

Probiotics in Aquaculture

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ABSTRACT

The skin, lateral line, gills and gastrointestinal tract or a combination of these organs are suggested to be infection routes in fish. This presentation will present some information on pathogenesis, protection against bacterial adhesion, autochthonous microbiota in the gastrointestinal tract and prebiotics. This information is important when discussing the use of probiotics in aquaculture.

Intensive fish production has increased the risk of infectious diseases and there is a growing need to find alternatives to antibiotic treatment for disease control as indiscriminate use of antibiotics in many parts of the aquaculture industry has led to the development of antibiotic resistance in bacteria. Today, a range of microorganisms have been suggested as or evaluated as fish probiotics. These include lactic acid bacteria, *Bacillus* species, *Pseudomonas* species, *Vibrio* species and other Gram-negatives. However, research in probiotics for aquaculture is at an early stage and much work is still needed.

Another aspect on fish health is the use of prebiotics to increase the population level of already beneficial bacteria colonizing the gastrointestinal tract and the effect of diet in disease resistance.

INTRODUCTION

Before presenting the hard facts on probiotics in aquaculture a short background about pathogenesis, protection against bacterial adhesion and the autochthonous microbiota in the gastrointestinal tract is first provided here. This background information is important when discussing the use of probiotics in aquaculture. Pathogenic microorganisms have evolved mechanisms to target skin, gills or gastrointestinal tract as a points of entry, and today it is generally accepted that the three major routes of infection in fish are through: (a) skin (Kawai *et al.*, 1981; Muroga and de la Cruz, 1987; Kanno *et al.*, 1990; Magarinos *et al.*, 1995; Svendsen and Bøggvald, 1997; Spanggard *et al.*, 2001); (b) gills (Hjeltnes *et al.*, 1987; Baudin Laurencin and Germon, 1987; Svendsen *et al.*, 1999); and (c) gastrointestinal tract (Sakai, 1979; Rose *et al.*, 1989; Chair *et al.*, 1994; Olsson, 1995; Grisez *et al.*, 1996; Olsson *et al.*, 1996; Romalde *et al.*, 1996; Jöborn *et al.*, 1997; Robertson *et al.*, 2000; Lødemel *et al.*, 2001).

Pathogenicity can be divided into four different phases (Birkbeck and Ringø, 2002): (1) the initial phase where the pathogen enters the host's environment, including the gastrointestinal tract; (2) the exponential phase where the pathogen adheres to and colonizes mucosal surfaces, replicates to sufficient numbers and translocate into host enterocytes; (3) the stationary phase where the pathogen replicates within the host and circumvents the host defence system. In this phase the host is moribund and this can quickly be followed by (4) the death phase.

In aquatic ecosystems, the intimate relationships between microorganisms and other biota and the constant flow of water through the gastrointestinal tract of fish and invertebrates will also affect their indigenous microbiota. Against this background, we may assume that the natural microbiota on eggs, larvae, fry, juveniles and adult fish may help to protect against colonization by a harmful microbiota. Another aspect seen from a microbial point of view is the fact that the microbiota of intensive rearing systems differs dramatically from that in the sea, and it is influenced by many factors such as; rearing techniques, nutrient, disinfection techniques and the use of antibiotics.

In order to adhere successfully, colonize and produce disease the pathogen must overcome the host defence system. It is well known that stress from environmental factors, such as oxygen tension, water temperature and water salinity, are important in increasing the susceptibility of fish to microbial pathogens. The water milieu can also facilitate transmission of these pathogens.

The pathogenesis of *Vibrio* infections in mammals is primarily a gut infection, and it is therefore logical to ask whether the same is true in fish. Fish pathogenic bacteria, such as *Vibrio salmonicida* and *V. anguillarum*, have been shown *in vivo* to adhere to the intestinal epithelium of fish larvae and to promote severe destruction of microvilli (Olafsen and Hansen, unpublished results, cited in Knudsen *et al.*, 1999). Severe damage with loss of cellular integrity was also noted in midgut of spotted wolffish (*Anarhichas minor* Olafsen) fry infected by *V. anguillarum* (Ringø, Mikkelsen and Myklebust, unpublished data, cited in Ringø *et al.*, 2002a) (Fig. 1), compared to normal enterocytes (Fig. 2) (Olsen, Myklebust and Ringø, unpublished data). Scanning electron microscopy investigations of human intestinal mucosa infected with enteropathogenic *Escherichia coli* (EPEC), showed that EPEC adhere intimately in microcolonies and cause gross alterations at the apical surface of infected enterocytes (Knutton *et al.*, 1987; Knutton, 1995).

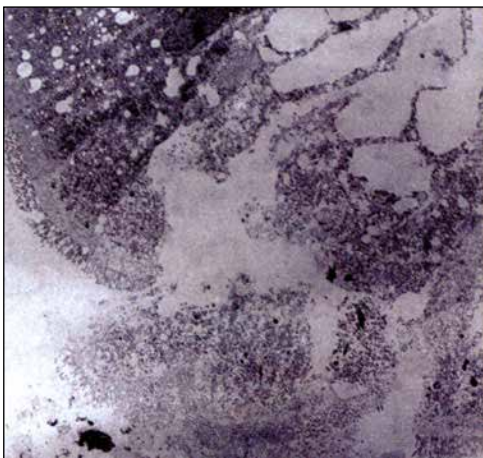
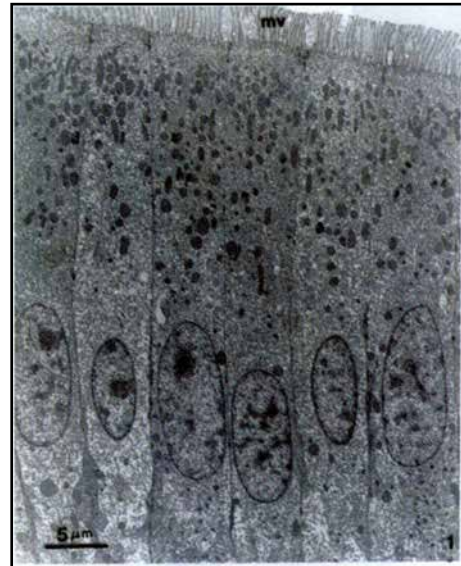


Figure 1. Spotted wolffish (*Anarhichas minor* Olafsen) fry infected by *Vibrio anguillarum*. Notice the severe cellular damage. (after Ringø, Mikkelsen and Myklebust, unpublished data)

Figure 2. Normal enterocytes. Cells display the normal columnar arrangement with intact intracellular junctions and a regular, well-defined microvillous brush border (mv) at the cell apex. (after Olsen, Myklebust and Ringø, unpublished data)



The susceptibility of early life stages of turbot and Atlantic halibut (*Hippoglossus hippoglossus* L.) to *Aeromonas salmonicida* ssp. *salmonicida* was studied in challenge experiments (Bergh *et al.*, 1997). Larvae of both species experienced high mortality during the yolk sac stage, and the authors suggested that this was as a result of the challenge test. However, the bacterium could not be recovered from the larvae by culture, but the pathogen was shown to be present in the intestinal lumen of some turbot larvae examined using immunohistochemical techniques (Fig. 3). Based on this result, the authors (Bergh *et al.*, 1997) proposed that *A. salmonicida* ssp. *salmonicida* may persist in the larvae.

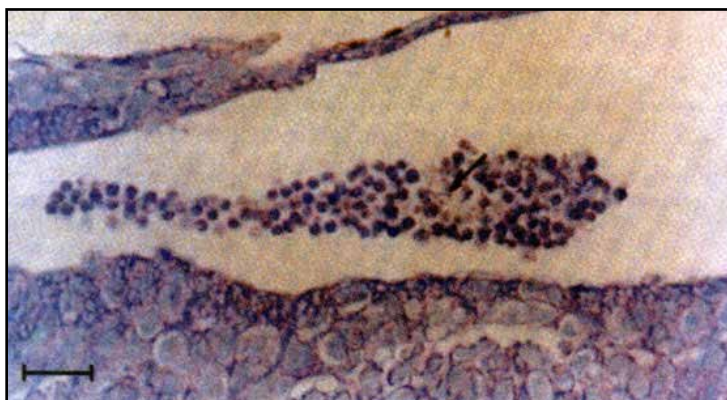


Figure 3. Semithin sections from turbot (*Scophthalmus maximus* L.) larva of group As-A, challenged with *Aeromonas salmonicida* ssp. *salmonicida* stained with Giemsa. Cell-like structures can be seen associated with positively red-stained bacteria-like structures (arrow). Scale bar is 10 μm. (after Bergh *et al.*, 1997)

Endocytosis of bacteria by enterocytes has been observed in the hindgut of several fish species (Ringø *et al.*, 2002a). In their study on turbot (*Scophthalmus maximus* L.) larvae, Grisez *et al.* (1996) reported free *V. anguillarum* from an endosome in the lamina propria by immunohistochemical staining (Fig. 4).

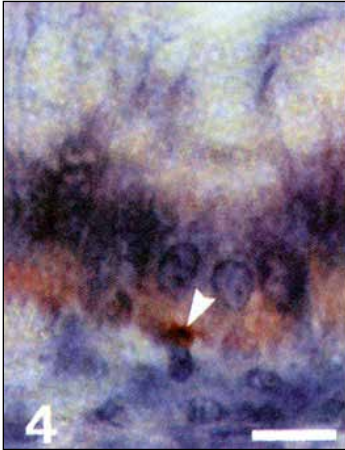


Figure 4. *Vibrio anguillarum* infection in turbot (*Scophthalmus maximus* L.) after oral challenge. *Vibrio anguillarum* attached to the microvilli (brush border) of the intestinal epithelium. (after Grisez *et al.*, 1996)

Readers with special interest in pathogenesis and the gastrointestinal tract of growing fish are referred to the review of Birkbeck and Ringø (2002).

PROTECTION AGAINST BACTERIAL ADHESION MUCUS

The internal surface of the host is the first defence barrier to infection. Intestinal mucins secreted by specialized epithelial goblet cells located in the intestinal enterocytes form a viscous, hydrated blanket on the surface of the intestinal mucosa that protects the delicate columnar epithelium. This is thought to be a vital component of the intestinal mucosal barrier in prevention of colonization by pathogens in both fish and endothermic animals (Florey, 1962; Forstner, 1978; Westerdahl *et al.*, 1991; Maxson *et al.*, 1994; Mims *et al.*, 1995; Henderson *et al.*, 1999). Gastrointestinal mucus is thought to have three major functions: (1) protection of the underlying mucosa from chemical and physical damage; (2) lubrication of the mucosal surface; and (3) to provide a barrier against enteroadherence of pathogenic organisms to the underlying mucosal epithelium.

It is well known that intestinal mucus is composed almost entirely of water (90-95%) and the electrolyte composition is similar to plasma, accounting for about 1% of the mucus weight. The remaining 4-10% is composed of high-molecular-weight glycoproteins (mucins), consisting of a protein core with numerous carbohydrate (fucose and galactose) sidechains. Hydrolysis of intestinal mucus material of rainbow trout liberated increased amounts of N-acetylgalactosamine and N-acetylglucosamine (O'Toole *et al.*, 1999), indicated that these carbohydrates may be present as mucin-bound moieties in fish intestinal mucus as the case for mucus from other animal species (Roussel *et al.*, 1988). The majority of intestinal mucus-associated lipids in rainbow trout partitioned to the organic phase during extraction with chloroform/methanol and was found to contain

saturated and unsaturated free fatty acids, phospholipids, bile acid, cholesterol, and monoglycerides and diglycerides (O'Toole *et al.*, 1999).

Olsson *et al.* (1992) put forward the hypothesis that the gastrointestinal tract is a site of colonization of *V. anguillarum* as the pathogen could utilize diluted turbot intestinal mucus as sole nutrient source. In a later study, Garcia *et al.* (1997) concluded that Atlantic salmon (*Salmo salar* L.) intestinal mucus is an excellent growth medium of *V. anguillarum*. This result is an important aspect of the pathogenesis of this pathogen.

The mucous blanket is constantly renewed by the secretion of high molecular weight glycoproteins from individual goblet cells throughout the epithelium. Goblet cells differentiate in the lower portion of the crypts of both small and large intestine and gradually migrate onto the villi or mucosal surface.

In an early study on histopathology changes caused by *V. anguillarum*, Ransom *et al.* (1984) found large amounts of goblet (mucus producing) cells in the anterior part of gastrointestinal tract of infected chum salmon (*Oncorhynchus keta* Walbaum). The first reaction of Arctic charr (*Salvelinus alpinus* L.), a salmonid fish, infected by pathogenic bacteria (*A. salmonicida* ssp. *salmonicida*) the causative agent of furunculosis is to peel off the infected mucus by increased goblet (mucus producing) cell production compared to uninfected fish (Lødemel *et al.*, 2001) (Fig. 5). A similar reaction to that found in infected Arctic charr, is also observed in rabbit and rats infected by pathogenic bacteria (Mantle *et al.*, 1989, 1991; Enss *et al.*, 1966), and this reaction may be considered a normal host response to particular intestinal infections (Mims *et al.*, 1995).

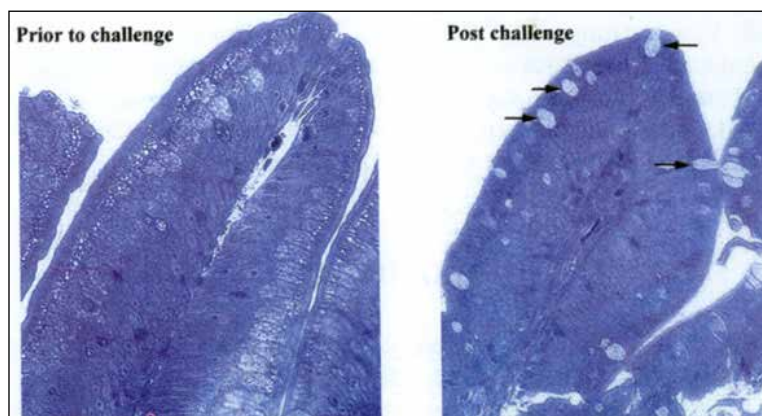


Figure 5. Light microscopic view of villi in the midgut from Arctic charr (*Salvelinus alpinus* L.) fed soybean oil prior to challenge and post challenge with the fish pathogen *Aeromonas salmonicida* ssp. *salmonicida*. Note the substantially more conspicuous goblet (mucus producing) cells (arrows) along the villi of infected fish. (after Lødemel *et al.*, 2001)

Furthermore, the peel off of mucus might lead to loss of the autochthonous (indigenous) microbiota closely associated with the intestinal epithelium forming one of the first defence to limit colonization of pathogenic bacteria.

AUTOCHTHONOUS MICROBIOTA

Savage (1983) defined bacteria isolated from the digestive tract as either autochthonous (indigenous) or allochthonous (transient). Recently, Ringø and Birkbeck (1999) presented a list of criteria (found in healthy animals, colonize early stages and persist throughout life, found in both free-living and hatchery-cultured fish, grow anaerobically, and found associated with epithelial mucosal in the digestive tract) for testing autochthony of bacteria from the gastrointestinal tract of fish. To define the presence of autochthonous microbiota in fish, electron microscope investigations are a useful tool (Ringø *et al.* 2002a). One might put forward the hypothesis that autochthonous microbiota associated closely with the intestinal epithelium form a barrier serving as the first defence to limit direct attachment or interaction of pathogenic bacteria to the mucosa as reported for endothermic animals (van der Waaij *et al.*, 1972; Forstner, 1978; Slomiany *et al.*, 1994; Henderson *et al.*, 1996). A remarkable feature of the indigenous intestinal microbiota of fish is that situations like stress, antibiotic administration, and even small dietary changes, affect the microbial community of the digestive tract. The stability of the intestinal flora is an extremely important factor in the natural resistance of fish to infections produced by bacterial pathogens in the digestive tract. Interest in the phenomenon of resistance provided by flora components against colonization by pathogens has existed for many years in the endothermic literature (for review see Hentges, 1983; Hentges, 1992; Tancrede, 1992; Salminen *et al.*, 1996). A fundamental question when discussing translocation from the gastrointestinal tract in fish is if the autochthonous microbiota has a protective role against pathogenic bacteria by producing antibacterial substances. Information about the existence of antibacterial substances produced by bacteria isolated from the digestive tract of fish has been demonstrated in several comprehensive reviews (Ringø and Gatesoupe, 1998; Gatesoupe, 1999; Hansen and Olafsen, 1999; Ringø and Birkbeck, 1999; Gomez-Gill *et al.*, 2000; Gram and Ringø, 2002; Ringø *et al.*, 2002b). However, it is not yet known to what extent the natural microbiota of fish may be protective towards pathogen colonization. This is an important subject to clarify as the aquaculture industry is plagued by many disease problems, and an important goal for the microbiologist should therefore be to increase colonization of the gut by bacteria with an antibacterial potential against fish pathogens.

PROBIOTICS

The use of food containing live microorganisms with beneficial properties has been known for centuries. O'Sullivan *et al.* (1992) referred to Plino who advocated the use of fermented milk products in the treatment of various gastrointestinal infections as early as 76 BC. In modern time the term "probiotic" was first used in 1954 by Vergin, but since then, many different variations of the definition have been proposed (Table 1).

Antibiotic treatment to prevent infection is not recommended because of selection for strains resistant to chemotherapy but also because of concern about environmental risk and that resistant strains in the environment may transfer R plasmid to human intestinal microbiota. Nevertheless, its use is still a practical measure even methods do not prevent pathogenic proliferation in the system. Furthermore, the use of antibiotics may dramatically change the intestinal microflora of the fish and, thus impair its first-line defences (Austin and Al-Zahrani, 1988; Strøm and Ringø, 1993). An alternative method to antibiotic treatment would be the use of

Table 1. Definitions of probiotics by various authors (after Gram and Ringø, 2002)

Definition	Reference	Comments
microbial compounds which promotes body functions and beneficial microorganisms microbially produced "factors" which promote growth of other organisms	Vergin, 1954	products - not live culture?
animal feed supplements - organisms and substances that have a beneficial effect on the host animal by contributing to its intestinal microbial balance	Lilly and Stillwell, 1965 Parker, 1974	growth promotion only feed what is "microbial balance"
live microbial feed supplements which beneficially affect the host animal by improving its intestinal microbial balance	Fuller, 1989	only feed what is "microbial balance"
a microbial dietary adjuvant that beneficially affects the host physiology by modulating mucosal and systemic immunity, as well as improving nutritional and microbial balance in the intestinal tract	Naidu <i>et al.</i> , 1999	only feed what is "microbial balance"
live microorganisms supplemented in food or feed which give beneficial effects on the intestinal microbial balance	Gildberg <i>et al.</i> , 1997	only feed what is "microbial balance"
a live microbial supplement which beneficially affects the host animal by improving its microbial balance is based on elimination of harmful microflora from the animal's digestive tract	Gram <i>et al.</i> , 1999	what is "microbial balance"
mono- or mixed cultures of live microorganisms which when applied to animal or man, beneficially affect the host by improving the properties of the indigenous microflora	Bogut <i>et al.</i> , 1998	elimination? only dietary tract
viable microorganisms (bacteria or yeasts) that exhibit a beneficial effect on the health of the host when they are ingested	Havenaar <i>et al.</i> , 1992	what are "properties of indigenous microflora"
beneficial bacteria which may override pathogens by producing inhibitory substances, or by preventing pathogenic colonization of the host	Conway, 1996	
beneficial bacteria that displace pathogens by competitive processes or by release of growth inhibitors	Holzapfel <i>et al.</i> , 1998	
live intestinal bacteria that are added to promote the viability of the host, but the term is also proper for bacteria able to regulate colonization of the outer surfaces	Salminen <i>et al.</i> , 1998	what is "override"
live microbial adjunct which has a beneficial effect on the host by modifying the host-associated or ambient microbial community, by ensuring improved use of the feed or enhancing its nutritional value, by enhancing the host response towards disease, or by improving the quality of its ambient environment	Riquelme <i>et al.</i> , 2000	
oral probiotics are living microorganisms, which upon ingestion in certain numbers, exert health benefits beyond inherent basic nutrition	Moriarty, 1997	
live microbial cultures added to feed or environment (water) to increase viability (survival) of the host	Skjermo and Vadstein, 1999	how is "colonization" regulated?
	Verschuere <i>et al.</i> , 2000	
	Guarner and Schaafsma, 1998	
	Gram and Ringø, 2002	

probiotics, or beneficial bacteria, which override pathogens by producing inhibitory substances, or by preventing pathogenic colonization in the host.

Table 2 shows the effect of addition of probiotic microorganisms (*Vibrio pelagius*, *V. mediterranei* Q40, *Aeromonas media*, *Pseudomonas* ssp. *Vibrio* ssp. and *Thalassobacter utilis*) on fish and crustacean larval survival.

Table 2. Effect of addition of probiotic microorganisms on fish and crustacean larval survival (after Gram and Ringø, 2002)

Presumed probiont	Pathogen	Host organisms	Effect on survival	Reference
<i>Vibrio pelagius</i>	not known	Turbot larvae	Increase accumulated survival from 6-9% on day 12 and from day 0-3% on 16 after hatching	Ringø and Vadstein, 1998
<i>Vibrio mediterranei</i> Q40	not known	Turbot larvae	Increase accumulated survival (5 days post hatching) in 5 separate experiments (e.g. 14 to 55% in trial 1 or 75 to 81% in trial 4).	Huys <i>et al.</i> , 2001
<i>Aeromonas media</i>	<i>Vibrio tubiashii</i>	Oyster larvae	Increase survival after 6 days from 4-100%	Gibson <i>et al.</i> , 1998
<i>Pseudomonas</i> and unknown strain	<i>Vibrio anguillarum</i> like	Scallop larvae	Increase survival from 5-60% after 14 days	Riquelme <i>et al.</i> , 1997
<i>Pseudomonas</i> and <i>Vibrio</i>	field trial? not known	Scallop larvae	Same survival after 48 hours as antibiotic treated tanks	Riquelme <i>et al.</i> , 2001
<i>Thalassobacter utilis</i>	field trial not known (<i>Vibrio</i> spp.)	Crab larvae	Increase survival from 16 to 26%	Nogami and Maeda, 1992 Nogami <i>et al.</i> , 1997

Gatesoupe (1997) tested in his study a siderophore-producing *Vibrio* as probiotic by feeding infected turbot larvae with rotifers enriched by the *Vibrio*. He reported improved larval survival 48 hours after infection. However, 10 days post-infection, no difference was seen in survival. In contrast to these results, Ringø and Vadstein (1998) reported that addition of *V. pelagius*, originally isolated from turbot larvae, to early developing turbot larvae had no short term effect, but caused a slight increase in survival after 12-16 days compared to larvae exposed to *Aeromonas caviae*. In an earlier study, Ringø *et al.* (1996) suggested by using an enzyme-linked immunosorbent assay that *V. pelagius* seems to colonize the larval gut when the bacteria was added to the tank water at the day of hatching but it was, however, not shown whether the

bacterium could persist in the gut if the larvae were removed from the *V. pelagi*-containing tank water.

In a recent study, Huys *et al.* (2001) searched for beneficial bacterial strains for turbot larviculture and reported increased survival of larvae by addition of a *V. mediterranei* Q40 originally isolated from sea bream larvae and an unknown organism isolated from turbot larvae at a concentration of 10^5 bacteria per ml water.

Some information is available on bacterial probiotics in culturing of Pacific oyster (*Crassostrea gigas* Thunberg) larvae. Gibson *et al.* (1998) used an *Aeromonas* media-like strain, originally isolated from Koi carp (*Cyprinus carpio*) from the Hawksbury River (Gibson, pers. com., 2001 to Lone Gram, Danish Institute for Fisheries Research, Denmark), by adding the probiont to waters of oyster larvae which had been challenged with the pathogen *V. tubiashii*. The challenge test caused an increase in numbers of the pathogen and a complete kill of the oyster population within five days, but by adding the *Aeromonas* media strain together with the pathogen, reduced numbers of *V. tubiashii* and resulted in complete survival of the population. These results are in accordance with earlier results demonstrating that additions of both algae (McCausland *et al.*, 1999) and bacteria (strain CA2) (Douillet and Langdon, 1994) improved growth of the Pacific oyster larvae.

Riquelme *et al.* (1997) investigated 506 bacterial isolates, obtained from laboratory and hatchery sources for their potential probiotic effect in Chilean scallop (*Argopecten purpuratus* Lamarck 1819) larval culture. Initially, both a *Pseudomonas* isolate showing *in vitro* activity against a larval pathogen a *V. anguillarum*-related strain and an unidentified isolate with no *in vitro* inhibitory activity were found to improve survival from 5% in the non-probiotic treated to 60% in the probiotic treated over a 14 day period. Screening the 506 bacterial strains, the authors found that only 11 isolates were able to inhibit growth of a *V. anguillarum* related bacterium associated with mortality of scallop larvae. However, several strains showing *in vitro* activity increased mortality of scallop larvae. Thus, this study clearly demonstrates the importance of *in vivo* testing, as strains with *in vitro* effect may be dangerous to the animals, and strains with no *in vitro* effect may have probiotic effects *in vivo*.

Recently, Riquelme *et al.* (2001) reported that growth and survival in field trials with Chilean scallop larvae treated with pathogen-antagonizing bacteria (*Vibrio* sp. C33 and *Pseudomonas* sp. 11) at 10^3 colony forming units per ml (CFU/ml) were the same as when the larvae were treated with antibiotics. The antagonizing bacteria were added to the water at the initiation of the experiment and again after 48 hours. Controls with no treatment were not included as the commercial producer experienced rapid mortality when no treatment was used. In an earlier investigation, Riquelme *et al.* (2000) studied the uptake of pathogen-inhibiting bacterial cultures in Chilean scallop larvae, and found that an *Arthrobacter* was ingested in significant numbers. This can be a way of continuously adding the probiotic culture to the scallop larvae. The *Arthrobacter* strain was not tested in *in vivo* infection trials.

In two studies, Nogami and Maeda (1992) and Nogami *et al.* (1997) added 10^5 - 10^6 CFU/ml of bacterial culture isolated from shrimp pond to seawater used for crab (*Portunus trituberculatus*) culture. The strain was a Gram-negative, non-fermentative, motile rod identified as *Thalassobacter utilis* (Maeda and Liao, 1992; Nogami *et al.*, 1997). The culture, which was added once every

seven days, was selected based on its ability to improve survival in *in vivo* infection trials. The organism also inhibited growth of *V. anguillarum*, *in vitro*. By adding the culture, a decline in concentration of *Vibrio* spp. in the seawater occurred and crab survival was significantly improved. It should be emphasized that the addition of five other microbial cultures (e.g. a *Bacillus subtilis*) accelerated mortality of the larvae (Nogami and Maeda, 1992).

Prevention of bacterial disease in growing fish is somewhat easier than in larvae, fry and juveniles. However, vaccination of fish smaller than 35 g (Intervet International, The Netherlands; Aqua Health Ltd, Canada) is not recommended, making the smaller stages of fish still susceptible to infection. For a range of bacterial pathogen-host combinations, good vaccines have been developed, and their optimization and use will be facilitated as further understanding of the pathogen virulence factors and of the host immune system emerges. The studies described in Table 3 should be regarded as trials of the probiotic concept rather than as suggestions for actual use of probiotics. Both the bacteriophage, *Tetraselmis*, Gram-positive bacteria (bacilli and caryobacteria, a lactic acid bacteria) and Gram-negative bacteria such as *Vibrio* and *Pseudomonas* have been evaluated as potential probiotics and the experiments cover both additions to the rearing water or incorporation in the feed (Table 3).

The pathogens which are most frequently used include; *Pseudomonas plecoglossicida*, *anguillarum*, *V. ordalli*, *Yersinia ruckeri*, and *A. salmonicida*. In most of the cited studies in Table 3 increased survival of the host organisms were observed.

Readers who want more information about probiotics in aquaculture are referred to the reviews of Gatesoupe (1999), Gomez-Gil *et al.* (2000), Verschuere *et al.* (2000) and Gram and Ringø (2002).

LACTIC ACID BACTERIA (LAB)

It is suggested that lactic acid bacteria (LAB) along with other bacteria that belong to the autochthonous (indigenous) microbiota of aquatic animals might be an important part of the defence mechanism against colonization of fish pathogens in the gastro-intestinal tract. In addition to the antagonistic microorganisms colonizing the mucus surface in the natural microbial defence mechanisms, it has been shown that the surface mucus also plays a role in the prevention of colonization by parasites, bacteria and fungi.

Effects of LAB administration on intestinal microbiota

It is well known that LAB under normal circumstances are not numerically dominant in the digestive tract of fish (Ringø and Gatesoupe, 1998). In order to increase the proportion of LAB, some investigations have attempted to increase their population level by dietary factors such as: (1) chromic oxide (Ringø, 1993); (2) different oils (Ringø *et al.*, 1998; Ringø *et al.*, 2002b); (3) high and low dietary lipids (Ringø and Olsen, 1999); and (4) inulin (Ringø, Myklebust and Olsen, unpublished results). Another important criterion for the use of LAB in commercial aquaculture, is the colonization potential of LAB in the fish gut, as Vibrionaceae may also persist

Table 3. Effect of addition of probiotic microorganisms on fish survival. (after Gram and Ringø, 2002)

Presumed probioten	Pathogen	Host organisms	Effect on survival	Reference
bacteriophage ¹	<i>Pseudomonas plecoglossicida</i>	ayu	increase survival from 35-75%	Park <i>et al.</i> , 2000
<i>Tetraselmis suecica</i>	Several Gram-negatives	salmon	increase survival from 0-15% to 20-100%	Austin <i>et al.</i> , 1992
<i>Bacillus</i> spp.	field trial not known	channel catfish	increase survival from 56-80%	Queiroz and Boyd, 1998
<i>Carnobacterium</i> sp.	<i>Vibrio anguillarum</i>	salmon	no effect on survival	Robertson <i>et al.</i> , 2000
	<i>Vibrio ordalii</i>		increase survival from 23-74%	
	<i>Yersinia ruckeri</i>		increase survival from 42-71%	
	<i>Aeromonas salmonicida</i>		increase survival from 0-20%	
	<i>Aeromonas salmonicida</i>	trout	increase survival from 32-74%	
<i>Vibrio alginolyticus</i>	<i>Aeromonas salmonicida</i>	salmon	increase survival from 0-82%	Austin <i>et al.</i> , 1995
	<i>Vibrio anguillarum</i>		increase survival from 10-26%	
	<i>Vibrio ordalii</i>		increase survival from 0-26%	
	<i>Yersinia ruckerii</i>		no effect on survival	
<i>Pseudomonas fluorescens</i>	<i>Aeromonas salmonicida</i>	salmon	increase healthy fish from 25-70 to 90-100%	Smith and Davey, 1993
<i>Pseudomonas fluorescens</i>	<i>Vibrio anguillarum</i>	trout	increase survival from 50-70%	Gram <i>et al.</i> , 1999
<i>Pseudomonas</i> spp.	<i>Vibrio anguillarum</i>	trout	increase survival (some strains)	Spanggaard <i>et al.</i> , 2001
			no effect (some strains)	
<i>Pseudomonas fluorescens</i>	<i>Aeromonas salmonicida</i>	salmon	no effect on survival	Gram <i>et al.</i> , 2001

¹ It is debatable whether or not a bacteriophage qualifies as a probioten "a live microbial...." due to the inert nature of viruses

for days or weeks in fish (Austin *et al.*, 1995; Munro *et al.*, 1995; Ringø and Vadstein, 1998). Some recent studies have demonstrated that carnobacteria strains are able to survive for several days in the intestine of larval and juvenile fish (Strøm and Ringø, 1993; Jöborn *et al.*, 1997; Gildberg and Mikkelsen, 1998; Ringø, 1999). Three of these studies (Jöborn *et al.*, 1997; Gildberg and Mikkelsen, 1998; Ringø, 1999) have suggested that there is apparently no host specificity with regard to colonization of the fish gut with carnobacteria, contrary to endothermic animals where adhesion of LAB appears to be complicated by host specificity (Lin and Savage, 1984; Fuller, 1986, 1989; Conway, 1989). However, the colonization site in the fish gut is also an important criterion. In a recent study, Gildberg and Mikkelsen (1998) administered two *Carnobacterium divergens* strains originally isolated from the intestine of mature Atlantic cod (*Gadus morhua* L.) and Atlantic salmon, to Atlantic cod juveniles via the food. When the Atlantic cod isolate was used, the authors only detected *C. divergens* in pyloric caeca, while the concentration of the bacteria was approximately ten fold higher in the pyloric caeca than in the intestine when the salmon isolate was used.

Transient bacteria may also be efficient if the cells are introduced at high dose. Moreover, as LAB may exert antibacterial effects against undesirable microbes, some investigators have attempted to increase the proportion of LAB associated with the fish digestive tract. In a study with four days old Atlantic cod larvae, Strøm and Ringø (1993) used an antagonistic LAB strain which, when added to the rearing water, favourably influenced the intestinal microbiota of the larvae by increasing the proportion of LAB from approximately 5% up to 70% and by a subsequent decrease in the proportion of the bacteria genera *Pseudomonas*, *Cytophaga/Flexibacter* and *Aeromonas* (Table 4). These results indicate that the LAB are able to colonize and may comprise a major part of the autochthonous microbiota in the gut of the larvae. A similar increase in intestinal LAB was also found in Atlantic cod fry fed a diet containing *C. divergens* (Gildberg *et al.*, 1997) (Table 4). In a study with Atlantic salmon fry, Gildberg *et al.* (1995) demonstrated that administration of LAB reported as *Lb. plantarum*, but later reclassified as *C. divergens* (Ringø *et al.*, 2001a) increased the proportion of adherent LAB to intestinal wall from nil to 100% (Table 4).

Recently, Byun *et al.* (1997) evaluated the effect of LAB (*Lactobacillus* sp. DS-12) administration via the feed on the intestinal microbiota of flounder (*Paralichthys olivaceus*) after one month of feeding (Table 4). *Lactobacillus* sp. DS-12 was not detected in the intestine of the control group, but 10^7 /g LAB were found in the GIT when the fish were fed a LAB supplemented feed.

In a recent study, Bogut *et al.* (2000) evaluated the effect of *Enterococcus faecium* on the intestinal microbiota of Sheat fish (*Silurus glanis*). In this study, the fish were exposed to *E. faecium* by including the bacteria in the diet. After approximately two months of feeding, some interesting differences in the intestinal microbiota were observed between the two rearing groups. *Enterococcus faecium*-administration decreased the population level of *Staphylococcus aureus*, *Escherichia coli* and other bacteria of the family Enterobacteriaceae, and resulted in complete elimination of *Clostridium* spp. (Table 4).

Only one investigation has evaluated the influence of a commercial LAB preparation on the allochthonous intestinal microbiota. Supplementation of one gram of *E. faecium* M74 per 100 kg feed influenced the intestinal microbiota of 0+ Israeli carp (*Cyprinus carpio*) to some extent (Bogut *et al.*, 1998). While *E. coli* disappeared from the intestinal microbiota of the fish after 14

Table 4. Effect of lactic acid bacteria (LAB) administration on intestinal microbiota. (after Ringø *et al.*, 2002)

Fish species	LAB used	Bacterial genera isolated and proportion of microflora population before administration (control)	Bacterial genera isolated and proportion of microflora population after administration	after challenge	References
Atlantic cod - larvae	<i>C. divergens</i>	<i>Pseudomonas</i> 42.5; <i>Cytophaga</i> /Flexibacter 42.5	<i>C. divergens</i> 70; <i>Pseudomonas</i> 20	*	Strøm and Ringø, 1993
Atlantic cod - fry	<i>C. divergens</i>	<i>Aeromonas</i> 10; <i>C. divergens</i> 5 No information was given	No information was given	<i>C. divergens</i> 75 <i>Pseudomonas</i> -like 25 <i>A. salmonicida</i> 90 <i>C. divergens</i> 10	Gilberg <i>et al.</i> , 1997 Gjildberg <i>et al.</i> , 1995
Atlantic salmon-fry	<i>C. divergens</i>	<i>Pseudomonas</i> , Enterobacteriaceae Gram positive cocci	<i>C. divergens</i> 100	*	Ringø, 1999 Byun <i>et al.</i> , 1997
Turbot - larvae Flounder ¹	<i>C. divergens</i> <i>Lactobacillus</i> sp. DS-12	<i>C. divergens</i> n.d. Enterobacteriaceae 4.3 (5/5); G(+) 4.6 (5/5) Yeast 4.6 (5/5); Hemolytic bacteria 5.8 (2/5) Mucoid colony form 4.8 (1/5); Aerobes 8.5 (5/5) Anaerobes 7.6 (5/5)	<i>C. divergens</i> (8x10 ³) Enterobacteriaceae 4.8 (5/5); G(+) 4.3 (5/5) <i>Lactobacillus</i> sp. DS-12 7.0 (3/5) <i>Clostridium</i> 4.3 (1/5); Yeast 4.3 (1/5) Hemolytic bacteria 5.1 (1/5)	*	
Carp ^b	<i>Ent. faecium</i>	Aerobes 7.3 (5/5); Anaerobes 6.6 (5/5) Enterobacteriaceae 6.2; <i>E. coli</i> 4.2	Enterobacteriaceae 6.2; <i>E. coli</i> n.d.	*	Bogut <i>et al.</i> , 1998
Sheat fish ^c	<i>Ent. faecium</i>	<i>Ent. faecalis</i> 3.3; <i>Staph. aureus</i> 3.7 <i>Bacillus</i> spp. 7.0; <i>Clostridium</i> spp. 2.9	<i>Ent. faecalis</i> 3.5; <i>Staph. aureus</i> 4.0 <i>Bacillus</i> spp. 7.0; <i>Clostridium</i> spp. 2.7 <i>Escherichia coli</i> 1.1; Enterobacteriaceae 1.9 <i>Staph. aureus</i> 1.4 <i>Bacillus</i> 5.6; <i>Clostridium</i> n.d.	*	Bogut <i>et al.</i> , 2000

^a - Data are presented as log 10 and frequency are shown in parentheses; ^b - Data are presented as log 10 after 4 weeks of feeding

^c - Data are presented as log 10 after 58 days of feeding; n.d - not detected; * - challenge test not done

days and onwards by feeding the probiotic preparation (Table 4), the population level of Enterobacteriaceae, *E. faecalis*, *S. aureus*, *Bacillus* spp. and *Clostridium* spp. were not reduced as a result of including *E. faecium* into the diet (Bogut *et al.*, 1998). The authors suggested a high adhesive ability in the epithelium of carp digestive tract for *E. faecium*. However, as they isolated the allochthonous (transient) intestinal microbiota, convincing experimental evidence was not provided.

When dealing with the potential of probiotics (for example LAB) in aquaculture the fundamental question arises whether it is possible to colonize and maintain the probiotic bacteria within the digestive tract. This is particularly important when long-term exposure may be required for the probiotic effect. In this respect, electron microscope investigations are a useful tool (Ringø *et al.*, 2001b, 2002a).

During the last decade some reports have been published on the nutritional contribution of LAB to the production rate of rotifer *Brachionus plicatilis* (Gatesoupe, 1990; Gatesoupe, 1991), while the control of the microbiota of rotifer cultures has received less attention.

Challenges in vivo

The major factors involved in the biocontrol of bacterial pathogens in the gastrointestinal tract are primarily those regulating the composition, functions and interactions of indigenous microbial populations with the animal tissues. This concept is supported by repeated observations that strains of transient enteropathogens can colonize intestinal habitats of endothermic animals. The fact that fish contain intestinal microbiota with antagonistic effects against fish pathogens has prompted investigators to conduct challenge experiments with LAB during the last decade (Gatesoupe, 1994; Gildberg *et al.*, 1995, 1997; Gildberg and Mikkelsen, 1998; Harzevili *et al.*, 1998). However, in these studies some conflicting results on the mortality were reported when the control group was compared with probiotic treatment (Table 5).

Gatesoupe (1994) suggested that *in vivo* experiments with turbot larvae using rotifers grown on LAB strains (resembling those of *Lactobacillus plantarum* or *Carnobacterium* sp.) improved the disease resistance in challenge tests with pathogenic *Vibrio* (*V. splendidus* strain VS 11). However, the results reported in this study were registered after 48 and 72 hours, beyond which the mortality pattern was not discussed. In three papers, Gildberg and Mikkelsen (1998) and Gildberg *et al.* (1995; 1997) have used two LAB strains originally isolated from Atlantic salmon and Atlantic cod by Strøm (1988). These two isolates were recently identified by 16S rDNA and AFLP™ fingerprinting as *C. divergens* (Ringø *et al.*, 2001a). In challenge trials with cohabitants with *A. salmonicida*, Gildberg *et al.* (1995) in contrast to the expectations, registered highest mortality of Atlantic salmon fry with fish given the diet containing *C. divergens*, originally isolated from Atlantic salmon intestine. In their study with Atlantic cod fry, Gildberg and Mikkelsen (1998) observed the same cumulative mortality independent whether the *C. divergens* isolates supplemented to the commercial feed were originally isolated from the digestive tract of Atlantic cod or Atlantic salmon, when the fish were bath exposed to *V. anguillarum*. On the other hand, an improved disease resistance of Atlantic cod fry was observed by supplementing a commercially dry feed with a strain of *C. divergens* originally isolated from the cod (Gildberg *et al.*, 1997). The

Table 5. Challenge tests of fish with lactic acid bacteria (LAB) (after Ringø *et al.*, 2002)

LAB isolate used	Host	Pathogen	Way of administration	Effect in challenge test	Suggested mode of action	Reference
<i>Carnobacterium</i> spp. ^a	Turbot - larvae	<i>V. splendidus</i>	Enrichment of rotifers	+	Antagonism and/or improved nutritional value of the rotifers	Gatesoupe, 1994
<i>C. divergens</i> ^b	Atlantic salmon - fry	<i>A. salmonicida</i>	Addition to the diet	-		Gildberg <i>et al.</i> , 1995
<i>C. divergens</i> ^c	Atlantic cod - juveniles	<i>V. anguillarum</i>	Addition to the diet	+	Not specified	Gildberg <i>et al.</i> , 1997
<i>C. divergens</i> ^b	Atlantic cod - fry	<i>V. anguillarum</i>	Addition to the diet	+ ^e	Antagonism	Gildberg and Mikkelsen, 1998
<i>C. divergens</i> ^c	Atlantic cod - fry	<i>V. anguillarum</i>	Addition to the diet	-		Gildberg and Mikkelsen, 1998
<i>Lb. rhamnosus</i> ^d	Rainbow trout	<i>A. salmonicida</i>	Addition to the diet	+		Nikoskelainen <i>et al.</i> , 2001

+ improved disease resistance; - no significant effect

* - isolated from rotifer; b. isolated from intestine of Atlantic salmon (Strøm, 1988); c - isolated from intestine of Atlantic cod (Strøm, 1988); d - a probiotic for human use

e - Twelve days after infection significant reduced cumulative mortality was recorded in fish given feed supplemented with *C. divergens* isolated from Atlantic salmon, but no effect was detected four weeks after infection

explanation for these conflicting results has not been elucidated. Gildberg and Mikkelsen (1998) put forward a hypothesis that bacteriocin production can be inducible and may not occur if the bacteria are not frequently challenged with inhibitors as previously demonstrated by Schröder *et al.* (1980). Furthermore, a recent study by Nikoskelainen *et al.* (2001) used the human probiotic *Lactobacillus rhamnosus* in a challenge test with *A. salmonicida* with promising results (Table 5). These results should stimulate fish microbiologist to use human probiotic LAB in future studies.

If the intestine is involved in infection as suggested by several authors, the fundamental question arises whether there are differences between the posterior part of the intestine and the hindgut region of the intestine? It is well established that the intestine in an immature or inflammatory state has an enhanced capacity to absorb intact macromolecules (for review see Olsen and Ringø, 1997). Furthermore, some studies report endocytosis of bacteria by enterocytes in the epithelial border of hindgut of herring (*Clupea harengus*) larvae (Hansen *et al.*, 1992; Hansen and Olafsen, 1999), herring and Atlantic cod larvae (Olafsen and Hansen, 1992) and 36 days old juvenile turbot (Grisez *et al.*, 1996). It is generally accepted that mature and non-inflammatory intestines of adult salmonids are not permeable to microparticulates in contrast to the mammalian gastrointestinal tract where M-cells are active in phagocytosis. However, a recent study demonstrated endocytosis of bacteria by enterocytes in the epithelial border of hindgut of adult salmonid fish (Fig. 6a), as well as in the posterior part of the intestine (pyloric caeca) (Fig. 6b) (Ringø *et al.*, 2002b). These results are in accordance with observations made by Vigneulle and Laurencin (1991) and Tamura *et al.* (1993) who measured phagocytosis of fixed *V. anguillarum* in the posterior intestine of rainbow trout (*Oncorhynchus mykiss*), sea bass (*Dicentrarchus labrax*), turbot (*Scophthalmus maximus*) and eel (*Anguilla anguilla*).

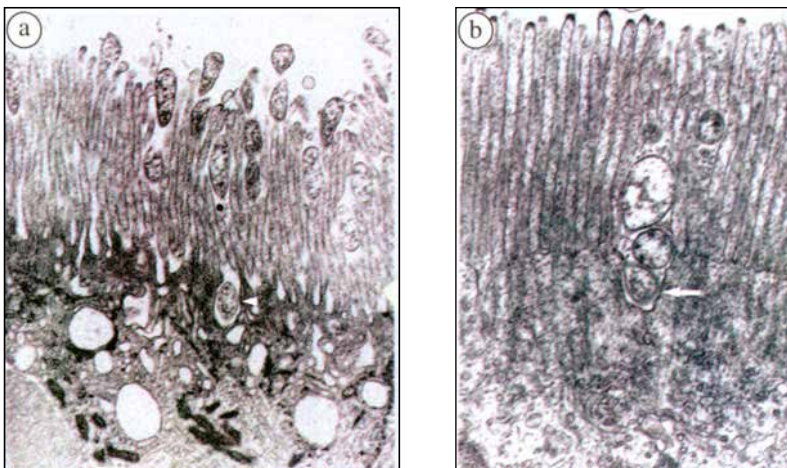


Figure 6. Endocytosis of bacteria demonstrated in the hindgut region (a) and pyloric caeca (b) of Arctic charr (*Salvelinus alpinus* L.). (after Ringø *et al.*, 2002b)

The observations of Vigneulle and Laurencin (1991), Tamura *et al.* (1993) and Ringø *et al.* (2001b, 2002a, 2002b) indicate that the intestine is involved in bacterial translocation. Yet no clear evidence is available on possible differences between different parts of the intestine with regard to bacterial infection.

It is well known that rotifers are often suspected of being a vector for bacterial infections to the predating organisms (Perez-Benavente and Gatesoupe, 1988; Tanasomwang and Muroga, 1988; Nicolas *et al.*, 1989). It is therefore surprising that studies dealing with the proliferation of larval pathogens in rotifer cultures are so scarce (Gatesoupe, 1991; Hazevili *et al.*, 1998). Gatesoupe (1991) reported that the proliferation of *A. salmonicida* that accidentally appeared in the experimental rotifer culture was inhibited by treatment with *Lb. plantarum*. Hazevili *et al.* (1998) reported that administration of the probiotic strain *Lac. lactis* AR21 under sub-optimal feeding regime, counteracted the growth inhibition of the rotifers due to *V. anguillarum*.

Readers with special interest in lactic acid bacteria in fish are referred to the comprehensive reviews of Ringø and Gatesoupe (1998) and Ringø *et al.*, 2002c.

PREBIOTICS

Specific bacterial pathogens can be an important cause of mortality as intensive husbandry practices often result in breakdown of natural barriers between the host and pathogens. Nowadays, the prevention and control of these diseases has concentrated on good husbandry practices and the use of vaccines and antibiotics. However, treatment by feeding antibiotics may cause the development of resistant bacteria through plasmids or bacteriophages (Towner, 1995). Therefore, there is an increased interest within the aquaculture industry in the control or elimination of antimicrobial use. Alternative methods need to be developed to maintain a healthy microbial environment. Two such methods that are gaining acceptance within the industry are the use of probiotic bacteria or prebiotics to control potential pathogens.

During the last decade, several reviews have dealt with the potential of probiotics in aquaculture (Ringø and Gatesoupe, 1998; Gatesoupe, 1999; Hansen and Olafsen, 1999; Ringø and Birkbeck, 1999; Gomez-Gill *et al.*, 2000; Verschuere *et al.*, 2000; Gram and Ringø, 2002; Ringø *et al.*, 2002c). It is therefore a pertinent question whether it is possible to colonize and maintain probiotic bacteria within the digestive tract. This is particularly important when long-term exposure is required for the probiotic effect. However, to date, there is no real evidence demonstrating the preventive effect of probiotics against colonization and adherence of fish pathogenic bacteria in aquaculture. The reason for this may be that the probiotics used are unable to colonize the mucus layer of the digestive tract or external surfaces. Examination of adhesion has become a standard procedure for selecting new probiotic strains for human application (Salminen *et al.*, 1996), but it is less common in aquaculture.

The addition of high doses of probiotic strains (for example, lactic acid bacteria) to established microbial communities of fish provoked a temporary change in the composition of the intestinal microbial community. However, within a few days after administration had stopped, the added strains showed a sharp decrease and were lost from the gastrointestinal tract (Jöborn *et al.*,

1997; Ringø and Gatesoupe, 1998). Another way to colonize and increase the population level of beneficial bacteria with antagonistic ability is the use of prebiotics. The modern concept of prebiotics implies the use of selective agents to favour growth of the protective indigenous gut microbiota. Dietary fiber is a prebiotic that belongs to the broad category of carbohydrates. Burkitt *et al.* (1972) defined dietary fiber as “the sum of polysaccharides and lignin which are not digested by the endogenous secretions of the human gastrointestinal tract.” They can be classified into soluble (*e.g.*, inulin and oligofructose), insoluble (*e.g.*, cellulose) or mixed (*e.g.*, bran). It is well known from endothermic investigations that dietary fibers are fermented by the anaerobic intestinal microbiota, primarily those colonizing the large intestine (Roberfroid, 1993; Gibson *et al.*, 1995; Roberfroid, 1995; Gibson, 1998; Rumessen and Gudmand-Høyer, 1998), leading to the production of lactic acid, short chain fatty acids (SCFA-acetate, propionate, and butyrate) and gases (H₂, CO₂ and CH₄) (Roberfroid, 1993) that are utilized by the host (Schneeman, 1999). Inulin, is a polydisperse carbohydrate consisting mainly of β (2→1) fructosyl-fructose links, generally referred to as fructan and is found in various edible fruits and vegetables such as wheat, onions, leeks, garlic, asparagus, artichokes and bananas (Roberfroid, 1993; Van Loo *et al.*, 1995). Although inulin is not a natural fiber in fish diet, the prebiotic potential of inulin and other dietary fibers may also have interesting applications in aquaculture. Some information is available about fermentation of inulin by fish gut microbiota, notably, *Carnobacterium piscicola* (Ringø *et al.*, 1998), *C. mobile* (Ringø and Olsen, 1999), and *Carnobacterium* spp. (Ringø and Olsen, 1999; Ringø *et al.*, 2001a). It is also known that dietary inulin resulted in damage to intestinal enterocytes of the salmonid fish Arctic charr (Fig. 7) (Olsen *et al.*, 2001) compared to normal enterocytes (Fig. 8), and that dietary inulin alters the adherent gut microbiota of Arctic charr (Ringø, unpublished results). However, the effect of dietary inulin on fish welfare is not yet known.

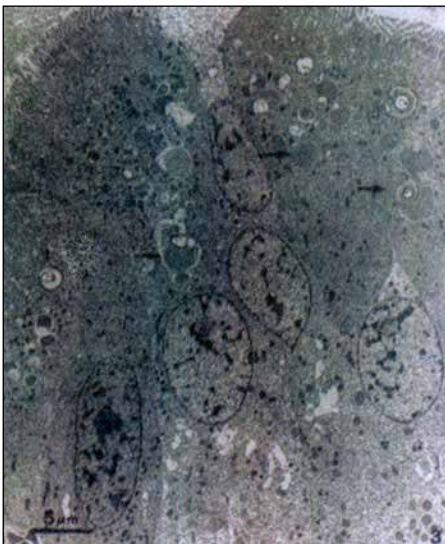
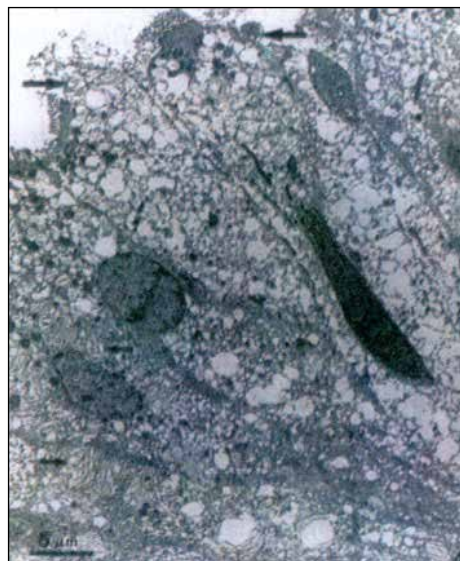


Figure 7. The epithelium in the hindgut of Arctic charr (*Salvelinus alpinus* L.) fed dietary inulin. The cells are highly vacuolated and many of the vacuoles have lamellar content (small arrows) which may be inulin. The apical surface of these cells shows sign of damage including loss of membrane and microvilli (large arrows). (after Olsen *et al.*, 2001)

Figure 8. Epithelial cells in the hindgut of Arctic charr (*Salvelinus alpinus* L.) fed control diet (dietary dextrin). The columnar cells typically show numerous vacuoles varying in size and electron density (arrows). Cytoplasm, microvilli and intracellular organelles appear normal. In the upper middle part of the field, an effete enterocyte is probably being shed as part of normal epithelial turnover. (after Olsen *et al.*, 2001)



ACKNOWLEDGEMENTS

Thanks to Drs. Bergh, Grisez and Olsen and Mr. Lødemel for providing their photographs.

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