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Review Article

Bacterial Interactions in Early Life Stages of Marine Cold Water Fish

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Abstract

The intensive rearing of various fish species in aquaculture has revealed intimate relationships between fish and bacteria that eventually may affect establishment of a "normal" mucosal microflora or result in disease epizootics. Interactions between bacteria and mucosal surfaces play important roles both at the egg and larval stages of marine fish. Bacterial adhesion and colonization of the egg surface occur within hours after fertilization. The diverse flora which eventually develops on the egg appears to reflect the bacterial composition and load of the ambient water, but species-specific adhesion at the egg surface may also play a role in development of the egg epiflora. Proteolytic enzymes produced by members of the adherent epiflora may cause serious damage to the developing egg and may also affect further adhesion of the epiflora.

Ingestion of bacteria at the yolk sac stage results in establishment of a primary intestinal microflora which seems to persist beyond first feeding. Establishment of a gut microflora is likely to undergo several stages, resulting in an "adult" microflora weeks to months after first feeding. Ingested bacteria may serve as an exogenous supply of nutrients or essential factors at an early life stage. Early exposure to high bacterial densities is probably important for immune tolerance, and thus for the establishment of a protective intestinal microflora.

Successful rearing of early life stages of several marine fish species depends on knowledge of the complex interactions among the cultured organisms and the bacterial communities which develop at the mucosal surfaces and in the ambient water and rearing systems. The routine use of antibiotics during rearing of fish larvae is not advisable, since it may increase the risk of promoting antibiotic resistance and adversely affect the indigenous microflora of the larvae. The use of probiotics has proven advantageous in domestic animal production, and the search for effective probiotics may have a great potential in aquaculture of marine organisms. Bacteria with antagonistic effects against fish pathogens have been successfully administered to several fish species, resulting in decreased mortality or increased growth rate.

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Introduction

Most marine organisms inhabit environments that are relatively rich in bacteria and other microorganisms. Seawater may function as a medium for both transport and growth of microorganisms, as distinct from air, which has been thought only to function as a transport medium. Thus, marine organisms share an ecosystem with microorganisms responsible for their disease. The majority of bacteria causing disease in marine fish are opportunistic pathogens that are present as part of the normal seawater microflora. Few are obligate pathogens, i.e., dependent on a living host for their propagation, e.g., *Renibacterium salmoninarum* and *Mycobacterium* spp. Altered health conditions caused by various environmental stressors such as alterations in temperature, oxygen concentration, or various pollutants [207, 262] may weaken the first-line defenses and allow bacteria to colonize, penetrate, and invade host tissues. Chemical or abrasive forces may also impair the integrity of the mucus layer and facilitate bacterial access to host epithelial surfaces. Environmental parameters may also, directly or indirectly, influence opportunistic bacteria. Larsen [134] reported a 10^3-fold increase in *Vibrio anguillarum* counts in seawater off the Danish coast due to discharge of carbohydrate-containing waste water. Virulence determinants of pathogenic bacteria may be "regulated" by environmental factors or may only be expressed under specific conditions [82]. Among the environmental variables known to have such effects are temperature, pH, osmotic strength, oxygen levels, and availability of iron [82].

The regulation of bacterial populations are complex processes that are not yet fully understood. Less than 1% of the total number of bacteria in natural seawater systems may be active and able to grow on or in laboratory media and the discrepancy between total bacterial counts and viable counts has been proposed to be due to the existence of a large number of dormant, nonactive, or viable but nonculturable bacteria [40, 123]. However, lately it has been questioned whether a large portion of the nonculturable bacteria may in fact be dead, non-nucleoid-containing bacterial ghosts, inferred from the fact that the widely used DAPI-stain bound poorly to DNA in seawater, and thus more likely stained bacterial surface ligands created by formaldehyde fixation [275]. The existence of a viable but nonculturable state in enteric bacteria has also been questioned by Bogosian et al. [29].

In addition to grazing by eukaryotic organisms, bacterial degradation by viral lysis may also participate in the complex regulation of bacterial populations. The number of free bacteriophage particles in coastal seawater may exceed 10^8 particles per ml [18, 264, 265] and the fraction of marine bacteria containing mature phage particles may range from 2 to 20% [94, 240]. Proctor and Fuhrman [190] estimated a mortality of 30 to 60% due to viral lysis from examination of sectioned marine bacteria. Bacteriophages have been described in the majority of bacterial genera, among which are various *Vibrio* spp. [14] and other fish pathogenic bacteria such as *Aeromonas salmonicida* [200], *Cytophaga* spp. [192], and *Flexibacter ovolyticus* [84] (Fig. 1). Chinese workers reported that vibriosis in milkfish (*Chanos chanos*) could be "controlled" by the bacteriophage AS10 [267], and thus suggested a possible means of biological moderation of a bacterial fish disease. So far, however, this approach has not been very successful.

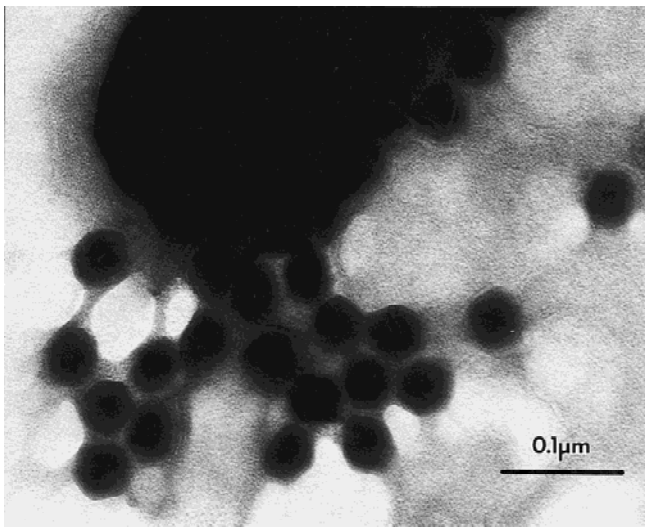

Fig. 1. *Flexibacter ovolyticus* NCIMB 13128 with free and attached phage particles. Prepared from a 4-day-old culture on Marine Agar (Difco). Bacterial growth were carefully suspended in particle-free (autoclaved and ultracentrifuged) distilled water, applied to carbon and Formvar-coated 400-mesh copper grids, air-dried, and stained in uranyl acetate (1.0%).

Strategies for growth, dispersal, and survival in seawater and at mucosal surfaces are essential for the establishment of a "normal" microflora as well as for opportunists and pathogens. Ruby and Morin [206] described how luminous enteric bacteria (*Photobacterium phosphoreum, Vibrio fischeri,* and *Vibrio* spp.), common members of the intestinal microflora of various seawater fishes, changed between an active, rapidly growing, and proliferating state in the nutrient-rich fish

intestine, and a less active, more oligotrophic way of life as members of the seawater bacterioplankton. Such transitions between "starvation survival" under oligotrophic conditions in the environment and a rapidly proliferating host adhesive state may be comparable to that of fish light-organ symbiosis [92], and similar shifts have also been described for fish pathogenic bacteria [57]. Quorum sensing—the phenomenon of low molecular weight signaling molecules eliciting population density dependent responses—may be involved in these transitions. This has been shown for *P. fischeri,* which does not emit bioluminescence when freeliving at low densities, but emits blue-green light at high densities as in the light organs of fish [241]. Bioluminescence in high-density populations of *P. fischeri* is dependent on accumulation of *N*-acyl-L-homoserine lactones (AHL) [241]. Conway et al. [44] found the mixed intestinal microflora to maintain its viability, with decreased cell volume and increased cell hydrophobicity, during starvation of the flounder (*Pseudopleuronectes* sp.), and it was inferred that members of the intestinal microflora possessed mechanisms for starvation survival.

In nature both the pelagic eggs of cod (*Gadus morhua*) and herring (*Clupea harengus*) eggs which are associated with seaweed in the tidal zone are exposed to relatively dense bacterial colonization and subsequent ongrowth. This situation becomes even more apparent for eggs incubated under intensive rearing conditions in aquaculture. Eggs of the Atlantic halibut (*Hippoglossus hippoglossus*), which normally spawns at depths of 200–300 m where the eggs may be exposed to bacterial direct counts in the order of 10^5 per ml [99], in aquaculture appear to be more sensitive to the effect of bacterial ongrowth as compared to eggs from other species that are distributed in the upper water layers. The gross effect of bacterial stress on yolk sac larvae depends on the maturity of the larvae at hatching and of the duration of the yolk sac stage, and may reflect the development of the immune system and the efficacy of nonspecific defense mechanisms of the particular species. Thus, halibut larvae, hatching in a very immature form with a yolk sac stage lasting for up to 50 days, appear to be very sensitive to the bacterial composition and load in the ambient water under intensive rearing conditions.

Bacterial Survival Strategies in the Marine Environment

Many fish pathogenic bacteria have developed strategies that allow them to survive for months or even years in water and sediment. Such survival strategies may help them to adapt to fluctuations in nutrient availability or to sustain life in extreme and variable environments. Stressed or starved cells may undergo morphological and biochemical changes resulting in dormancy—the viable but nonculturable state [40]. Dormant cells are viable and in most instances probably also virulent, but unable to grow on conventional culture media. A state of viable but nonculturable has been recognized for a variety of marine bacterial species, including *Vibrio cholerae* [40], *V. vulnificus* [175], other *Vibrio* spp. [147], and *A. salmonicida* [Enger, Ø. 1992. PhD thesis, Univ. of Bergen, Norway] [4]. Characteristics of dormancy may be the formation of ultramicrocells [9, 151], decrease in rRNA content [131], synthesis of starvation survival proteins [166], increased resistance to heat and UV radiation [166], and/or an increase in number of genomes [247]. Viable but nonculturable cells may be detected by direct viable count methods [128], using either fluorescence microscopy or flow cytometry.

Vibrios are readily isolated as dominant proportions of the culturable microflora from marine animals, seaweed, and plankton, and the number of marine vibrios has been observed to coincide with variations in planktonic particles in the sea [107, 185, 225]. During short-term starvation experiments *V. anguillarum* showed a greater survival potential when particles were present in the water [172]. A long-term starvation survival-potential has been described for *V. anguillarum, V. salmonicida* [100], and *Yersinia ruckeri* [247]. Both *V. anguillarum* and *V. salmonicida* were able to survive for more than 60 weeks in autoclaved 31‰ seawater at 6–8°C [100], and *Y. ruckeri* was reported to survive for 4 months in unsupplemented water at salinities ranging from 0‰ to 20‰ [247]. Although these experiments were performed under sterile conditions, thus excluding competition and predation, a high potential for survival in the environment may be implied for these fish pathogens. However, prolonged survival by *A. salmonicida* in various aquatic environments, including water, sediments, seaweeds, and various invertebrates, has been reported [54], and Enger et al. [58] detected *V. salmonicida* in fish farm sediments more than 18 months after an outbreak of cold water vibriosis.

The aim of this review is to elucidate various aspects of interaction among bacteria and developing fish eggs and larvae. Thus, establishment of a normal microflora and opportunistic and pathogenic interactions will be discussed. Emphasis, however, will be on the former types of interactions.

Host Defense Mechanisms

Fish spend all their life stages in an aqueous environment that allows intimate contact with a varied microflora, including pathogenic and opportunistic bacteria, that may colonize their various external and internal body surfaces. Thus, it is tempting to assume that the indigenous mucosal microflora of fish may play an equally important role in the "first-line defense" as that demonstrated for land-living vertebrates. The establishment of a normal or protective microflora is regulated by factors such as suitable receptors for adhesion and conditions for proliferation of the bacteria, and by nonspecific defense factors and specific immunity of the host. Both the outermost layer of the eggshell (the *chorion*) and the intestinal mucosa of fish possess receptors of a glycoproteinaceous nature, and species-specific differences in bacterial colonization of fish eggs have been observed [86]. Heterogeneity of the surface sugar moieties may probably exercise a selection pressure in the establishment of an indigenous microflora, which then in turn may act as a barrier against overgrowth by potentially harmful bacteria.

Nonspecific Defense Mechanisms in Eggs and Larvae

The presence of bacteria in fish eggs has provoked an interest in the presence of endogenous defense factors. Extracts from fish eggs may exhibit bactericidal activity [133], and immune molecules, such as immunoglobulins [68, 93, 118], are also present in fish eggs. Various agglutinating or lectin-like substances have also been demonstrated in fish eggs [116, 129, 182, 255, 259].

Lectins are a heterogenous group of glycoproteins with the capacity to agglutinate various cells and microorganisms by binding to carbohydrate ligands. Lectins are found in all organisms, from viruses and bacteria to mammals. They may act as recognition, adhesion, or signaling molecules. In invertebrates lectins may take part in defense against bacteria by acting as inducible opsonins that increase the rate of phagocytosis [170, 173], whereas in mammals various lectin-like molecules play complex roles in signaling and immune regulation [121]. It has been proposed that lectins participate in defense mechanisms against particulate pathogens in fish [28], although their roles are not fully understood. The presence of lectins has been reported in developing and mature eggs of various fresh- and seawater fishes, such as the *Salmonidae, Percidae, Cyprinidae,* and *Clupeidae* [10, 130, 133, 164, 165, 272], catfish (*Silurus asotus*) [105], and plaice (*Pleuronectes platessa*) [28]. Lectin activity has also been demonstrated in eggs from cod (*G. morhua* L.), wolffish (*Anarhichas lupus* L.), and Arctic charr (*Salvelinus alpinus* L.) [169]. Krajhanzl et al. [130, 164, 165] proposed that the agglutinating effect of lectins released into the perivitelline space, i.e., the space between the eggshell and the cytoplasmic membrane, would immobilize pathogens and prevent them from entering the egg at fertilization. The released lectins could also have a more general protective function in agglutinating bacteria at the egg surface, thus preventing them from colonizing the eggshell. Kudo and Inoue [133] reported presence of lectins in extracts of eggshells from fertilized eggs of rainbow trout (*Oncorhynchus mykiss*) and *Tribolodon hakonensis,* and they found these extracts to have a strong bactericidal effect on *Aeromonas hydrophila.* However, the exact functions of lectins in fish eggs are still disputed, and it has even been proposed that egg lectins are also involved in the fertilization process, preventing polyspermy [165].

Many lectins from invertebrates and upwards are heterogeneous, multiunit molecules that may exert a range of ligand-binding activities; for a review, see Olafsen [169]. Some lectins are functionally and structurally related to members of the pentraxin-family of acute-phase proteins, C-reactive protein (CRP), and serum amyloid P component (SAP). Pentraxin-like proteins are evolutionary conserved and are found from the *Mollusca* [253] to mammals. They have also been reported in several teleost and elasmobranch fish species such as plaice (*P. platessa* L.) [186], rainbow trout (*O. mykiss*) [112], channel catfish (*Ictalurus punctatus*) [242], Atlantic salmon (*Salmo salar* L.), cod (*G. morhua*), halibut (*H. hippoglossus*), wolffish (*A. lupus*) [139], and dogfish (*Mustelus canis*) [199]. In eggs of the lumpsucker (*Cyclopterus lumpus*), an extremely high level of CRP has been reported [61], and CRPs have also been demonstrated in eggs of *Channa punctatus* [149]. The literature concerning acute phase proteins in fish have recently been reviewed, but the biological functions of the pentraxins are still not fully understood [111].

Lysozyme, either alone or in conjunction with complement and specific antibodies, exerts an important bacteriolytic mechanism in higher vertebrates and has also been demonstrated in various tissues and in mucus secretions of fish [63, 70]. Yousif et al. [271, 273] have reported occurrence of high levels of lysozyme in eggs of various salmonids, coho salmon (*Oncorhynchus kisutch*), chinook salmon (*O. tshawytscha*), and rainbow trout (*O. mykiss*). They propose that the lysozyme activity, which has effect against both Gram-positive and Gram-negative fish pathogenic bacteria,

may explain why only certain bacterial fish pathogens are transmitted vertically from mother to progeny. Thus, the presence of lysozyme in eggs of new fish species for aquaculture might reveal important information regarding disease resistance.

In addition to the above-mentioned lectins, lectin-like molecules, and lysozyme, macrophages and various types of lysins and agglutinins are reported to play important roles in the nonspecific defense in larval and juvenile fish [62, 246]. In rainbow trout, active endocytic macrophages are found in the gills and connective tissues of the skin and gut of 4-day-old larvae [81]. Thus, macrophages may provide the larvae with an effective means by which pathogens are prevented from entering systemic circulation. Whether other known nonspecific defense mechanisms such as peroxidase, catalase [61], chitinase [70, 79], transferrin and lactoferrin [3], trypsin-like proteases [31, 97], or interferon [61], which are reported to occur in skin mucus and in various tissues of adult fish, are present and active in the egg and larval stages is presently unknown.

Immunological Aspects of Host–Microbe Interactions

Nonspecific mechanisms constitute the most significant part of egg and larval defense, because of the immature state of the immune system at these stages [62, 145]. Passive immunity, in the form of transmission of maternally developed immunoglobulins against various antigens, has been demonstrated in eggs of tilapia (*Oreochromis aureus* and *O. mossambicus*) [150, 244], carp (*Cyprinus carpio*) [256], channel catfish (*I. punctatus*) [93], Atlantic salmon (*S. salar*) [136], plaice (*P. platessa*) [28], and red seabream (*Pagrus major*) [117, 118] and may serve as protection in early life stages in at least some fish species. In chum salmon (*Oncorhynchus keta*) the egg immunoglobulin was found to be of a peculiar IgM-related type, serologically identical to serum IgM, but with a smaller H chain [68]. A more curious observation by Hildemann [96] was that newly hatched larvae of the Amazon discus fish (*Symphysodon discus*) fed on mucus derived from the parental skin, suggested as a means of securing the larvae passive immunity from their parents, while in Midas cichlids (*Cichlasoma citrinellum*), feeding on parental mucus was claimed to furnish the larvae with growth hormones [220].

Immune competence, i.e., the ability to produce specific antibodies to introduced antigens, appears to be related more to weight than to age, or more precisely to a critical number of immune competent cells. Grace et al. [81] have reported immune competence in 4-day-old yolk sac larvae of trout, in which they demonstrated an effective system for particle trapping by macrophages under the skin and on the gills before maturation of the lymphoid tissues. At what developmental stage larvae of marine, cold-water fish are able to elicit an immune response to antigen stimulation is uncertain. There are great species differences in immune development, and factors such as temperature, time from fertilization until hatching, and duration of the yolk sac stage are important. A specific immune system is probably not fully mature until several weeks after hatching [56]. The fact that fish eggs hatch and develop into healthy larvae and juveniles in an environment often sustained with high numbers of potential pathogenic microorganisms supports the assumption that larvae have to "recognize" antigens at a very early stage, resulting in immune tolerance, which is necessary for the establishment of a protective microflora.

Ingestion of bacteria by fish larvae may be of immunological importance by presenting antigen determinants to the developing immune system [48, 204]. This may result either in antigen priming or in development of immune tolerance to the sequestered bacterial strains, thereby aiding the establishment of an indigenous microflora. In juvenile and adult fish, local mucosal and secretory immune responses are important in protection against bacterial pathogens [89, 90, 250], but at what developmental stage these kinds of defense mechanisms start to function is not yet clear. It has been suggested that absorptive enterocytes in the intestinal epithelium may function as an antigen-sampling device, thereby presenting antigenic determinants to intraperitoneal lymphoid cells [48, 201, 203]. It is thus possible that the observed endocytosis of bacterial antigens in intestinal enterocytes of cod (*G. morhua*) and herring (*C. harengus*) larvae [87, 174] are involved in stimulation of a developing immune system.

Although mucosal accumulations of lymphoid cells, the so-called Peyer's patches, have not been described in fish, all cells necessary for a local or mucosal immune response appear to be present in the intestinal mucosa [50, 90, 202]. In yolk sac larvae of cod, uptake of bacterial antigens has also been demonstrated in the foregut by immunohistochemistry [174]. Bacterial antigens were demonstrated in columnar epithelial cells of the developing foregut. Some degree of specificity among the test bacteria used (*V. fischeri, V. salmonicida,* and a *Flavobacterium* sp.) was observed, i.e., with a preferential uptake of *V. fischeri* and *V. salmonicida* as compared to *Flavobacterium* sp. [174]. It appears evident that the early establishment of an intestinal microflora in

close association with the gut mucosa will affect larval immune development.

Adhesion of Bacteria to Mucosal Surfaces

The epidermal mucus layer constitutes the primary biological interface between fish and the aqueous environment. The mucus coat may be an adhesion site for bacteria [43, 228], but it has also been suggested that it may function to prevent firm attachment of bacteria to the skin [45]. In halibut (*H. hippoglossus*) larvae, the number of mucous cells increases, and the chemical composition of the mucus changes, during development from pelagic larvae to bottom-dwelling flatfish [181], and such changes may affect bacterial adhesion and colonization. The mucus coat forms the primary barrier against infection [109, 187, 188], and it has been demonstrated that fish skin mucus has inhibitory effects on bacteria [64, 88]. Various defense factors in the mucus, such as immunoglobulins, complement, lysozyme, and agglutinins, may aid in protection [62, 88], and the continuous shedding of mucus may defer microbial colonization. In the rearing of fish, traumatic skin damages or erosion of the mucous layer may impair the first-line defense, and inadequate mucus shedding may result in bacterial proliferation on the body surface. In yolk-sac larvae of halibut (*H. hippoglossus*) a positive correlation has been demonstrated between the bacterial load in the water and the number of saccular, mucus-producing cells in the epidermis [181].

Bacterial adhesion to mucosal surfaces is a crucial stage in the establishment of a protective "normal" microflora, and also the first step in an infective process. Various factors may influence the processes of adhesion and colonization, reflecting properties related to the bacteria, to the epithelial cell surface, and to exogenous factors in the microenvironment in which the process occurs. The first major step in the adhesion process is the chemotactic attraction of bacteria to the surface of the mucus [67]. This is followed by penetration and trapping within the mucus either passively or actively promoted by bacterial motility and chemotaxis. Eventually adhesion to receptors in the mucus or the epithelial surface occurs [65]. These reactions may be modified, either enhanced or reversed, by the action of adhesion inhibitors, enzymes, or lectins. An infective process eventually proceeds by action of secreted bacterial toxins or exoenzymes.

Microbial Adhesion to Cells and Tissues

The adhesion process and mechanisms involved are well described for various mammalian systems, but few reports have described adhesion of bacteria to mucosal surfaces of fish [11, 102, 114, 132, 215]. Most of these appear to be descriptive, rather than elucidating mechanisms of adhesion. Krovacek et al. [132] reported the presence of adhesive factors in the adhesion of *V. anguillarum* and *A. hydrophila* to cultured fish cells, rainbow trout liver cells and chinook salmon embryo cells, and to mucus-coated glass slides. Balebona et al. [11] examined the influence of salinity and pH on the adhesion of *Vibrio* spp. to *Sparus aurata* skin mucus and suggested that receptor-specific interactions were involved in adhesion processes. In vitro adhesion of *V. anguillarum* to rainbow trout gut sections was reported by Horne and Baxendale [102], who reported adhesion to be more predominant in the upper and midgut sections than in the esophagus, stomach, and lower gut. However, in vivo experimental challenge did not reveal differences in adhesion to the various gut segments, but in vaccinated fish adhesion of *V. anguillarum* to the midgut region was significantly lower than in nonvaccinated fish. This difference may probably be accounted for by secreted mucus antibodies, as observed in the inhibition of *V. cholerae* adhesion to rabbit intestinal tissues by antibodies to heat-stable *V. cholerae* antigens [66].

Cell surface hydrophobicity is an important factor in the adhesion process of various pathogenic bacteria [1, 34, 101, 184], but its importance has been questioned for fish pathogens such as *Aeromonas sobria, A. hydrophila, A. salmonicida, V. anguillarum,* and *Y. ruckeri* [215]. Of the 42 strains tested by Santos et al. [215], 78% were pathogenic for fingerling rainbow trout, but hydrophobicity was weak or absent in all *Y. ruckeri* strains and only moderate in the *Aeromonas* and *Vibrio* isolates. This is in accordance with Balebona et al. [11], who found that cell surface hydrophobicity was not related to the adhesion abilities of *Vibrio* spp. to *S. aurata* skin mucus. However, they found a strong positive correlation between the ability to form pellicles in liquid broth and adhesion to skin mucus. Adhesion of various strains of pathogenic and nonpathogenic bacteria isolated from fish to cryosections of mucosal surfaces of Atlantic salmon (*S. salar*) has been studied [127]. The majority of strains tested, both pathogenic and nonpathogenic, were adhesive to mucus in all epithelial surfaces. In contrast, *V. anguillarum* serotype O2 were not adhesive to mucus, but bound well to all other tested parts of the tissues. Hence, adhesion to mucus was not restricted to pathogens and was not demonstrated for all pathogenic strains.

Receptor-specific interactions, i.e., specific recognition mediated by cell surface sugars of the host, are well documented from mammalian models. Colonization of the small

intestine of mammals by enterotoxigenic *Escherichia coli* is mediated by pili-like structures that exhibit adhesins and colonization factor antigens [60]. These lectin-like surface structures may alter the surface properties of the cells, and the hydrophobicity of enteric organisms may be a function of type and number of pili on the cell surface [60]. Thus, *E. coli, Salmonella typhi* and *V. cholerae* bind specifically to sugar residues on mammalian cell surfaces [167], and in vitro attachment of *V. cholerae* to rabbit intestinal epithelial cells is mediated by a bacterial lectin with specificity for *N*-acetyl-D-glucosamine [217]. Chen and Hanna [38] reported *V. anguillarum, V. ordalii,* and *V. parahaemolyticus* to adhere to various cells of rainbow trout. For all three bacterial species tested, adhesion was inhibited by incubating the bacteria with monoclonal antibodies against the respective bacterial strains, indicating that receptor sites on the bacterial surface were blocked. Such studies indicate that blocking of adhesion may be a suitable way of preventing pathogen colonization. Experiments with uptake of intact bacterial antigens in cod larvae [174] revealed a preferential uptake of *V. fischeri* and *V. salmonicida* as compared to *Flavobacterium* sp., thus indicating specificity in the bacteria–epithelium interaction. Bacterial lipopolysaccharides (LPS) have been demonstrated to take part in host adhesion for a variety of mammalian pathogens. We have found that LPS, and in particular its carbohydrate moiety, is involved in the adhesion of marine vibrios. The great variation in the carbohydrate proportion of LPS may reflect variation in adhesive properties among various *Vibrio* strains [127]. Thus, adhesion specificity may be an important mechanism in colonization of mucosal surfaces in fish.

Colonization of External Surfaces

Reports that describe microbial colonization of mucosal surfaces of gills and integument of healthy fish are relatively sparse, and little information covers the early life stages of marine fish (Fig. 2). Yoshimizu et al. [270] examined the surface of yolk sac larvae of salmonids (*Oncorhynchus masou* and *O. keta*) and found that the bacterial flora was mainly composed of *Pseudomonas, Flavobacterium/Cytophaga,* and *Achromobacter.* The microflora was to some extent affected by the bacterial flora in the water. In yolk sac larvae of halibut (*H. hippoglossus*), a morphological diverse bacterial flora has been demonstrated by means of scanning electron microscopy (SEM) [189].

In adult marine fish of various species a variety of bacterial genera and species have been isolated from slime and external surfaces [5, 103, 104]. These include members of *Pseudomonas, Vibrio, Achromobacter, Flavobacterium/Cytophaga, Moraxella, Micrococcus, Acinetobacter,* and *Aeromonas.* For a review, see [36, 209]. Austin [5], in a survey of bacteria isolated from a coastal marine fish rearing unit, revealed bacteria from the skin of healthy turbot (*Scophthalmus maximus*) to exhibit greater taxonomic diversity than both the incoming and released seawater and the slime covering the inside of the incubators. *Photobacterium angustum, Vibrio logei, Alcaligenes faecalis, Pseudomonas fluorescens,* and *Bacillus firmus* were isolated exclusively from the surface of healthy turbot, and this skin microflora was distinct from the bacterial flora in the ambient water. On the external surfaces of freshwater fish the same taxonomic bacterial groups seem to prevail, with *Aeromonas* being more and *Vibrio* less abundant [45, 103, 104, 208].

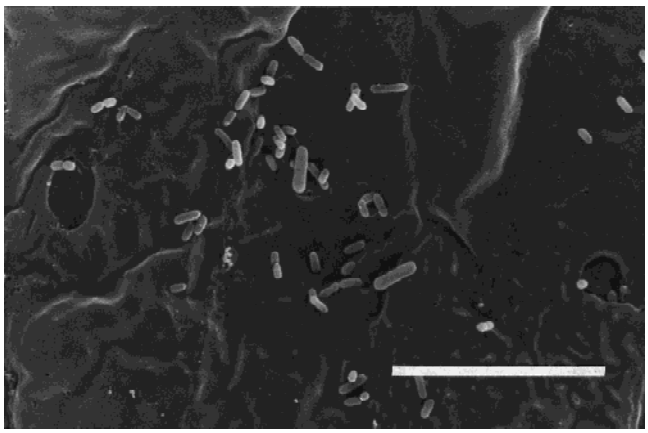

Fig. 2. Diverse bacterial colonization at the ventral surface of a cod larva 0.5 h after hatching. Fixation and preparation as described in Fig. 3. Bar = 10 μm.

Discrepancies are reported between quantitative bacterial determination and examination of the skin surface by means of SEM [36, 45, 119]. Healthy skin surfaces often appear devoid of bacteria, although cultured samples from the same skin area give rise to high numbers of CFU. However, bacterial colonization and propagation may be readily detected in skin ulcerations by SEM. It is likely that bacteria are trapped or closely associated with the skin mucus and will therefore be washed off in preparation for SEM. However, bacteria tightly associated with the skin of fast-swimming fish have been examined for their hydrophobicity and ability to produce drag-reducing hydrophobic slime [22, 216], thus suggesting a hitherto undescribed ecological niche in which bacteria assist in fish locomotion by secreting drag-reducing slime.

The bacterial microflora of gill surfaces of turbot (*S. maximus*) has been examined by Mudarris and Austin [154]. They found the gill microflora to be distinct from the microflora in the ambient water and composed of the following major genera and species: *Janthinobacterium lividum, Vibrio* spp., *Hyphomicrobium, P. fluorescens, Asticacaulis* spp., and *Prostecomicrobium.* Many of the isolated bacterial types possessed attachment structures and the bacteria tended to colonize protected niches rather than exposed surfaces on the gill filaments. Colonization of gill surfaces in freshwater fish, mostly salmonids, has been reviewed by Cahill [36].

Microbiology at the Egg Stage

The eggshell consists of a thick lamellar inner layer, *zona radiata,* and a thinner outer layer, *chorion* or *zona pellucida.* The thickness and lamellar structure varies between teleost species; generally the eggshell of pelagic eggs is thinner (typically 2.5 to 10.0 μm) than demersal eggs (typically 15 to 60 μm) [140, 141]. The chorion and zona radiata of marine fish eggs are composed of a highly hydrophobic protein aggregate, with the main component *ichthulokeratin,* a pseudokeratin resembling fibrin, and glycoproteins with 2 to 3% carbohydrate dominated by galactose, mannose, *N*-acetylglucosamine, and *N*-acetylneuraminic acid (Oppen-Berntsen, D.O., 1986. MSc thesis, Univ. of Bergen, Norway) [179]. The glycoproteinaceous nature of the eggshell is well suited for adhesion and colonization by bacteria. The eggshell is permeable to water, gases, and inorganic nutrients [47, 194]. The osmotic barrier is the perivittelin membrane situated beneath the zona radiata. The fish embryo will secrete inorganic and low molecular weight organic compounds which diffuse through the eggshell and establish a gradient outside the eggshell. This gradient may act as a chemoattractant for bacteria able to utilize the secreted compounds. On the basis of the egg surface sugar moieties, and through the phases of adsorption, adhesion, and colonization, a primary microflora will eventually become established on the egg surface. A review on factors affecting the quality in fish eggs has been published recently by Brooks et al. [32].

It has been known for decades that fish eggs, as well as other biotic and abiotic submersed surfaces, are readily colonized by bacteria (Fig. 3). As early as in 1919, Dannevig demonstrated bacterial growth on cod eggs resulting in high mortality [46]. He described a filamentous bacterium, probably *Leucothrix mucor,* which at present also causes problems in cod hatcheries. Since then researchers have been aware of the positive correlation between bacterial growth, not necessarily of pathogens, and increased mortality [180, 223]. SEM of cod (*G. morhua*) eggs collected from surface waters revealed that major areas of the egg surface were colonized by large rod-shaped bacteria [86]. In contrast, SEM of halibut (*H. hippoglossus*) eggs collected from spawning depths of 200–250 m revealed eggs almost devoid of bacteria (Hansen, Olafsen, and Haug, unpublished data). However, published information concerning taxonomy of the bacterial epiflora of marine fish eggs is still scanty [86, 122, 152].

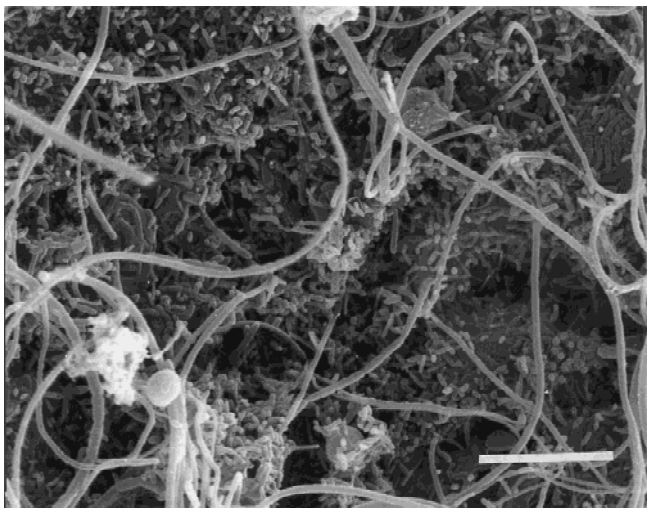

Fig. 3. The surface of a cod egg close to hatching. The egg was incubated in sand-filtered seawater and was heavily overgrown by bacteria at the time of hatching. The long fungus-like threads are filaments of *Leucothrix mucor.* Eggs were fixed in formaldehyde–glutaraldehyde (2.5 and 2.0%, vol/vol, respectively), postfixed in osmium tetroxide (1.0%), dehydrated in ethanol, critical-point dried, and coated with gold–palladium. Bar = 10 μm.

Colonization and bacterial growth on fish eggs pose great problems for the rearing of several marine fish species. The intensive incubation techniques, often resulting in eggs overgrown by bacteria, may affect the commensal relationship between the indigenous microflora and opportunists, and subsequently hamper egg development, hatching, larval health, and ongrowth. Knowledge of the adherent bacterial epiflora on fish eggs is sparse, and most published papers are concerned with eggs of salmonids [12, 13, 15, 16, 39, 249]. The microbial community of the ambient environment of developing fish eggs will indisputably influence the composition of the bacterial egg epiflora (Figs. 4 and 5). It is likely that the glycoproteinaceous nature of the eggshell will selectively favour colonization of specific bacterial groups. This is

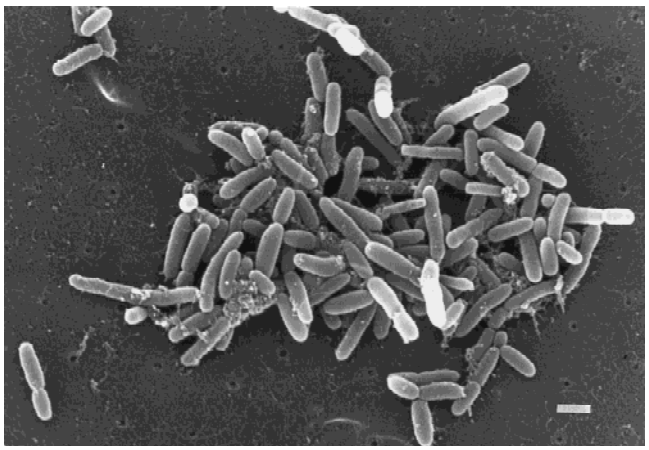

Fig. 4. Bacterial colonization on the surface of a cod egg. Eggs were aseptically dissected from the ovaries, fertilized under sterile conditions, and incubated in a diluted culture of *Vibrio fischeri* (strain TEE 005) for 135 h. After incubation the eggs were washed in sterile filtered seawater (0.22 μm), fixed, and treated as described in Fig. 3. Bar = 1.0 μm.

to some extent reflected in the species–specific differences found between bacterial groups colonizing cod and halibut eggs [86]. It has been demonstrated that the primary egg epiflora may alter the egg surface, probably by exoenzymatic activity, resulting in a very homogenous secondary colonization when the primary epiflora has been removed [86]. It is tempting to assume that a large variation in surface receptors acting as adhesion sites for a varied microflora on the egg surface could act as a barrier against potential pathogenic bacteria and thus be part of the "first-line" defense at the egg stage.

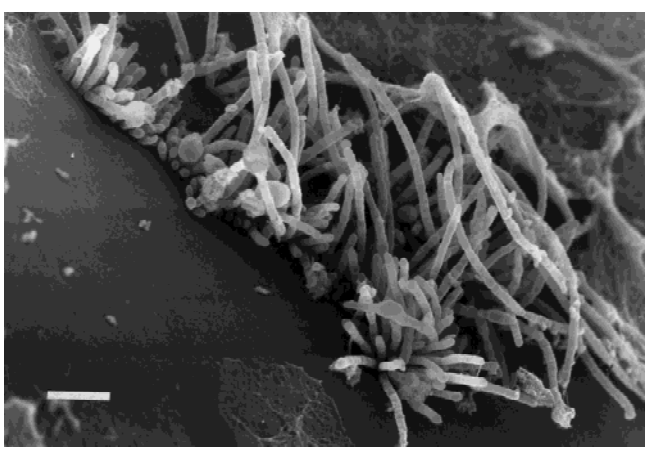

Fig. 5. *Leucothrix mucor* colonizing the surface of a cod egg. Eggs were incubated in a diluted culture of *L. mucor* for 140 h and treated as described in Fig. 3. Bar = 10 μm.

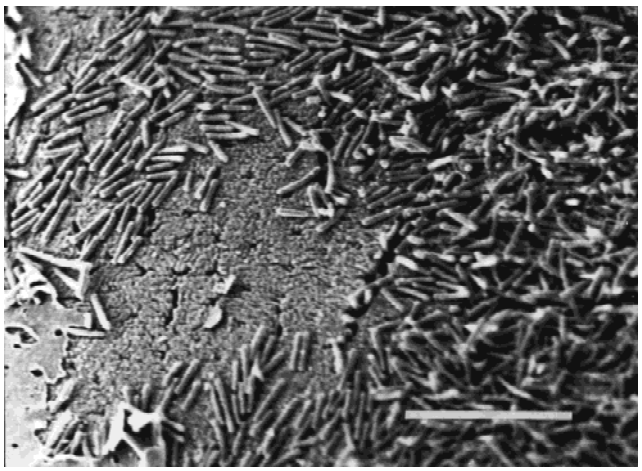

Fig. 6. Scanning electron micrograph of the surface of a halibut egg demonstrating ulceration due to colonization by *F. ovolyticus.* Remains of the outermost layer of the eggshell, the chorion, are shown to the left, and severe attack on the inner part of the eggshell, the zona radiata, to the right. Fixation and preparation as described in Fig. 3. Bar = 10 μm.

Bacterial colonization may have adverse effects on the egg and on the developing embryo. It is evident that colonization of the egg surface by pathogens or opportunists may be detrimental and cause disease of eggs (Fig. 6) [84] or larvae [19, 20]. Additionally, the bacterial epiflora may cause problems other than disease for the developing embryo. Massive overgrowth by obligate aerobic bacteria may result in hypoxia in the developing embryo. However, at an early embryonic stage the oxygen demand is partly covered by the oxygen stores in the yolk and perivitelline fluid, and anoxic external conditions are unlikely to affect embryonic development at this stage. As the oxygen demand increases toward hatching, the effect of oxygen deficiency may result in accumulation of lactic acid, retarded development, and possible neural injuries [95]. Oxygen deficiency is probably the major cause of death in cod eggs heavily colonized by the obligate aerobic, filamentous *L. mucor,* which is not known to penetrate the eggshell or to produce exotoxins or toxic metabolites [85, 113]. In some fish species hatching is induced as a result of insufficient oxygen supply, whereas in halibut hypoxic conditions seem to have the opposite effect, namely hatching delay (Helvik, J.V. 1991. PhD thesis, Univ. of Bergen, Norway). Since the oxygen concentration is critical for successful hatching, bacterial overgrowth on the egg surface may also affect hatching. Moreover, exoproteolytic enzymes released by the adherent bacterial epiflora may damage the chorion [86] or even destroy zona radiata, resulting in release of hatching enzymes or leakage of cell

constituents [19, 84]. Eventually, bacterial exotoxins or toxic metabolites, e.g., NH_3 and H_2S, released by the epiflora may harm the developing embryo [12]. In marine fish eggs a negative correlation has also been demonstrated between bacterial colonization and the physical strength of the eggshell [125].

Major Bacterial Groups Colonizing Fish Eggs

The adherent microflora of cod and halibut eggs from various hatching facilities in western and northern Norway have been examined [84, 86]. The egg epiflora of both cod and halibut eggs were dominated by members of the genera *Pseudomonas, Alteromonas, Aeromonas,* and *Flavobacterium.* Members of the genus *Vibrio* were present only in minor amounts. *V. fischeri* was isolated from cod eggs, while *V. anguillarum* was not detected. *Moraxella* and *Alcaligenes* spp. were isolated only from halibut eggs. Species of *Cytophaga* and *Flexibacter,* and *L. mucor,* were detected on eggs of both fish species. There were marked differences in the growth of *L. mucor* on the two species of fish eggs. On cod eggs, massive growth of *L. mucor* gave rise to eggs with a macroscopic "hairy" appearance, while *L. mucor* was scarcely detectable on the surface of halibut eggs, possibly indicating differences in adhesion sites on the egg surface. The universal tidal zone epibiont, *L. mucor,* has also been isolated from the eggs of winter flounder, *Pseudopleuronectes americanus* [113]. On both cod and halibut eggs the incidence of known opportunistic pathogens was sparse. Bolinches and Egidius [30] found *Actinomyces*-like bacteria and *Flavobacterium, Vibrio, Pseudomonas–Moraxella,* and *Photobacterium* spp. to prevail in a halibut rearing unit, whereas in larval incubators *Actinomyces*-like bacteria proved to be the overall component of the bacterial community. In a turbot (*S. maximus*) brackish water hatchery, Keskin et al. [122] isolated *A. hydrophila, Moraxella* sp., *Pseudomonas aeruginosa,* and *P. fluorescens* from turbot eggs. In a taxonomic survey of bacteria in a marine fish-rearing unit, Austin [5] found *Vibrio* spp., members of the *Cytophaga/Flexibacter* and *Cytophaga/Flavobacterium* groups, *Acinetobacter calcoaceticus,* and *P. phosphoreum* to be the dominant bacteria. The egg epiflora, however, was not examined by Bolinches and Egidius, or by Austin. The prevailing bacterial groups encountered in the epiflora of marine fish eggs are presented in Table 1. Somewhat unexpectedly, members of the genus *Vibrio* were not among the dominating bacterial groups in the epiflora of egg species so far examined.

Table 1. Major bacterial genera/species demonstrated in the epiflora of marine fish eggs

Fish species	Bacteria	Reference
Cod (*Gadus morhua*) and Atlantic halibut (*Hippoglossus hippoglossus*)	*Pseudomonas* spp. *Alteromonas* spp. *Aeromonas* spp. *Flavobacterium* spp.	86, 152
Turbot (*Scophthalmus maximus*)	*Aeromonas hydrophila* *Moraxella* sp. *Pseudomonas aeruginosa* *P. fluorescens*	122

The percentage of pigmented bacteria appear to vary among eggs of different fish species. Bacterial pigment production is an important means by which bacteria are protected against harmful ultraviolet radiation [52, 163]. Norkrans [163] reported that 95% of the bacteria isolated from marine surface microlayers were pigmented and that carotenoid pigments containing more than eight conjugated double bonds were the most effective in protection against biologically active UV (UV-B) radiation. The ratio of pigmented bacteria decreases downward in the water column. Pigmented bacteria (mainly yellow and orange pigments) were found to constitute about 40%, 30%, and 16% of the bacterial isolates from eggs of herring, cod, and halibut, respectively (Hansen and Olafsen, unpublished data). Cod eggs and eggs of the herring strain used (Balsfjord-strain) [124] are distributed in the surface and tidal zone, respectively, and are thus exposed to high levels of UV radiation. Halibut eggs, however, are normally situated at depths of 200 to 250 m [91] and are hence exposed to minimal levels of UV radiation. Adaptation to surface conditions with elevated UV radiation plays an important ecological role in nature and is probably also reflected in the colonization of egg surfaces.

Eggs in incubators for aquaculture will often be more exposed to bacterial colonization than most eggs in nature, although there is a great potential for microbial growth in natural spawning areas due to the ample amounts of male gonad materials released [53], and the composition and nature of the ambient microflora may vary widely. There also appear to be species-specific differences in adhesion sites between eggs from different fish species. In conclusion it appears that the relatively dense, nonpathogenic adherent bacterial epiflora on the eggs characterized by high diversity most likely will function as an effective barrier against possible pathogens found in the ambient water.

Table 2. Bacterial genera/species reported to occur inside fish eggs (intraovum infection)

Fish species	Seawater or freshwater	Bacteria	Reference
Cod (*Gadus morhua*)	seawater	*Enterobacteriaceae* spp.	Hansen, unpubl. data
Atlantic halibut (*Hippoglossus hippoglossus*)	seawater	*Corynebacterium-Nocardia-Mycobacterium* spp.	Hansen, unpubl. data
Atlantic salmon (*Salmo salar*), rainbow trout and coho salmon (*Oncorhynchus mykiss* and *O. kisutch*)	freshwater	*Renibacterium salmoninarum*	35, 59
Rainbow trout (*O. mykiss*)	freshwater	*Lactobacillus* sp.	42
		Aeromonas spp.	108
Chinook salmon (*Oncorhynchus tshawytscha*)	freshwater	*Gram negative: Aeromonas hydrophila, Pseudomonas* spp., *P. fluorescens, Vibrio fluvialis, V. parahaemolyticus, Enterobacter* spp., *Hafnia alvei,* and *Serratia liquefaciens* *Gram positive: Listeria* sp., *Staphylococcus aureus, Corynebacterium hoffmannii, Bacillus* sp., and *Aerococcus viridans*	218
Rainbow and brown trout (*O. mykiss* and *Salmo trutta*)	freshwater	*Pseudomonas* sp. *Aeromonas hydrophila*	12
Steelhead trout (*O. mykiss*)	freshwater	*Flavobacterium psychrophilum*	33

Intraovum-Infected Fish Eggs

Vertical transmission and intraovum infections have been suspected for fish pathogenic viruses such as IPNV and IHNV. Until the mid-1980s, the only bacteria reported to occur inside fish eggs were *R. salmoninarum* in salmonid eggs [59] and a *Lactobacillus* sp. in eggs of diseased, post-spawning rainbow trout (*O. mykiss*) [42]. In 1987 Sauter et al. [218] reported the presence of bacteria from several genera inside eggs of chinook salmon (*Oncorhynchus tshawytscha*). Both established pathogens and bacteria not known to cause disease were demonstrated, including members of the genera *Vibrio, Listeria, Corynebacterium,* and *Staphylococcus.* In addition, *Enterobacter* spp., *Pseudomonas* spp., and *A. hydrophila* were present. The authors were unable to demonstrate a positive correlation between the presence of any specific of the above-mentioned bacterial species and increased mortality rate, but reported members of the genera *Listeria, Corynebacterium,* and *Staphylococcus* to occur more frequently in high-mortality egg groups. From the work of Sauter et al. [218], a very high frequency of intraovum infection may be calculated, 40% to 100%, while Barker et al. [12, 13] only reported a 3% to 8% incidence of intraovum infection in eggs of rainbow trout (*O. mykiss*) and brown trout (*Salmo trutta*). They did, however, examine single eggs, and the results were not correlated to individual females. Recently, Brown et al. [33] have demonstrated vertical transmission of the causal agent of bacterial coldwater disease, *Flavobacterium psychrophilum,* from broodstock females to eggs of steelhead trout (*O. mykiss*).

The presence of intraovum bacteria has also been demonstrated in unfertilized eggs of cod and halibut (Hansen, unpublished data), revealing an incidence of 16% and 20% in cod and halibut eggs, respectively. Bacteria belonging to the *Enterobacteriaceae* were isolated from cod eggs and *Corynebacterium–Nocardia–Mycobacterium* from halibut eggs. No positive correlation was demonstrated between the presence of intraovular bacteria and bacteriemia of the mother fishes. The infected halibut eggs were followed through fertilization, hatching, and larval metamorphosis, and they developed normally. However, long-term effects were not examined. Hatching and development of the infected cod eggs were not monitored. The presence of intraovular bacteria in halibut eggs indicates that problems associated with poor egg quality may arise from this kind of infection. Bacterial groups reported to occur inside fresh- and seawater fish eggs are presented in Table 2.

Bacteria have also been demonstrated on the surface of unfertilized cod eggs, aseptically dissected from the ovaries [86]. A *Caulobacter* sp. and a *Seliberia*-like bacterium have been visualized by means of SEM. It is not clear in what way or at what developmental stage eggs are infected in the ovaries, but the assumption that unshed eggs are sterile [248] does not seem to be valid.

In conclusion, it is clear that many of the problems associated with intensive rearing of early life stages of marine

fish are due to bacteriological activities, and it is evident that both the community composition and the bacterial numbers in egg incubators are of importance. It is therefore crucial not only to keep strict control on pathogens and opportunists, but also to keep the total bacterial load at a minimum. In order to control bacterial colonization of the eggs, the search for probiotics, i.e., "harmless" bacteria that may restrain the adhesion of potentially harmful bacteria, should be encouraged. The presence of intraovum-infected eggs in both cod and halibut—although the importance of such infections is not clear—should encourage routines for managing healthy broodstocks of marine fish, and monitoring eggs not only for viral agents (e.g. IPNV), but also for the occurrence of intraovular bacteria.

Microbiology at the Larval Stages

In intensive rearing the early life of fish larvae takes place in incubators with hatching eggs, debris, and released inorganic and organic substances, which provides suitable substrates for bacterial growth. This may result in a 10^3-fold increase in the bacterial counts of the water in the incubators, measured as CFU per ml [86]. In order to osmoregulate, fish larvae start "drinking" before the yolk sac is consumed [144, 193, 254], and bacteria thus enter the digestive tract before active feeding commences. It has been demonstrated that newly hatched yolk sac larvae of cod ingest substantial amounts of bacteria and that older larvae graze on eggs and debris [171, 174]. It is therefore evident that the egg microflora will be an important factor in the first establishment of an indigenous larval microflora. Active ingestion of suspended bacteria has also been demonstrated in fry of the common carp (*C. carpio*) [24] and tilapia (*Oreochromis niloticus*) [23]. The incubation conditions, with large biomasses per volume of water, are exposing the yolk sac larvae to dense bacterial populations. Viable counts of the epiflora of halibut eggs just prior to hatching have been found to be in the order of 2×10^2 to 8×10^4 bacteria per egg (Hansen and Olafsen, unpublished data). In turbot (*S. maximus*), Keskin et al. [122] reported a mean total bacterial count of 3.7×10^6 per egg the day before hatching. In these kinds of intensive rearing systems, the number of bacteria entering the larval digestive tract will probably be higher than in nature, at least with respect to pelagic eggs and larvae.

Establishment of an Intestinal Microflora

Fish larvae are hatched into and participate as a part of the same ecosystem as opportunistic bacteria that cause serious diseases in marine aquaculture, e.g., *Vibrio* spp. [41, 55]. The functions of the indigenous intestinal microflora are well understood in warm-blooded animals. In fish the roles or even existence of an indigenous intestinal microflora have been disputed [221, 266]. However, it now appears accepted that a primary transient microflora will become established at the larval stage and develop into a persistent flora at the juvenile stage or after metamorphosis (Fig. 7). As in higher vertebrates the intestinal microflora must adapt to varying conditions of nutrient composition, pH, anaerobiosis, concentration of bile salts and digestive enzymes, the host's immune system, and the mutual influences of other members of the intestinal bacterial community. Complex interactions exist between the host and the intestinal microflora, allowing some bacterial strains to become established, while others are digested or expelled, or may bring about infection. In turbot (*S. maximus*) larvae challenged with *V. anguillarum*, Grisez et al. [83] demonstrated that the route of infection was through endocytosis in the gut epithelium. In addition to the ability to cope with the above-mentioned physiological factors in the intestine (e.g., capsule or slime production), members of the intestinal microflora will have to adapt to extremely different conditions—fluctuating between the nutrient-rich intestinal tract and the compar-

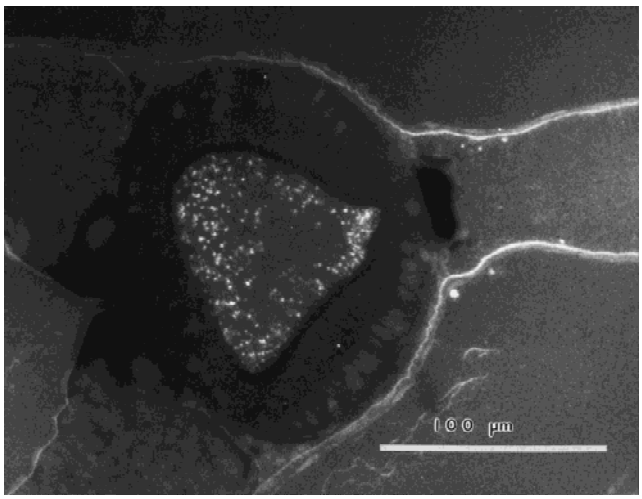

Fig. 7. Cross-section through the anterior part of the hindgut of a 14-day-old herring larva kept in sand-filtered seawater, demonstrating fluorescent bacteria in the lumen and in close association with the microvilli layer. The larva was fixed in formaldehyde–glutaraldehyde (2.5 and 2.0%, vol/vol, respectively), postfixed in osmium tetroxide (1.0%), dehydrated in ethanol, and embedded in Epon–Araldit. Semithin sections were double-stained with 4′,6-diamidino-2-phenylindole (DAPI) and primulin. Fluorescence microscopy.

atively oligotrophic ambient seawater. It has been shown that luminous bacteria, e.g., *P. phosphoreum, V. fischeri,* and *V. logei,* are commonly present in the intestinal tracts of many marine fishes. They are capable of extensive proliferation in the gut and on fecal particles before subsequent distribution into the water [206]. Thus, these bacteria seem to be well adapted for the transition between the intestinal tract and a freeliving mode of life.

Because of the immature nature of the immune system in fish larvae, they have to rely on nonspecific defense mechanisms, of which a protective intestinal microflora intimately associated with the gut mucosa will constitute a primary barrier. An understanding of the characteristics or features and the role of the indigenous microflora of marine fish larvae may help to improve diets and incubation conditions for the intensive mass rearing of healthy fish.

The intestinal microflora of various fresh- and seawater fishes have been described in a number of investigations [6, 138, 142, 168, 178, 210, 211, 214, 235]. Although the majority of studies has concentrated on adult stages, several reports exist on the microflora of larvae and juveniles [21, 26, 37, 72, 87, 122, 155, 156, 158, 159, 232, 234, 239, 270]. The collective term *gut group Vibrios,* introduced by Liston in 1957 [138], indicated that members of the genus *Vibrio* dominated in the intestine of marine fish. Since then it has been reported that *Vibrio* and *Pseudomonas* prevail in the intestinal tract of a variety of species of marine fish [178, 211, 222]. Members of the genera *Achromobacter, Corynebacterium/Nocardia, Flavobacterium/Cytophaga,* and *Micrococcus* are also frequently encountered [103, 143]. There are, however, problems arising in interpretation and comparison of results from the various authors. This is due to the fact that only a minor part of the reported studies discriminates between the bacterial load in the intestinal content and the "true" intestinal microflora, i.e., bacteria intimately associated with the intestinal mucosa [178, 209, 269].

Lactic acid bacteria have been isolated from the intestinal mucosa of cod (*G. morhua*), saithe (*Pollachius virens*), capelin (*Mallotus villosus*), herring (*C. harengus*), and Atlantic salmon (*S. salar*) in seawater (Strøm, E., 1988. MSc thesis, Univ. of Tromsø, Norway) [219, 232]. They did all belong to the genus *Lactobacillus,* resembling *L. plantarum.* In freshwater salmonid fry, lactic acid bacteria were isolated as the major part of the adherent intestinal microflora, while in marine species they constituted only a minor part. These lactic acid bacteria produced growth-inhibiting factors resulting in inhibition of various *Vibrio* spp., especially *V. anguillarum*. It is tempting to think of these strains as indigenous bacteria which in function might be comparable to the lactic acid bacteria in the intestine of homeothermic higher vertebrates. For a review of lactic acid bacteria in fish, see Ringø and Gatesoupe [196]. Obligate anaerobic bacteria, predominantly *Clostridium* and *Bacteroides* spp., have been isolated as minor parts of the intestinal microflora of various marine fish species [146, 208, 209].

In contrast to what is reported for seawater fish, the intestinal microflora of freshwater fish species tends to be dominated by members of the genera *Aeromonas, Plesiomonas,* representatives of the family *Enterobacteriaceae* [209, 210, 252, 268], and obligate anaerobic bacteria of the genera *Bacteroides, Fusobacterium,* and *Eubacterium* [213, 251]. Sugita et al. [239] have examined development of the gut microflora in goldfish (*Carassius auratus*) from hatching until adult stages. As a model system they described three stages in the development of the intestinal microflora: (1) the transient flora, consisting of *Plesiomonas shigelloides, Enterobacteriaceae, Moraxella,* and *Bacteroidaceae,* which were present for a relatively short period of time in low frequencies of occurrence and which were also detected in the diets, water and on the egg surface; (2) the permanent indigenous flora, consisting of *Aeromonas punctata, A. hydrophila, Pseudomonas* spp., and *Clostridium* spp., which occurred abundantly at all stages in the goldfish development; and (3) the adult flora, dominated by *Bacteroides* type A, first detected approximately 2 months after hatching. The late establishment of *Bacteroides* type A in the goldfish intestine was related to the sequential changes in the structure and function of the digestive tract. This model may be suitable for the development of the intestinal microflora in several marine fish species. Major bacterial genera/species encountered in the intestinal microflora of various seawater fishes are summarized in Table 3.

Several reports describe bacteria firmly attached to the intestinal mucosa [177, 178, 209, 232] (Fig. 8). It is now accepted that fish possess a specific intestinal microflora consisting of aerobic, facultative anaerobic, and obligate anaerobic bacteria, and that the composition may change with age, nutritional status, and environmental conditions [26, 143, 212, 237, 238, 251]. Conway et al. [44] found the intestinal microflora of flounder (*Pseudopleuronectes* sp.) to undergo changes such as decreased cell volume and increased hydrophobicity when the fish was deprived of food. This was accompanied by a rapid response to renewed supplies of nutrients, indicative of a persistent microflora with a starvation-survival strategy.

In conclusion, it is evident that an intestinal microflora will become established very soon after hatching in marine

Table 3. Major bacterial groups isolated from the intestinal microflora of marine fish species[a]

Fish species	Fish developmental stage	Bacteria	Reference
Cod (*Gadus morhua*)	larval/juvenile	*Vibrio, Lactobacillus, Bacillus*	232
	adult	*Vibrio, Photobacterium phosphoreum, Pseudomonas nautica, Alteromonas putrefaciens, Vibrio iliopiscarius*	137, 178
Herring (*Clupea harengus*)	larval	*Pseudomonas/Alteromonas, Flavobacterium*	87
Atlantic halibut (*Hippoglossus hippoglossus*)	yolk sac	*Cytophaga/Flexibacter/Flavobacterium, Pseudomonas*	21, Hansen and Bergh, unpubl. data
	larval	*Vibrio/Aeromonas*	21
Turbot (*Scophthalmus maximus*)	larval	*Vibrio* spp. including *V. fluvialis, V. alginolyticus, V. pelagius, V. scophthalmi, V. splendidus, V. alginolyticus,* and *V. parahaemolyticus, Aeromonas* spp., *A. hydrophila, Chromobacterium violaceum, Achromobacter* sp., *Acinetobacter calcoaceticus, Pseudomonas fluorescens, P. putida*	26, 72, 122, 155, 159
Yellowtail (*Seriola quinqueradiata*)	adult	*Vibrio, Flavobacterium, Aeromonas*	211
Dover sole (*Solea solea*)	yolk sac/ metamorphosed larvae	*Pseudomonas/Alcaligenes, Vibrio*/anaerogenic *Aeromonas, Moraxella*	37
	juvenile/adult	*Vibrio*/anaerogenic *Aeromonas, Moraxella, Flavobacterium, Alcaligenes, Photobacterium, Micrococcus, Staphylococcus*	37, 142
Red seabream (*Pagrus major*)	larval/juvenile	*Vibrio, Pseudomonas, Enterobacteriaceae, Cytophaga, Aeromonas*	156
Black seabream (*Acanthopagrus schlegeli*)	larval/juvenile	*Vibrio, Pseudomonas, Enterobacteriaceae, Cytophaga, Aeromonas*	156
Striped bass (*Morone saxatilis*)	juvenile	*Vibrio, Aeromonas, Flavobacterium, Enterobacteriaceae, Pseudomonas*	143
	adult	*Aeromonas, Pseudomonas, Enterobacteriaceae, Vibrio*	143

[a] Wherever possible, the bacterial genera/species are listed in decreasing order of incidence.

larvae. The need for marine larvae to "drink" in order to osmoregulate will probably accelerate this establishment compared to that of larvae of freshwater fish. The transient, primary intestinal microflora will develop into a more diverse and specialized flora due to impacts from developments in the intestinal structure and function. The bacterial load and composition of the diet and ambient water, together with external environmental factors, will also undoubtedly influence the development of the gut microflora. It is likely that bacterial "pioneer" strains, having adapted to the ecological niche formed by the larval gut, will persist and develop into components of the "adult" microflora.

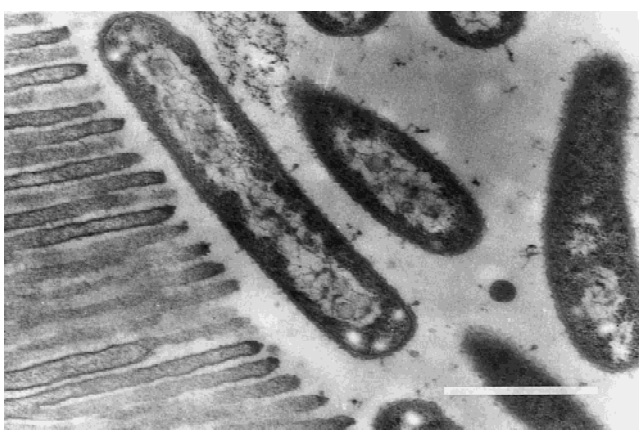

Fig. 8. Bacteria associated with the microvilli layer in the anterior part of the hindgut in a 14-day-old herring larva incubated in sand-filtered seawater. Larvae were washed in sterile filtered seawater, fixed, and embedded as described in Fig. 7. Ultrathin sections were stained in uranyl acetate (1.0%) and lead citrate (2.6%). Bar = 1.0 μm.

Endocytosis of Bacteria—Nutritional Aspects

Bacteria as food for marine invertebrates and also for fish have been the subject of a number of investigations [25, 142, 171, 221, 261, 274]. Bacteria may play an important role for

marine animals by furnishing cell substances or micronutrients such as essential fatty acids [115, 197], vitamins [120, 236], minerals, or even enzymes [79, 137]. At the yolk sac stage, the level of digestive enzymes in the gut is low [98], and therefore surface structures of ingested bacteria should not be affected to the same extent as in older fish. The sequestration of intact proteins from the gut lumen may be nutritionally advantageous to larval and juvenile fishes [76], but it has been found to have little or no nutritional relevance to adult gastric fish [148]. It has been suggested that intracellular protein digestion is nutritionally important in fish larvae before gastric gland differentiation, and that this digestive process is restricted to the hindgut [174, 258, 260].

The alimentary canal of teleostean fish larvae is histologically and functionally undifferentiated at the time of hatching [80, 229]. Prior to first exogenic feeding, the intestinal tract of cod larvae differentiates into the fore-, mid-, and hindgut; the intestine then becomes histologically and functionally distinct [87, 126, 171]. The straight, noncoiled larval gut thus resembles that of stomachless or agastric fish characterized by intracellular digestion as opposed to that of gastric fish, in which extracellular digestion is the main digestive mechanism [80]. The gastrointestinal tract in yolk sac larvae of herring is essentially tubular and is divided into two segments, the fore- and the hindgut, of approximately equal length [87]. The ontogeny of the alimentary canal differs both morphologically and functionally in different taxa [80]. The intestinal tract of herring larvae differs both in relative length and in gross morphology from yolk sac larvae of cod. In both larval species, however, the gut has a straight, noncoiled form, and it is generally conceded that in fish larvae having a straight gut, ingested particles pass rapidly to the posterior part of the gut and accumulate [27, 87, 110]. In cod larvae, fixed red sheep blood cells and fluorescent latex spheres (1.0 and 3.9 μm) have been demonstrated to accumulate in the hindgut [171, 174], whereas bacteria are readily endocytosed.

Ciliated epithelial cells, rich in mitochondria, were found in the posterior part of the foregut in herring larvae and in the esophagus/foregut area in cod larvae [87, 174]; they have also been demonstrated in halibut (*H. hippoglossus* L.) larvae [153]. In general, ciliated cells are believed to propel water, food particles, and mucus along the gut [227]. The origin and function of these cells in the gastrointestinal tract of fish are not clear, but Al-Hussaini [2] discussed the possibility of an evolutionary development from a ciliated border in lower chordates to a brush border in higher teleosts. In the lungfish (*Protopterus aethiopicus*), Purkerson et al. [191] suggested that these cells could represent a secondary oxygen uptake system through the gut wall. It is likely that the ciliated epithelial cells demonstrated in marine fish larvae are involved in the process of propelling particles and bacteria toward the posterior part of the gut.

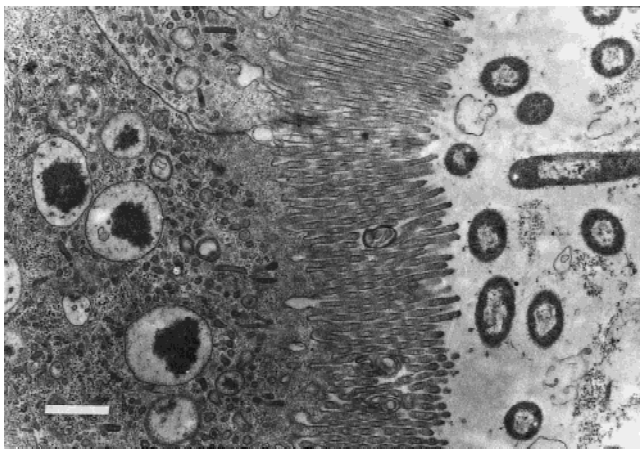

Fig. 9. Endocytosis of bacteria demonstrated in enterocyte in the posterior part of the hindgut in a 14-day-old herring larva. The larva was fixed and treated as described in Fig. 7. Bar = 1.0 μm.

By means of electron microscopy, endocytosis of bacteria by enterocytes in the epithelial border of the hindgut of cod [174] and in the posterior part of the hindgut of herring larvae [87] has been demonstrated (Fig. 9). In embryos and newborn larvae of *Sebastes* (*S. schlegeli, S. melanops,* and *S. taczanowskii*) endocytosis of high molecular weight protein complexes, such as glycoproteins and lipoproteins, has been demonstrated in rectal epithelial cells [224, 245]. Endocytosis was observed in embryos with a functional mouth, and the ingested material probably arose from ovarian fluid. Absorption of macromolecular proteins has also been demonstrated in the posterior part of the intestine in yolk sac larvae of the sea bass, *Dicentrarchus labrax* [49]. These findings are in accordance with observations in a number of adult fish species, where sequestering of proteins has been demonstrated to take place in the posterior part of the intestine [74, 75, 157, 160, 161, 205, 230, 231].

Ingestion of bacteria by "drinking" and mucosal movements, propulsion of bacteria by ciliated epithelial cells, low levels of extracellular enzymes in the digestive tract, and subsequent endocytosis in the hindgut could be strategies of nutritional value, sustaining the larvae with exogenous nutrients before active feeding commences. This way of securing exogenous nutrition differs markedly from the later larval and juvenile stages in which high levels of digestive en-

Table 4. Some beneficial effects of probiotic bacteria on marine organisms

Effect	Organism	Reference
Growth promotion	Turbot (*Scophthalmus maximus*) larvae	71, 73
	Crab (*Portunus trituberculatus*)	162
	Pacific oyster (*Crassostrea gigas*)	51
Disease prophylaxis	Molluscs and penaeid prawns (pathogenic *Vibrio* spp.).	7, 198
	Atlantic salmon *Salmo salar (Aeromonas salmonicida, Vibrio anguillarum,* and *V. ordalii)*	8

zymes are present and extracellular digestion will be the major route of nutritional uptake.

Probiotics: Potential Use in Aquaculture

The use of food containing live microorganisms with assumed beneficial properties has been known for centuries. As early as 76 B.C., Plinio advocated the use of fermented milk products in the treatment of various gastrointestinal infections [183]. In domestic animal production the search for proper probiotics to minimize use of antibiotics as health- and growth-promoting substances has been extensive [257]. The term "probiotics" was originally used to describe the effects of growth stimulating substances in protozoans [69], but has since been used in a broader sense to denote living cells with beneficial effects on the host by improving the microbial balance or properties of the indigenous microflora [183]. Some beneficial effects of probiotics used in various marine organisms are presented in Table 4.

Several demands have to be fulfilled for a candidate organism to be characterized as probiotic. The organisms (i) should be nonpathogenic and biochemically and physiologically well characterized, (ii) should be normal inhabitants of, and capable of surviving and growing at the site of application exerting their beneficial effect(s) on the host, and (iii) should maintain their viability and activity throughout product manufacture and storage. Thus, candidate probiotic strains intended for use in the gastrointestinal tract have to cope with the various biotic and abiotic factors of this environment, including physiochemical factors such as pH and redox potential, digestive enzymes, bile salts, and local immune responses. Beneficial effects and potential risks related to the use of probiotics in general are discussed by Huis in't Veld et al. [106].

Table 5. Some factors responsible for the inhibition/establishment of nonindigenous bacteria at mucosal surfaces

Factors	Effect on nonindigenous bacteria
Nutritional competition	Inhibits growth
Antibiotics	Inhibit growth or kills
Inhibitory metabolites; volatile organic acids, lactic acid, hydrogen peroxide, H_2S	Inhibits growth, especially at low oxidation–reduction potential; lowers local pH
Motility	Motile bacteria compete better for access to space and nutrients
Enhance immunological responses	Prevents colonization
Synergism with local immunological responses	Prevents colonization

To become established, the probiotic bacteria have to successfully compete against already settled microorganisms in the same ecological niche. This includes competition for nutrients and site of adhesion and colonization. In order to screen candidate probiotic organisms, examination of colonization potential, including hydrophobic properties and the presence of specific adhesion factors, is important. Cultured cell lines, which have been used for the study of attachment of pathogens to gastrointestinal cells, would provide defined systems for characterizing adhesive properties of probiotic bacteria. However, such systems do not guarantee that the tested bacteria are able to be successfully established in the gastrointestinal tract. Some factors affecting the establishment of probiotic bacteria in competition with already present microorganisms are presented in Table 5.

In recent years increased interest has focused on the search for probiotics that may improve health conditions in intensive rearing of marine organisms. This research has been spurred by an intention to minimize the use of antibiotics in aquaculture. Apart from the potential hazard of increased antibiotic resistance [243], the extensive use of antibiotics on fish larvae may result in dramatic changes in the indigenous microflora [6, 87]. As in agriculture, lactic acid bacteria have in many instances been the organisms of choice, as has also been the case in marine aquaculture. Lactic acid bacteria have been fed to rotifers, *Brachionus plicatilis,* which then in turn were used as a food source for turbot (*S. maximus*) larvae [71–73]. Thus, the rotifers served as a vector for introducing probiotic bacteria into the gastrointestinal tract of the fish larvae. The added *Streptococcus thermophilus, Lactobacillus helveticus,* and *L. plantarum* seemed to hamper development of pathogens such as *A. salmonicida* and *Vibrio*

alginolyticus in the rotifer cultures. Gatesoupe reported a significant increase in the mean weight of turbot larvae fed rotifers enriched in lactic acid bacteria at day 20 compared to control larvae [73]. In experiments with 5-day-old cod (*G. morhua*) larvae, it was demonstrated that addition of *L. plantarum* to the water affected both the bacterial counts and the composition of the bacterial flora [233]. Gilberg et al. [77, 78] reported a certain improvement in disease resistance in cod (*G. morhua*) fry given a dry feed containing *Carnobacterium divergens* three weeks before challenge with *V. anguillarum,* as compared to fry that were given feed without lactic acid bacteria. Skjermo et al. [226] reported significantly higher growth rate in turbot larvae reared in microbially matured water than in membrane filtered water. They concluded that microbial maturation resulted in selection of nonopportunistic bacteria, thereby hampering opportunists to establish and proliferate.

Vibrios are common members of the indigenous microflora of healthy fish, and commensal vibrios with inhibitory activity against pathogens have been described from various fish species [17, 176, 263]. Bacterial isolates from the intestinal tract and external surfaces of turbot have been screened for inhibitory effects against fish pathogenic bacteria, especially *V. anguillarum* [263]. Selected strains were investigated for their "intestinal colonization potential," that is, their capacity to adhere to and to grow in turbot intestinal mucus [176]. Since vibriosis caused by *V. anguillarum* has been a major disease problem in rearing larval and juvenile stages of turbot, prophylactic supply of probiotic bacteria with inhibitory effect against *V. anguillarum* seemed to be a tempting alternative to vaccination or eventually therapeutic treatment. Olsson et al. [176] concluded that adult marine flatfish, turbot and dab (*Limanda limanda*), harbour intestinal and skin-mucus-associated bacteria with the capacity to suppress growth of *V. anguillarum*. However, preliminary experiments trying to establish these strains in the intestine of larval turbot, and then subsequently challenge with *V. anguillarum,* have so far not been very successful (A. Westerdahl, University of Göteborg, personal communication). Bacteria which inhibit growth of pathogenic *Vibrio* species have also been isolated from halibut (*H. hippoglossus*) larvae [17] and Atlantic salmon (*S. salar*) [114]. *Vibrio iliopiscarius* and nonpathogenic strains of *V. salmonicida* have been isolated in various amounts as parts of the autochthonous intestinal microflora in salmon (*S. salar*), cod (*G. morhua*), saithe (*P. virens*), and herring (*C. harengus*) [177, 178]. These *Vibrio* species produce antibacterial substances with inhibitory effects against *V. anguillarum, V. ordali,* and pathogenic strains of *V. salmonicida* in vitro, and may thus have the potential of in vivo inhibition of these pathogens in the fish intestine [177, 178]. Addition of a potential probiotic bacteria, *Vibrio pelagius,* isolated from healthy turbot (*S. maximus*), to turbot larvae demonstrated that this bacterium was established in the intestinal microflora [195]. However, the effect on survival of the turbot larvae was not investigated.

By using aseptically dissected and artificially fertilized cod eggs, we tried to manipulate and control bacterial colonization on the egg surface [86]. Eggs were primed with diluted cultures of antibiotic-producing bacterial strains [135] and transferred to cultures of environmental isolates with presumed good adhesive properties. Gnotobiotic eggs incubated directly in the diluted environmental cultures were used as controls. Within a few days colonization in all experimental groups equalled that of the controls. The antibiotic-producing strains thus failed to prevent colonization by the environmental strains used.

In conclusion, use of probiotics in intensive rearing of various marine organisms has a great potential, not least taking into account the environmental effects of the extensive use of antibiotics and the expenses associated with vaccination programs. The use of host-specific strains appears essential when evaluating potential probiotic strains. Based on the successful use of probiotics in domestic animals, this mode of disease prophylaxis and health-promoting effort could be a promising approach in aquaculture as well.

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