and Liu, 1993).

Thus, it has been of interest to identify and characterize acute-phase proteins from fish as potential early indicators for infection or inflammation. In plaice (Pepys et al., 1982), rainbow trout (Murata et al., 1994; Murata et al., 1995), and dogfish (Robey et al., 1983), both CRP- and SAP-like proteins have been described. In plaice (Pepys et al.,

1982) and rainbow trout (Kodama et al., 1989), a moderate increase in CRP levels had been reported in response to *Escherichia coli* LPS injection or *V. anguillarum* infection. In catfish that had high levels of CRP in normal serum, a decrease in serum CRP was reported during *Saprolegnia* infection (Szalai et al., 1994). We isolated and partially characterised pentraxin-like proteins from Atlantic salmon, common wolffish, cod, and halibut (Lund and Olafsen, 1998b); however, none of these exhibited any acute phase response (Lund and Olafsen, 1999). Thus, there appears to be a family of pentraxin-like, or phosphorylcholine-reactive proteins (PRP) in fish. Some of these demonstrate heterogenous binding, and we have also isolated a plasminogen-alike PRP from salmon (Lund and Olafsen, 1998a).

In this context, it is of interest that the levels of C-reactive proteins from carp were elevated 2.8–3.5 times following metal intoxication (Paul et al., 1998), observed as a unique molecular variant. This variant coexists with normal CRPs and appears to differ significantly in carbohydrate content. Thus, microheterogenous forms of CRP appeared to be induced by metal pollution, possibly by altered glycosylation. The differences in glycosylkation of CRP following such stimuli may suggest new avenues for pentraxins and the not yet fully understood control of the acute phase response (Paul et al., 1998). Thus, multispecific molecules that occur in distant groups, such as fish and mammals, may be related in structure or receptor binding, but differ in their functional roles in the immune system. The biological roles of such defense molecules in fish and invertebrates should be further elucidated.

7. Microflora manipulation in aquaculture

The use of probiotic microorganisms has proven advantageous in domestic animal production (Vanbelle et al., 1990). In aquatic ecosystems, the intimate relationships between microorganisms and other biota and the constant flow of water through the digestive tract of fish and invertebrates will also affect their indigenous microflora. Against this background, we may assume that the natural microbiota on eggs and larvae may help to protect against colonisation by a harmful microflora. The microflora of intensive larval rearing systems, however, differs dramatically from that in the sea, and is influenced by rearing techniques, nutrients, disinfection techniques, and use of antibiotics.

It is not yet known to what extent the natural microflora of fish may be protective towards pathogen colonisation (for a review, see Hansen and Olafsen, 1999). However, there is increasing evidence that microflora manipulation, or addition of "probiotic" microorganisms, may improve health conditions and survival of larvae in intensive rearing. The term probiotic describes living cells that exert beneficial health effects on the host by improving the microbial balance or properties of the indigenous microflora (Fuller, 1989; O'Sullivan et al., 1992; Huis in't Veld et al., 1994). Such characteristics may be antagonism or colonisation prevention towards pathogens, stimulation of natural defenses, or health benefits from released factors. The use of probiotics in aquaculture has recently been reviewed (Gatesoupe, 1999; Gomez-Gil et al., 2000).

In aquaculture, it would be tempting to improve rearing conditions by microbial control or manipulation of eggs in incubators. Severe microbial problems occur at the

egg stage, microflora manipulation of the egg epiflora would appear relatively easy, and the larval microflora are affected by the eggs. We tried to manipulate the egg epiflora by incubating bacteria-free (gnotobiotic) eggs with defined cultures of selected bacteria (Hansen and Olafsen, 1989). Cod eggs were aspetically dissected, fertilised, and kept in sterile seawater. The sterile eggs were primed with antibiotic-producing bacterial strains, originally isolated and characterised by Lemos et al. (1985). After incubation for 3 days, the eggs were transferred to natural seawater and, thus, exposed to the environmental microflora. Germ-free eggs incubated in seawater or diluted environmental cultures were used as controls. Within a few days, colonisation in all experimental groups equalled that of the controls. The antibiotic-producing isolates, thus, failed to prevent colonisation by a heterogenous flora of environmental strains. It, thus, appears that a high colonisation potential for a diverse natural microbiota could furnish protection against overgrowth by opportunistic single strains (Hansen and Olafsen, 1989).

It has been observed and well documented that *Artemia* and rotifer cultures may transfect bacteria that are harmful to the fish larvae during start-feeding, and various attempts at microbial control of the start-feeding cultures have been reported. An approach to this would be microbial control of the microflora of live food organisms (*Artemia* and rotifers) used for larvae. Microbial control of *Artemia* juveniles has been achieved by preemptive colonisation by selected bacterial strains (Verschuere et al., 1999), and the resulting changes in the *Artemia* microflora appeared stable. Management of the microflora of food organisms may be achieved by introduction of selected bacterial strains to axenically hatched rotifers (Rombaut et al., 1999) by controlled use of cultured microalgae in cultivation of *Artemia* diets (Olsen et al., 2000) or by successful incubation of axenic rotifer (*Brachionus*) cultures with selected bacterial strains (Douillet, 2000a,b). These approaches also usually had positive effects on rotifer production. Manipulation of the larval microflora could be aimed at suppressing specific pathogens. The introduction of a probiotic *Lactococcus lactis* strain to rotifers (*Brachionus*) was demonstrated to inhibit *V. anguillarum* and enhance rotifer growth rate (Shiri Harzevili et al., 1998). In contrast, preincubation in bacterial suspensions could be aimed at suppressing opportunistic bacteria in *Brachionus* or *Artemia* cultures (Makridis et al., 2000), as an example of an ecosystems approach to suppress opportunistic bacteria. Successful attempts at controlling the microflora by manipulating the ecosystem have been reported, using microbially matured water in a system that competitively selects against opportunistic and potentially pathogenic bacteria. Significantly higher growth rates were observed in turbot larvae reared in matured water than in membrane filtered water and, thus, this appears to be a promising method for ecosystem management (Skjermo and Vadstein, 1999; Skjermo et al., 1997; Salvesen et al., 1999, 2000a,b).

Thus, it appears that improved microbial control of larval food organisms is feasible through microflora or ecosystem manipulation. Stable transfection of microorganisms between hosts, or from food organisms to hosts, however, is a difficult procedure and such systems still have to be tested for long-term effects. The bacteria that have so far been tested are either established probionts, like lactic acid bacteria, specific antagonists or competitiors to pathogens, such as some apathogenic vibrios or bacteria that may stimulate the host or positively affect the ecosystem.

Lactic acid bacteria are widely used as probiotics (Conway, 1989). Species resembling *L. plantarum* have been isolated from the intestinal mucosa of several fish species (Strøm, 1988; Strøm and Olafsen, 1990), and results indicate that they produce growth inhibiting factors towards various pathogenic *Vibrio* spp. Lactic acid bacteria have been fed to rotifers used as food for turbot larvae (Gatesoupe, 1994). The added bacteria were inhibitory towards pathogens in rotifer cultures, and also resulted in a significant increase in the weight of the turbot larvae (Gatesoupe, 1991). Improvement in disease resistance of cod (*G. morhua*) fry given a dry feed containing *Carnobacterium divergens* 3 weeks before challenge with *V. anguillarum*, has also been observed (Gildberg et al., 1997; Gildberg and Mikkelsen, 1998), and recently, the use of a *Carnobacterium* sp. as a probiotic for Atlantic salmon and rainbow trout has been reported (Robertson et al., 2000). The potential of lactic acid bacteria as probiotics in aquaculture has been reviewed (Ringø and Gatesoupe, 1998).

Vibrios are natural members of the indigenous microflora of healthy fish (Rødsæther et al., 1977; Colwell, 1984; Kanno et al., 1990; Olafsen, 1993; Bergh, 1995), and also dominate in the intestinal tract of marine fish, reviewed in Hansen and Olafsen (1999). Fish pathogenic vibrios may be present on wild fish or healthy fish in aquaculture (Rødsæther et al., 1977; Olafsen et al., 1981; Enger et al., 1989; Hoff, 1989a; Enger et al., 1991; Olafsen, 1993). Commensal vibrios with inhibitory activity against pathogens have been isolated from mucosal surfaces of healthy fish (Westerdahl et al., 1991; Olsson et al., 1992; Bergh, 1995). Adult marine flatfish harboured intestinal and skin-mucus-associated bacteria with the capacity to suppress growth of *V. anguillarum* (Olsson et al., 1992), but attempts to establish these strains in the intestine of larval turbot to protect against subsequent challenge with *V. anguillarum* have not been successful. Addition to turbot larvae of a potential probiotic bacterium, *V. pelagius*, isolated from healthy turbot, *Scophthalmus maximus* L., demonstrated that this bacterium was established in the intestinal microflora (Ringø et al., 1996) with a positive effect on the larvae (Ringø and Vadstein, 1998). Liston (1957) described "gut group Vibrios" from marine flatfish. These indigenous vibrios are dominated by two typical groups (Sera and Ishida, 1972; Onarheim and Raa, 1990), originally classified as *V. iliopiscarius* and nonpathogenic strains of *V. salmonicida*, and identified as parts of the autochthonous intestinal microflora of salmon (*S. salar*), cod (*G. morhua*), saithe (*P. virens*), and herring (*Clu. harengus*) (Onarheim and Raa, 1990; Onarheim et al., 1994). These *Vibrio* species produced inhibitory substances against *V. anguillarum*, *V. ordali*, and pathogenic strains of *V. salmonicida* in vitro and, thus, had the potential of in vivo inhibition of these pathogens (Onarheim and Raa, 1990; Onarheim et al., 1994). In wild-captured cod juveniles, the intestinal microflora was dominated by vibrios (50%), and of these 30% crossreacted with antibodies against a pathogenic *V. salmonicida* strain (Strøm and Olafsen, 1990). In groups of the juveniles that had been fed an artificial diet for 1 year, the apathogenic gut-vibrios could not be detected. It was, thus, assumed that the diet could influence the persistence of commensal, apatogenic strains that may help to confer protection against related pathogenic strains.

In agreement with the above concept, we observed that survival of halibut (*H. hippoglossus*) larvae was affected by incubation with indigenous bacteria (Ottesen and Olafsen, 2000). The bacteria used were all isolated from fish, and were two types of

"gut-group vibrios", an apathogenic *V. salmonicida* strain and *V. iliopiscarius* (Onarheim et al., 1994), and a *L. plantarum* strain (Strøm, 1988). Larval survival in the first 2 weeks was significantly higher following addition of apathogenic *V. salmonicida* and *L. plantarum* strains to the incubation water, while *V. iliopiscarius* reduced survival compared to control groups. Thus, introducing a strain related to *V. salmonicida* increased survival, possibly by competition. Introduction of any presumed apathogenic strain, however, may not be unequivocally beneficial. In our case, a competitor to a pathogen (*V. salmonicida*) or an established *L. plantarum* strain proved effective. We also demonstrated an increase in the number of epidermal saccular mucus cells (Ottesen and Olafsen, 1997) of halibut larvae following incubation of the larvae in seawater with increased numbers of bacteria and following addition of apathogenic bacteria to the incubators (Ottesen and Olafsen, 2000). This finding suggests that changes in the microflora may induce the nonspecific defenses of the larvae.

8. Conclusion

The interaction between marine bacteria and various biota involved during production of fish larvae in aquaculture is still not well understood. To achieve improved microbial control in larval rearing systems, we still need information about bacterial colonisation factors, host regulation of the adherent microflora as well as interaction with egg surfaces and food organisms. Also, we still lack information about development of tolerance to the microflora and locally induced secretory mucosal immunity in fish.

Most marine bacteria adhere well to fish mucus; however, we still lack information about microbial adhesion factors as well as nonspecific defense systems in the mucus. Despite many fish pathogenic strains being relatively host-specific, little is known about receptors for bacterial adhesion. Moreover, for marine bacteria, there is a conspicuous lack of information about invasion strategies like antigen shift or phase variation, mechanisms that are known to be key factors in microbial pathogenicity.

The use of probiotics, or microbial manipulation, in intensive rearing of marine organisms may have a profound potential in health management. Based on the successful use of probiotics in domestic animals, such health-promoting efforts could be promising. It is not likely, however, that improved microbial control may be achieved by finding "ideal probiotics". Various stages and situations may call for different approaches, such as antagonism, competition, bacteriocin production, immune stimulation, or health promotion. Thus, it is likely that the use of a selected mixture of beneficial strains may prove more effective in different situations and more stable over time. Such bacterial "cocktails" are used in animal production, like the Food and Drug Administration approved Preempt®, a blend of 29 live, nonharmful bacteria naturally present in healthy adult chickens, used in the USA since 1998 for preventing *Salmonella* in chickens. Thus, a feasible approach could be the use of controlled bacterial communities at various critical stages of larval rearing. Such revolving multifunctional bacterial communities will have several advantages over single-strain probiotics. This will require a better basic understanding of various aspects of host–microbe interactions in aquaculture. At the same time, a set of protocols for practice of bacterial management in fish hatcheries is also required for the industry to draw on the collected knowledge.

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