The aerobic intestinal microflora of 2-week-old herring (Clupea harengus) larvae was characterized by using conventional microbiological methods and electron microscopy. Larvae were hatched and kept in filtered seawater or in seawater with penicillin and streptomycin. The gastrointestinal tract of herring larvae is essentially a straight tube divided into two compartments. Light microscopy revealed bacteria present in a progressively increasing amount throughout the length of the gastrointestinal tract from esophagus to anus. The posterior region of the intestinal lumen appeared completely occluded with bacteria. The intestinal microflora consisted mainly of members of the genera Pseudomonas and Alteromonas in the larvae incubated in filtered seawater, whereas Flavobacterium spp. dominated in larvae exposed to antibiotics. The intestinal microflora of untreated fish larvae was sensitive to all tested antibiotics, whereas multiple resistance was found in the intestinal microflora of the group given antibiotics. Thus, a dramatic change in the microflora resulted from incubation with antibiotics. Nonpigmented yeasts were detected in both larval groups. Ciliated epithelial cells were observed in the midgut, probably propelling bacteria towards the hindgut, where endocytosis of bacteria has been demonstrated. These findings suggest that transport and sequestering mechanisms resembling those of invertebrates may be found in the gut of fish larvae. The possible significance for larval health and nutrition is discussed.

Whereas the role of the commensal microflora of warm-blooded animals is firmly established, reports of that of fish have been conflicting. The role, or even existence, of a stable indigenous microflora in fish is not yet fully understood; neither is it known at what stage such a flora is established. The most serious diseases that affect fish in marine aquaculture are caused by opportunistic bacteria, such as Vibrio spp. (8, 11). An understanding of the characteristics or features and the role of the indigenous microflora of marine fish larvae may help to improve feed and conditions for the intensive mass rearing of healthy fish.

The intestinal microfloras of various fresh- and saltwater fishes have been described in a number of investigations (25, 30). Only a limited range of taxa appeared to be represented in some cases (38), whereas other investigations reported a wider diversity of the gut microflora (2). However, the majority of studies have been concerned with adult fish, and only a few reports exist on the microflora of fish larvae (7, 28, 45, 49, 55).

It has been inferred by some workers that the intestinal microflora of fish reflects the bacterial content of ingested food and of the environment (21, 41) and that the intestinal tract of nonfeeding fish is essentially sterile (53). Thus, the existence of a truly indigenous gut microflora in fish has been disputed, but several reports describe bacteria firmly attached to the gut mucosa of adult fish (33, 37, 44). It now appears that fish contain a specific intestinal microflora consisting of aerobic, facultatively anaerobic, and obligately anaerobic bacteria (6, 39, 47, 48, 50). Conway et al. (9) found the intestinal microflora of flounder (Pseudopleuronectes...

**TABLE 1. Morphological and biochemical characteristics of bacterial isolates from the two larval groups, STA (exposed to antibiotics) and STB (control larvae)**

<table>
<thead>
<tr>
<th>Test*</th>
<th>Result ( % of isolates positive from^b)</th>
<th>STA (n = 21)</th>
<th>STB (n = 27)</th>
</tr>
</thead>
<tbody>
<tr>
<td>O/F glucose, A</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>O/F glucose, NC</td>
<td>+ (100)</td>
<td>d (26)</td>
<td></td>
</tr>
<tr>
<td>O/F glucose, O</td>
<td>0</td>
<td>d (70)</td>
<td></td>
</tr>
<tr>
<td>O/F glucose, F</td>
<td>0</td>
<td>- (4)</td>
<td></td>
</tr>
<tr>
<td>Acid produced aerobically from:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fructose</td>
<td>0</td>
<td>- (4)</td>
<td></td>
</tr>
<tr>
<td>Sucrose</td>
<td>0</td>
<td>+ (96)</td>
<td></td>
</tr>
<tr>
<td>Mannose</td>
<td>0</td>
<td>+ (4)</td>
<td></td>
</tr>
<tr>
<td>Mannitol</td>
<td>0</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>Glycerol</td>
<td>0</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Ribose</td>
<td>0</td>
<td>- (0)</td>
<td></td>
</tr>
<tr>
<td>Starch</td>
<td>0</td>
<td>d (33)</td>
<td></td>
</tr>
<tr>
<td>N-Ac-Glu</td>
<td>0</td>
<td>- (4)</td>
<td></td>
</tr>
<tr>
<td>Methyl red</td>
<td>0</td>
<td>- (4)</td>
<td></td>
</tr>
<tr>
<td>Voges-Proskauer</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Indole</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Nitrate reductase</td>
<td>0</td>
<td>- (96)</td>
<td></td>
</tr>
<tr>
<td>Amylase</td>
<td>d (76)</td>
<td>+ (89)</td>
<td></td>
</tr>
<tr>
<td>Chitinase</td>
<td>0</td>
<td>- (7)</td>
<td></td>
</tr>
<tr>
<td>Caseinase</td>
<td>0</td>
<td>+ (100)</td>
<td></td>
</tr>
<tr>
<td>DNase</td>
<td>+ (100)</td>
<td>+ (96)</td>
<td></td>
</tr>
<tr>
<td>β-Galactosidase</td>
<td>0</td>
<td>+ (96)</td>
<td></td>
</tr>
<tr>
<td>Growth at:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25°C</td>
<td>d (38)</td>
<td>(100)</td>
<td></td>
</tr>
<tr>
<td>30°C</td>
<td>0</td>
<td>+ (100)</td>
<td></td>
</tr>
<tr>
<td>37°C</td>
<td>0</td>
<td>- (19)</td>
<td></td>
</tr>
</tbody>
</table>

* Abbreviations: O/F glucose, oxidative or fermentative metabolism of glucose; A, alkaline; NC, no pH change; O, oxidative; F, fermentative; N-Ac-Glu, N-acetylglucosamine.

^b +, 80% or more positive; d, 21 to 79% positive; -, 80% or more negative.

* Corresponding author.
† Present address: The Norwegian School of Safety Engineering and Management, Skåregaten 103, N-5500 Haugesund, Norway.
FIG. 1. Toluidine-stained cross section through the anterior part of the foregut of a herring larva not exposed to antibiotics (group STB). Bacteria are seen in the intestinal lumen.

FIG. 2. Toluidine-stained cross section through the posterior part of the foregut of a herring larva kept in filtered seawater (STB). The epithelial layers are shallowly folded, and bacteria are seen in the intestinal lumen.
FIG. 3. Toluidine-stained section through the posterior part of the hindgut of a herring larva kept in filtered seawater (STB), showing the intestinal lumen completely occluded with bacteria.

FIG. 4. Higher magnification of Fig. 3 demonstrating the dense bacterial masses in the intestinal lumen of the posterior part of the hindgut.
FIG. 5. DAPI-stained cross section through the anterior part of the foregut of a herring larva kept in filtered seawater (STB), demonstrating fluorescent bacteria in the intestinal lumen. The section is cut anterior to the one shown in Fig. 2.

FIG. 6. Epithelial microvilli of the anterior part of the foregut, visualized by transmission electron microscopy. The microvilli are undeveloped but apparently are able to sustain a glycocalyx. A longitudinal section through a rod-shaped bacterium is seen in the lower part of the photograph.
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 nutrients, indicative of a persistent microflora with a starvation survival strategy. An adherent microflora has also been observed in “wild” nonfeeding cod (43). On the other hand, Strøm and Olafsen (44) demonstrated that the composition of the intestinal microflora of wild-captured juvenile cod changed after subsequent feeding on a commercial diet. It is not known whether bacterial colonization is affected by differences in habitat, development, or morphological features of larvae from different fish species.

Aquaculture of marine fish species has resulted in intensive systems for egg incubation and hatching in incubators with large biomasses. In such systems, newly hatched larvae will be exposed to an environment with egg debris and high bacterial populations (18). Marine fish larvae ingest substantial numbers of bacteria, starting from hatching, by drinking seawater to osmoregulate and by ingestion of mucus (18, 31, 51). Thus, bacteria enter the digestive tract before active feeding commences. Ingested bacteria may affect the establishment of a primary intestinal microflora, furnish nutrients or enzymes (3, 25, 41), or affect early immune development by exposing antigenic determinants (10, 35).

Antibiotics are frequently used to control bacterial proliferation in these intensive systems. Despite this, little is known about the effect of chemotherapy on the indigenous microflora of adult marine fish (1, 2), and apparently no experiments with marine fish larvae have been reported. The aim of this study was to describe characteristics of the intestinal microflora of herring larvae and how hatching in the presence of antibiotics would affect this flora. The antibiotics used, penicillin and streptomycin, were those originally recommended to minimize bacterial growth on herring eggs (5).

MATERIALS AND METHODS

Herring larvae. Eggs from herring (Clupea harengus) were obtained from a local strain that spawns in the tidal zone of Balsfjord, southeast of Tromsø, Norway. The eggs were incubated in glass aquaria with sand-filtered seawater which was changed every third day. The water temperature was 3.5 to 4°C. Prior to hatching, eggs were incubated in sand-filtered seawater (group STB) or in sand-filtered seawater containing 50 IU of crystalline penicillin and 0.05 mg of streptomycin sulfate per ml (group STA). The water in both larval groups was changed every second day. Fourteen-day-old herring larvae were used in these experiments.

Light, fluorescence, and electron microscopy. Herring larvae were carefully transferred to sterile filtered seawater (0.22 μm) for 5 min and fixed in formaldehyde-glutaraldehyde (final concentrations, 2.5 and 2.0%, vol/vol, respectively) in 0.05 M cacodylate buffer, pH 7.2 (14). Larvae were postfixed in 1% osmium tetroxide in cacodylate buffer and dehydrated in ethanol. After propyleneoxide treatment, larvae were embedded in Epon-Araldite. Semithin sections (1.0 μm) were used for light and fluorescence microscopy. Sections for fluorescence microscopy were stained with toluidine blue for 1 min. Sections for fluorescence microscopy were stained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI; Sigma) (34), 10 μg/ml, for 8 min. DAPI reacts with DNA,

![Image](image_url)
resulting in bright-blue fluorescence (365 nm) with retarded fading as compared with acridine orange and ethidium bromide. An Olympus BH-2 microscope with epifluorescence equipment and Olympus OM-1 camera were used for light and fluorescence microscopy. For light microscopy Kodak Ektachrome 160 Professional film was used, and for fluorescence microscopy Kodak Ektachrome 400 and 1000 film was used. Ultrathin sections for electron microscopy were prepared with a Reichert-Jung Ultracut microtome (C. Reichert AG, Vienna, Austria) equipped with a diamond knife. Sections were stained for 1 h in 1.0% uranyl acetate and then for 5 min in 2.6% lead citrate. Transmission electron microscopy was performed with a 100S electron microscope (JEOL, Tokyo, Japan).

Microbiological isolation and characterization. Before dividing the eggs between the two experimental groups, STA and STB, viable counts of the adherent epiflora were made. Eggs were carefully removed from the substratum, Fucus serratus, and then rinsed four times in sterile 70% seawater, homogenized, and plated on Difco marine agar. In addition, water samples from the egg incubator were plated on marine agar. The plates were incubated at 12°C for up to 18 days to allow growth of slow-growing strains.

Isolation of larval intestinal microflora. Forty larvae from each of the experimental groups were surface disinfected in 0.05% potassium iodide for 3 min and carefully washed in sterile 0.9% NaCl for four 1-min periods (40). Aliquots consisting of 10 larvae were homogenized in 1 ml of sterile 0.9% sodium chloride in a Potter-Elvehjem homogenizer. Serial dilutions were plated on marine agar and tryptone soy agar (Difco) supplemented with 1.5% sodium chloride and 0.5% glucose. Incubation conditions were as for the egg epiflora viable counts. Colonies were picked at random and characterized by the methods of references 18 and 20. The incubation temperature for the various biochemical tests was 15°C unless otherwise stated.

RESULTS

Light and fluorescence microscopy. Light microscopy of a series of toluidine-stained semithin cross sections from the oral cavity through the alimentary canal demonstrated that the intestinal tract was colonized by bacteria in numbers increasing drastically towards the posterior part of the gut (Fig. 1 to 4). In the anal region of the gut the lumen was occluded with bacteria (Fig. 3 and 4). The gastrointestinal tract of herring larvae is essentially tube shaped, with a length of roughly three-quarters of the larval body. It is divided into two segments of approximately equal length, the fore- and the hindgut. In both larval groups (STA and STB) the bacterial flora appeared to be morphologically very homogeneous. Fluorescence microscopy of DAPI-stained sections revealed bacteria in the intestinal lumen (Fig. 5). No background fluorescence was observed when DAPI was applied directly on the Epon-Araldite sections. Bacteria in the gut lumen and on external surfaces fluoresced bright blue with essentially no tissue autofluorescence, as we have observed when using other fluorochromes (e.g., fluorescein isothiocyanate) on fish tissue (17). The DAPI staining technique is well suited for detecting bacteria in semithin Epon-Araldite sections of the intestinal tract.

Electron microscopy. Electron microscopy confirmed the
light microscopic findings with respect to distribution of bacteria along the gastrointestinal tract (Fig. 6 and 7) and also revealed the majority of cells to be intact, many of them undergoing division. Endocytosis of bacteria was demonstrated in the posterior part of the gut (Fig. 8), and ciliated cells were observed among the epithelial cells of the midgut (Fig. 9).

**Microbiological examination.** The adherent egg epiflora was characterized by great diversity with respect to colonial morphology and pigmentation. The egg epiflora viable counts were in the order of 2 × 10⁶ CFU per egg, and CFU in water were 4 × 10⁶ per ml. Viable counts of larva homogenates, measured as CFU on marine agar, were 1.3 × 10⁴ and 3.7 × 10² per larva in the STB and STA groups, respectively. In addition, two types of opaque nonpigmented yeasts appeared, one with a smooth surface and the other with a rough surface. Yeast cells were identified by microscopic examination, revealing 7- to 10-μm, slightly oval, refractile bodies. Budding was observed in some of the isolates. Yeast viable counts were 5.7 × 10² and 1.1 × 10¹ CFU per larva in the STB and STA groups, respectively.

The two larval groups exhibited distinctly different aerobic intestinal microfloras. Both larval microfloras were characterized by a very low bacterial diversity. The STB isolates were all gram-negative, Kovac’s oxidase-positive, motile, nonpigmented rods. They all contained one or more vacuoles when grown for 2 days at 16°C in marine broth (Difco). Some of them were able to produce acid aerobically from one or more of the tested carbohydrates (Table 1). These isolates were most probably members of the genus *Pseudomonas* or *Alteromonas*. Bacterial isolates from the STA group were all gram-negative, Kovac’s oxidase-positive, yellow-pigmented, nonmotile rods. They were not able to produce acid from any of the tested carbohydrates and did not exhibit gliding motility. Additional biochemical and physiological characteristics are given in Table 1. The results for the STA isolates indicate that they belong in the genus *Flavobacterium*. Neither STB nor STA isolates possessed urease, but they exhibited catalase and gelatinase activities and grew at 4 and 16°C. Of the enzymatic activities tested (Table 1), only nitrate reductase, caseinase, and β-galactosidase differed between the two groups, STB being positive and STA being negative. With respect to antibiotic susceptibility, isolates from the STB group were susceptible to penicillin, ampicillin, streptomycin, chloramphenicol, and sulfamethoxazole-trimethoprim, while STA isolates were resistant to all antibiotics tested, except O/129 (150 mg) and novobiocin, to which this group was partially susceptible (Table 2).

**DISCUSSION**

The results from herring larvae presented here confirm the existence of an intestinal microflora in the early life stages of marine fish. Also, in yolk sac larvae of cod, *Gadus morhua* L. (19, 31), and halibut, *Hippoglossus hippoglossus* L. (16), we have observed bacteria in the intestinal tract before active feeding had commenced. There are indications that the primary intestinal microflora in cod and halibut larvae hatched in intensive systems may originate in the resident egg epiflora at hatching. Whether this is the case with naturally hatched fish larvae remains to be investigated. We
also do not know whether the primary intestinal microflora is only transient, with the establishment of a resident fish after active feeding commences (27, 49). However, DAPI-stained sections and transmission electron microscopy of the intestinal tract in herring larvae revealed the majority of the bacteria to be intact and probably alive (Fig. 6 to 8). This supports the contention that these bacteria may be members of a metabolically active and proliferative microflora. Ingested bacteria could be important for establishment of a protective intestinal microflora and may be of nutritional or immunological significance for the larvae. It is known that bacteria in the intestine of fish may release polymer-degrading enzymes which could aid in prey digestion, e.g., chitinase and N-acetylglucosaminidase (15, 24, 25), and also furnish vitamins (46).

The alimentary canal of teleostean fish larvae is histologically and functionally undifferentiated at the time of hatching (42). In herring larvae the gut has a straight, noncoiled form. It is generally conceded that, in fish larvae having a straight gut, ingested food materials pass rapidly to the posterior part of the gut and accumulate (4). Bacteria are found to accumulate in the posterior part of the hindgut of herring larvae. Quantification of bacteria in the intestinal tract from semithin toluidine-stained cross sections revealed an increase in bacterial counts from about 102 in esophagus to more than 3 × 103 in the posterior part of the hindgut. Counts from DAPI-stained sections gave results corresponding to about 75% of the toluidine-stained sections, the majority of intestinal bacteria thus being intact with intracellular DNA. Ciliated epithelial cells were found in the posterior part of the foregut of herring larvae (Fig. 9) and have also been found in the esophagus-foregut area in cod (32) and other marine fish larvae (22). Such ciliated cells may take part in the cleansing of bacteria from the brush border or in the effective transport of bacteria towards the posterior part of the gut of marine fish larvae.

Electron microscopy demonstrated endocytosis of bacteria by enterocytes in the epithelial border of the posterior part of the hindgut (32), as observed in a number of juvenile and adult fish species (12, 13, 29, 52). McLean and Ash (26) argued that such protein uptake had little or no nutritional value in adult fishes. Several authors have proposed that fish absorptive enterocytes, capable of sequestering intact proteins from the gut lumen, might function as an antigen-sampling device (10, 35, 36). In the early larval stages of marine fish, endocytosis might provide a supply of essential factors not degradable by the gut bacteria. This supports the contention that ingested bacteria may have a nutritional or otherwise stimulating effect (3, 25, 41). Given the abundance of bacteria, the morphology of the gut, and the observed endocytosis by hindgut enterocytes of herring larvae (32), it appears that the gut microflora could play an important part for this fish species by producing essential nutrients, e.g., fatty acids or vitamins, or exposing antigenic determinants.

In other fish species it has been demonstrated that the gut microflora may produce essential unsaturated fatty acids (54), even from diets containing only saturated fatty acids (23). It is tempting to assume that the large numbers of living bacteria accumulating in the hindgut of herring are due to proliferation of selected bacterial strains with distinctive functional properties in the digestive tract of herring.

A characteristic feature of both intestinal microfloras (STB and STA) was the homogeneity and low diversity in the bacterial populations. The aerobic culturable bacterial flora of the gastrointestinal tract of larvae not exposed to antibiotics (STB) was dominated by Pseudomonas or Alteromonas strains, while the intestinal flora of larvae exposed to antibiotics (STA) consisted almost exclusively of three or four Flavobacterium species. Pseudomonas or Alteromonas strains could not be detected in this larval group. We found that the Flavobacterium spp. exerted a high degree of multiple resistance to the tested antibiotics. In addition, the STA isolates were apparently less biochemically versatile than the isolates from the STB larvae. While 70% of the isolates from STB metabolized glucose in an oxidative way, none of the isolates from STA attacked glucose. Of the isolates from STB, 96% produced acid aerobically from sucrose; 33%, from starch; and 19%, from mannitol. None of the STA isolates was positive in this trait. None of the STA isolates demonstrated nitrate reductase, caseinase, or β-galactosidase activities, while almost 100% of the STB isolates did. Screening of STA isolates did not reveal plasmids; thus, antibiotic resistance was most likely not plasmid coded. Addition of antibiotics to the incubating water thus entailed a dramatic alteration in the composition of the larval intestinal microflora.

The results presented clearly demonstrate an intestinal microflora in herring larvae at the yolk sac stage. From the biochemical characteristics of the bacterial isolates, it is clear that the two intestinal microfloras may have quite different influences on the larvae and on larval digestion after onset of exogenous feeding. These findings support the contention that routine use of antibiotics should be avoided in the mass rearing of marine fish larvae. Besides the possibility of selecting antibiotic-resistant strains or even opportunistic bacteria, dramatic changes in the microflora could affect bacteria important in nutrition or otherwise in the development of marine fish larvae.

**ACKNOWLEDGMENTS**

Eggs and larvae of herring were kindly provided by Elin Kjørvik and Inger Johanne Lurås, University of Tromsø. We thank Laina Dalsbø, University of Tromsø, and Randi Sunnfjord, University of Bergen, for sectioning the microscopic specimens and the Electron Microscopic Laboratory at the Faculty of Science, University of Bergen, for assistance with electron microscopy and photographic work.

Financial support from the Norwegian Fisheries Research Council is gratefully acknowledged.

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**TABLE 2. Antibiotic susceptibility of intestinal bacterial isolates from larvae in groups STA (exposed to antibiotics) and STB (control larvae)**

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Result (%) of isolates susceptible</th>
<th>STA (n = 21)</th>
<th>STB (n = 27)</th>
</tr>
</thead>
<tbody>
<tr>
<td>O/129, 10 µg</td>
<td>R (0)</td>
<td>R (0)</td>
<td>R (0)</td>
</tr>
<tr>
<td>O/129, 150 µg</td>
<td>d (71)</td>
<td>R (0)</td>
<td>R (0)</td>
</tr>
<tr>
<td>Penicillin G, 10 µg</td>
<td>R (0)</td>
<td>S (100)</td>
<td>S (100)</td>
</tr>
<tr>
<td>Ampicillin, 10 µg</td>
<td>R (0)</td>
<td>d (63)</td>
<td>d (52)</td>
</tr>
<tr>
<td>Tetracycline, 30 µg</td>
<td>R (0)</td>
<td>d (63)</td>
<td>d (52)</td>
</tr>
<tr>
<td>Polymyxin B, 10 µg</td>
<td>R (0)</td>
<td>S (100)</td>
<td>S (100)</td>
</tr>
<tr>
<td>Streptomycin, 30 µg</td>
<td>R (0)</td>
<td>S (89)</td>
<td>S (89)</td>
</tr>
<tr>
<td>Chloramphenicol, 30 µg</td>
<td>R (0)</td>
<td>S (100)</td>
<td>S (100)</td>
</tr>
<tr>
<td>Novobiocin, 5 µg</td>
<td>d (67)</td>
<td>d (37)</td>
<td>d (37)</td>
</tr>
<tr>
<td>Sulfamethoxazole + trimethoprim, 25 µg</td>
<td>R (0)</td>
<td>S (100)</td>
<td>S (100)</td>
</tr>
</tbody>
</table>

a S, 80% or more susceptible; d, 21 to 79% susceptible; R, 80% or more resistant.

b O/129, 2,4-diamino-6,7-diisopropyl-pteridine.

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Also see [other references](http://aem.asm.org/) for more detailed information.
REFERENCES


