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Effect of dietary components on the gut microbiota of aquatic animals. A never-ending story?

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Abstract

It is well known that healthy gut microbiota is essential to promote host health and well-being. The intestinal microbiota of endothermic animals as well as fish are classified as autochthonous or indigenous, when they are able to colonize the host's epithelial surface or are associated with the microvilli, or as allochthonous or transient (associated with digesta or are present in the lumen). Furthermore, the gut microbiota of aquatic animals is more fluidic than that of terrestrial vertebrates and is highly sensitive to dietary changes. In fish, it is demonstrated that [a] dietary form (live feeds or pelleted diets), [b] dietary lipid (lipid levels, lipid sources and polyunsaturated fatty acids), [c] protein sources (soybean meal, krill meal and other meal products), [d] functional glycomic ingredients (chitin and cellulose), [e] nutraceuticals (probiotics, prebiotics, synbiotics and immunostimulants), [f] antibiotics, [g] dietary iron and [h] chromic oxide affect the gut microbiota. Furthermore, some information is available on bacterial colonization of the gut enterocyte surface as a result of dietary manipulation which indicates that changes in indigenous microbial populations may have repercussion on secondary host–microbe interactions. The effect of dietary components on the gut microbiota is important to investigate, as the gastrointestinal tract has been suggested as one of the major routes of infection in fish. Possible interactions between dietary components and the protective microbiota colonizing the digestive tract are discussed.

KEY WORDS: antibiotics, aquatic animals, dietary components, intestine, microbiota

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Introduction

Numerous studies have reviewed the structure and function of the fish gut in relation to the diet (Bakke *et al.* 2011; Buddington *et al.* 1997; Olsen & Ringø 1997; Ray & Ringø 2014). As the gut microbiota has not been included in these papers, a more specific discussion is needed. Therefore, the main objective of the present review was to summarize the available information regarding the effect of dietary components on the gastrointestinal (GI) microbiota of fish.

Until the 1970s, controversy existed about the role, and even the existence, of an indigenous gut microbiota in fish. However, it is now generally accepted that fish and other aquatic animals have a microbiota in the GI tract (for review see; Yoshimizu & Kimura 1976; Horsley 1977; Cahill 1990; Ringø *et al.* 1995; Hansen & Olafsen 1999; Ringø & Birkbeck 1999; Ringø *et al.* 2003; Ringø 2004; Austin 2006; Izvekova *et al.* 2007; Merrifield *et al.* 2010a,

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2011a; Nayak 2010a; Pérez *et al*. 2010; Ringø *et al*. 2010a; Lauzon & Ringø 2012; Nya & Austin 2013; Llewellyn *et al*. 2014) which in turn has increased interest in their diversity and functional relationship (Clements *et al*. 2014; Zhou *et al*. 2014). However, the gut microbiota is modulated by dietary manipulations (Table 1) as well as by seasonal variations, stress, individual variations and different regions of the GI tract, cultured versus wild, triploid versus diploid, day-to-day variations, male versus female, developmental stages/life cycle, microbial aspects of live feed, fast versus slow growing, hierarchy formation, starvation, migration from fresh water into sea water and migration from sea water back to fresh water, water quality [pseudo-green water versus clear water, recirculation versus conventional flow-through, fish farms within a restricted area, environmental and ecological factors, and host ecology and environment (Table 2)]. To avoid the individual level variations of the gut microbiota, several studies have analysed the samples of pooled individuals (e.g. Hovda *et al*. 2007; Ringø *et al*. 1995; Roeselers *et al*. 2011; Spanggaard *et al*. 2000; Sullam *et al*. 2012; Zarkasi *et al*. 2014).

The intestinal microbiota of fish, as is the case of mammals, is classified as autochthonous (indigenous) or allochthonous bacteria (Kim *et al*. 2007; Ringø & Birkbeck 1999; Ringø *et al*. 2003). The autochthonous bacteria are those able to colonize the host's gut epithelial surface or are associated with the microvilli (Fig. 1), while the allochthonous bacteria are transient, associated with food particles or present in the lumen. In this context, it is important to evaluate the effect of dietary components on the intestinal microbiota of fish, as the gastrointestinal (GI) tract is one of the major ports of entry for some pathogens (Birkbeck & Ringø 2005; Burbank *et al*. 2011; Groff & LaPatra 2000; Harikrishnan & Balasundaram 2005; Ringø *et al*. 2007a,b).

In the 1970s, 1980s and 1990s, numerous investigations were conducted to determine the dietary effects on the intestinal microbiota, and the majority of these studies were based on culture-dependent techniques and the use of physiological and biochemical properties to characterize the gut microbiota. However, from 2000 to 2006, there was a shift to use molecular methods to characterize culturable gut bacteria (e.g. Huber *et al*. 2004; Jensen *et al*. 2002; Ringø *et al*. 2006a,b,c; Spanggaard *et al*. 2000), but nowadays culture-independent methods have become more common (Table 3). These recent investigations have widened our knowledge about the intestinal microbiota of fish and demonstrate that the microbial diversity of the fish gut is more complex than previously believed.

Table 1 Overview of studies investigated the effect of diet on gut microbiota of aquatic animals

Dietary component used	Aquatic animals	References
Levels of dietary lipid	Rainbow trout	Lesel *et al*. (1989)
	Arctic charr	Ringø & Olsen (1999)
Different dietary lipid sources	Arctic charr	Ringø *et al*. (2002)
	Gilthead sea bream	Montero *et al*. (2006)
	Atlantic salmon	E. Ringø, R.E. Olsen & S. Sperstad (unpublished data Table 4)
	Rainbow trout	Ringø *et al*. (unpublished data – Table 5)
	Grass carp	Huang (2008)
Dietary polyunsaturated fatty acids	Arctic charr	Ringø (1993a); Ringø *et al*. (1998)
	Brown trout	Manzano *et al*. (2012)
Thymus vulgaris essential oil	Rainbow trout	Navarrete *et al*. (2010b)
Levels of dietary fish protein hydrolysates	European sea bass	Kotzamanis *et al*. (2007)
Different diets	Gold fish	Sugita *et al*. (1988a)
	Atlantic cod	Strøm & Olafsen (1990)
	Arctic charr	Ringø & Olsen (1994)
	Rainbow trout	Mansfield *et al*. (2010)
	Puffer fish	Yang *et al*. (2007)
	Yellow grouper	Feng *et al*. (2010)
	Rohu	Ramachandran *et al*. (2005)
	Senegalese sole	Makridis *et al*. (2005)
	Tilapia	Kihara & Sakata (1997)
	Winter flounder	Seychelles *et al*. (2011)
	Common carp	Li *et al*. (2013)
	Threespine stickleback and Eurasian perch	Bolnick *et al*. (2014)
	Abalone	Tanaka *et al*. (2003, 2004)
Different binding agents	Plaice	Gilmour *et al*. (1976)
Marine protein hydrolysates	Sea bass	Delcroix *et al*. (2015)
Microalga (*Scenedesmus alimeriensis*)	Gilthead sea bream	Vizcaíno *et al*. (2014)
Soybean meal products	Atlantic cod	Refstie *et al*. (2006), Ringø *et al*. (2006b)

Table 1 (Continued)

Dietary component used	Aquatic animals	References
	Atlantic salmon	Bakke-McKellep *et al*. (2007), Ringø *et al*. (2008), Green *et al*. (2013), Navarrete *et al*. (2013)
	Gilthead seabream	Dimitroglou *et al*. (2010)
	Gilthead seabream	de Paula Silva *et al*. (2011)
	Goldfish	de Paula Silva *et al*. (2011)
	Grass carp	Huang (2008)
	Rainbow trout	Heikkinen *et al*. (2006), Merrifield *et al*. (2009a)
	Silver crucian carp	Cai *et al*. (2012)
	Different fish species	Merrifield *et al*. (2011a)
Plant-based diets	Rainbow trout	Desai *et al*. (2012)
Invertebrate meals	Mirror carp	Z.Y. Wan, S. Davies & D.L. Merrifield (unpublished data)
Casing meal	Grass carp	Huang (2008)
Rapeseed – and cottonseed meal	Grass carp	Huang (2008)
Wheat middling and corn meal	Grass carp	Huang (2008)
Wheat germ roots and distillers dried grains	Grass carp	Huang (2008)
Different protein sources	Atlantic salmon	Hartviksen *et al*. (2014, 2015)
Atlantic krill	Atlantic salmon	Ringø *et al*. (2006c)
Chitin	Red sea bream	Sera & Kimata (1972), Kono *et al*. (1987)
	Atlantic cod	Zhou et al. (2013a)
	Atlantic salmon	Askarian *et al*. (2012)
	Japanese eel	Kono *et al*. (1987)
	Giant fresh water prawn	Kumar *et al*. (2006)
Chitosan	Gibel carp	Chen *et al*. (2014)
Cellulose	Atlantic salmon	Ringø *et al*. (2008)
Cellulase	Grass carp	Zhou *et al*. (2013b)
Xylanase	Jian carp	Jiang *et al*. (2014)
Iron	Sea bass	Gatesoupe *et al*. (1997)
Copper	Nile tilapia	Hu *et al*. (2007)
Metal-nanoparticle	Zebra fish	Merrifield *et al*. (2013)
Chromic oxide (Cr_2O_3)	Arctic charr	Ringø (1993b,c, 1994)
Inositol	Jian carp	Jiang *et al*. (2009)

Table 1 (Continued)

Dietary component used	Aquatic animals	References
Acidic calcium sulphate	Pacific white shrimp	Anuta *et al*. (2011)
Sodium butyrate	Tropical catfish	Owen *et al*. (2006)
	Common carp	Liu *et al*. (2014)
Organic acid blend (formic-, lactic-, malic-, tartaric- and citric acid)	Red hybrid tilapia	Koh *et al*. (2016)
Organic acid (Mera™ Cid)	Rainbow trout	Jaafar *et al*. (2013)
Salt	European sea bass	Sun *et al*. (2013)
Alginic acid	Tilapia	Merrifield *et al*. (2011c)
Poly-β-hydroxybutyrate	Siberian sturgeon	Najdegerami *et al*. (2012, 2015)
	Giant fresh water prawn	Nhan *et al*. (2010)
Vitamin C	Jian carp	Liu *et al*. (2010)
Methionine	Jian carp	Tang *et al*. (2009)
Valine	Jian carp	Dong *et al*. 2013;
Inositol	Jian carp	Jiang *et al*. (2009)
Pantothenic acid	Jian carp	Wen *et al*. (2010)
Biotin	Jian carp	Zhao *et al*. (2012b)
Thiamine	Jian carp	Feng *et al*. (2011)
Phosphorus	Jian carp	Xie *et al*. (2011)
Betaine	Hybrid tilapia	He *et al*. (2012)
Probiotics	Different fish species	Ringø (2004), Gatlin *et al*. (2006), Ringø *et al*. (2005), Merrifield *et al*. (2010a,b,c), Dimitroglou *et al*. (2011), Lauzon & Ringø 2012, Daniels *et al*. (2013), Hoseinifar *et al*. (2014b), Cordero *et al*. (2015)
Prebiotics	Different fish species	Gatlin *et al*. (2006), Merrifield *et al*. (2010a), Ringø *et al*. (2010b), Dimitroglou *et al*. (2011), Daniels *et al*. (2013), Hoseinifar *et al*. (2014a,b), Ringø *et al*. (2014a)
Synbiotics	Different species	Abid *et al*. (2013), Daniels *et al*. (2013), Hoseinifar *et al*. (2014a,b), Ringø & Song (2016)
Yeast culture	Hybrid tilapia	Zhou *et al*. (2009c)

Table 1 (Continued)

Dietary component used	Aquatic animals	References
Inactive brewer's yeast	Beluga	Hoseinifar *et al.* (2011b)
	Common carp	Omar *et al.* (2012)
Potassium diformate	Hybrid tilapia	Zhou *et al.* (2009d)
Immunostimulants	Atlantic cod	Gildberg & Mikkelsen (1998), Skjermo *et al.* (2006)
	Atlantic salmon	Liu *et al.* (2008)
	Red tilapia	Merrifield *et al.* (2010c)
	Hybrid tilapia	He *et al.* (2010)
Different antibiotics	Different species	For review see the present study

Even though the traditional culture-based technique possesses rather low sensitivity for measuring the composition, structure and stability of bacteria colonizing the digestive tract of fish, it is able to indicate differences due to minor dietary alterations as observed by Refstie *et al.* (2006) in gut microbiota of Atlantic cod (*Gadus morhua* L.) fed standard or bioprocessed soybean meal (BPSBM) (Fig. 2).

The gut microbiota may function to prevent pathogens from colonization; it is likely that the gut microbiota might be of vital importance with regard to fish health. The objective of the present paper, to review present information on how dietary supplements affect the population level of gut bacteria and composition, is relevant. This is strengthened by the fact that during the last decade, the aquaculture industry is increasingly demanding sustainable alternative lipid and protein sources to reduce the use of fish meal (FM) and fish oil (FO) (Tacon & Metian 2008; Hemre *et al.* 2009; Merrifield *et al.* 2011a; Morais *et al.* 2012; Olsen & Hasan 2012; Hansen & Hemre 2013).

This review firstly presents a short overview of the GI tract of fishes and the techniques most often used for the study of GI microbiota as a background for the succeeding chapters covering impact of the nutrients sources, probiotics, prebiotics and antibiotics. The results cited in the present review include works published in peer-reviewed scientific journals, open access peer-reviewed scientific journals, books as well as minimally circulated investigations available as short communications, and abstracts presented in books from international conferences. The latter is done in order to indicate that there are numerous interesting investigations ongoing albeit not yet been published in scientific journals. Furthermore, in order to give the reader satisfactory information on dietary effect on the gut microbiota, the present authors include some information from endothermic animals when needed.

Table 2 Overview of studies investigated the effect on gut microbiota of fish

Factors	References
Seasonal variations	Sugita *et al.* (1981, 1983), Ringø (2000), Al-Harbi & Naim Uddin (2004), Hagi *et al.* (2004); Naviner *et al.* (2006), Hovda *et al.* (2012)
Stress	Olsen *et al.* (2005), Ringø *et al.* (2000, 2014b)
Individual variations and different regions of the GI tract	Sugita *et al.* (1990), Ringø *et al.* (1995), Spanggaard *et al.* (2000), Ringø *et al.* (2001b); Hovda *et al.* (2007), McDonald *et al.* (2012), Fjellheim *et al.* (2012); Star *et al.* (2013)
Cultured versus wild	Bacanu & Oprea (2013), Kim & Kim (2013)
Triploid versus diploid	Cantas *et al.* (2011)
Day-to-day variations	Sugita *et al.* (1987, 1990)
Male versus female	Iehata *et al.* (2015)
Different fish species fed similar diet	Li *et al.* (2014b)
Developmental stages/life cycle	Cao *et al.* (2012), Zhao *et al.* (2012a), Huang *et al.* (2014)
Microbial aspects of live feed	Conceição *et al.* (2010), Bakke *et al.* (2013)
Fast versus slow growing fish	Sun *et al.* (2009)
Hierarchy formation	Ringø *et al.* (1997)
Starvation	Xia *et al.* (2014)
Migration from fresh water to sea water and Migration from sea water back to fresh water	Ringø (2004)
Water quality	Gatesoupe *et al.* (2013)
Recirculation versus conventional flow-through	Attramadal *et al.* (2012)
Fish farms	Diler *et al.* (2000)
Environmental and ecological factors	Sullam *et al.* (2012)
Host ecology and environments	Wong & Rawls (2012)

The gastrointestinal tract – the dynamic environment of the microbiota

General considerations

The key function of the alimentary tract is its ability to dissolve foodstuffs and process nutrients to make them suit-

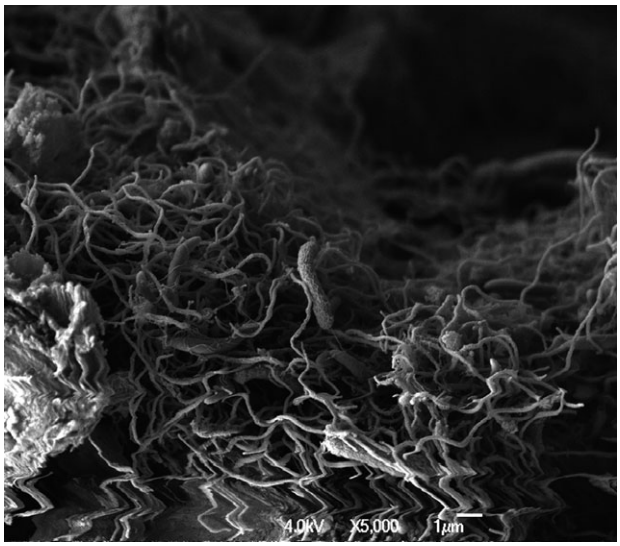

Figure 1 Bacteria associated with the microvilli in the hindgut chamber of Atlantic cod (*Gadus morhua* L.). After Løvmo (2007).

able for absorption by various transport mechanisms in the wall of the GI sections. Besides hydrolytic reactions catalysed by endogenous enzymes secreted by the pancreas and cells in the gut wall, considered to play the major roles in digestion, fermentation may also play key roles in digestive processes in fish as in many other monogastrics. The role of fermentation in fish is unclear, as research on microbiota in fish intestine is still in its early stages. However, its role is considered to be of minor quantitative importance for nutrient supply in cold-water species. The importance of the intestinal microbiota is highly significant for normal functioning of the immune apparatus of the GI tract and the general resistance of the fish towards pathogens and other foreign factors constantly influencing the fish via the intestine.

The characteristics of the microbiota, products of metabolism, etc. depend greatly on the conditions of the intestine, determined by species-specific parameters along the GI tract such as anatomy, endogenous inputs of digestive secreta, pH, osmolality, redox potential, compartment size and structure, passage rate and residence time (Ray & Ringø 2014).

Anatomy

The GI tract is a tube histologically differentiated in different segments that course through the body. This tube may have a few to several hundred subcompartments in which microbes may divide and grow. The GI tract is commonly divided in the following regions: mouth, gill arch, oesophagus, stomach, pyloric caeca, mid-intestine (MI), distal intestine (DI) and rectum. The GI tract of Atlantic cod is illustrated in Fig. 3. Some fish species lack a typical stomach which in these fish is replaced by a foregut. Pyloric caeca are finger-like extensions typical of most teleost fish. They are located in the proximal part of the intestine, MI, and, when present, number from a few, as in Atlantic halibut (*Hippoglossus hippoglossus* L.), to several hundred as in the Atlantic cod. The structure of the wall of the GI tract varies along the tract, but has in common a surface facing the lumen of mucus-producing (goblet) cells between enterocytes. The latter holds digestive and transport apparatus located in microvilli facing the lumen, and being responsible for the uptake of nutrients (Fig. 4a). The mucosa lining of the GI tract represents an interface between the external and internal environments and, in conjunction with the associated organs (e.g., pancreas, liver and gall bladder), provides the functions of digestion, osmoregulation, immunity, endocrine regulation of GI tract and systemic functions, as well as the elimination of environmental contaminants and toxic metabolites.

Just below the mucosa, we find the submucosa which is a layer of connective tissue, blood vessels and nerves. A single or double layer of muscles is located outside the submucosa. The serosa forms the outer layer of the GI tract. In some fish, the compartments may hardly be distinguishable macroscopically, while in other the sections are divided clearly and may be separated by valves or sphincters. The presence of valves and sphincters between the subcompartments of the intestine may greatly influence the residence time of the chyme in the compartment and hence for the possibilities of the microbiota to develop.

The oesophagus is, in most fish, short and of small diameter, with the possibilities to expand greatly, and with numerous goblet cells aiding in food passage. A common feature of carnivore fish species is great elasticity and strong musculature in the stomach wall. In some fish species, the muscles of the stomach seem to function as a grinder. Carnivore species show the shortest GI tract, typically less than the body length, whereas in herbivore, such as tilapia, the GI tract may be more than 20 times the body length (Ray & Ringø 2014). The rectum is usually separated from the rest of the tract by a sphincter and contains more goblet cells than the more proximal parts, but also has absorptive cells making the distal intestine of fish

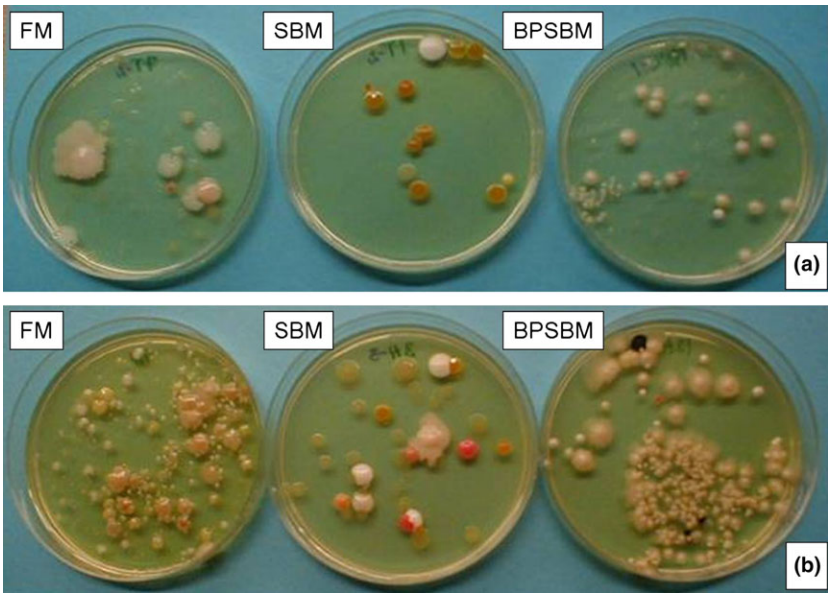

Figure 2 Indigenous adherent bacteria isolated from the gut wall of (a) the proximal intestine and (b) the distal fermentation chamber of Atlantic cod fed; FM, SBM or BPSBM. After Refstie *et al.* (2006).

different from that of mammals and birds, which do not have absorptive cells with microvilli (Rust 2002). The greatest microbial activity is usually reported in this compartment.

There is a great variability of the structure and functional characteristics of the GI tract among fish species (Ray & Ringø 2014; Suyehiro 1941) which seems to match to the wide diversity of feeding habits and environmental conditions exploited by fish. The variation is obvious by comparing the GI tract characteristics of carnivorous and herbivorous fish and those from fresh water and sea water.

Physiological characteristics

Fish have the ability to adapt the GI tract characteristics rapidly and reversibly to match the changes in functional demands that take place during the life history (e.g., metamorphosis, anadromous or catadromous migrations and from day-to-day due to seasonal shifts in diet or environmental conditions) (Karila *et al.* 1998). The mechanisms behind involve a wide diversity of hormones and other signalling molecules secreted by the various cells of GI tract. They modulate the composition of digestive gut wall, exocrine pancreas and liver and allow fish rapidly and reversibly to alter the characteristics of the GI tract and other organ systems to adapt to changes in the contents of the GI tract, such as amounts and types of nutrients, pH, ionic composition, and to environmental conditions (Holst *et al.* 1996).

Fluid is constantly secreted or leaking into the GI tract from the wall tissue and from the liver and pancreas. These fluids contain a great range of compounds that may stimulate or inhibit the growth and composition of the intestinal microbiota. Besides macromolecules such as a great number of proteins, for example digestive enzymes and mucopolysaccharides, these fluids contain phospholipids, bile acids, antimicrobial components acting on a great variety of species (Laubitz *et al.* 2003), antioxidants such as glutathione, minerals, waste products eliminated from the body through the faeces, for example bilirubins giving colour to the faeces, and bicarbonate to stabilize the pH of the luminal contents. Although our knowledge is limited for fish, it can be suggested that these fluids vary greatly in quantity as well as composition between intestinal segments, within species under different conditions not at least diet composition. Information has been reported in the scientific literature regarding quantities of water and material entering the GI tract of fish (Gonzalez 2012; Reitan *et al.* 1998). Alterations in composition have been observed, however. Examples are the alterations observed in digestive enzymes reported within the gut contents of salmonids induced by incorporation of plant material in the diet (Krogdahl *et al.* 1994; Nordrum *et al.* 2003; Romarheim *et al.* 2006; Santigosa *et al.* 2008) and alterations in content of bile acids caused by dietary fibre and soyasaponins (Chikwati *et al.* 2012; Romarheim *et al.* 2006). The various components of the digestive secreta to the intestine may serve as substrates for the microbes, but enzymes such as proteases and lipases and antimicrobial components meant

Table 3 Culture-independent methods used in studies evaluating the gut microbiota in aquatic animals

Method	Species	Microbiota: allochthonous (allo) or autochthonous (auto)	Part of the GI tract investigated	References
DGGE	Atlantic halibut[1]	Allo and auto	Whole larvae	Jensen *et al*. (2004)
	Atlantic cod	Allo and auto	Whole larvae	Brunvold *et al*. (2007)
	Atlantic cod	Allo and auto	Whole larvae	McIntosh *et al*. (2008)
	Atlantic cod	Auto	PI and DI	Zhou & Wang (2012)
	Atlantic salmon			Hovda *et al*. (2007, 2012)
	Atlantic salmon	Allo	Whole intestine	Liu *et al*. (2008)
	Brown trout	Allo	Whole intestine	Manzano *et al*. (2012)
	Rainbow trout	Allo and auto	PI and DI	Merrifield *et al*. (2009b)
	Rainbow trout	Allo	Whole intestine	Dimitroglou *et al*. (2009)
	Puffer fish	Allo	Whole intestine	Yang *et al*. (2007)
	Yellow grouper	Auto	Whole intestine	Zhou *et al*. (2009a)
	Emperor red snapper	Auto	Stomach and intestine	Zhou *et al*. (2009b)
	Hybrid tilapia	Auto	Whole intestine	Zhou *et al*. (2009c)
	Red tilapia	Allo	Whole intestine	Ferguson *et al*. (2010)
	Sterlet	Allo and auto	Whole intestine	Bacanu & Oprea (2013)
	Abalone	Allo and auto	Whole intestine	Tanaka *et al*. (2004)
	Queen conch	Allo and auto	Stomach, mid and DI	Carrascal *et al*. (2014)
Biolog Ecoplate™ and DGGE	Different fish species	Allo	Whole intestine	Mouchet *et al*. (2012)
PCR-TGGE	Rainbow tout	Allo and auto	Whole intestine	Navarrete *et al*. (2012)
Clone libraries				
	Atlantic salmon	Allo and auto	Whole intestine	Navarrete *et al*. (2009)
	Rainbow trout	Allo and auto	Whole intestine	Kim *et al*. (2007)
	Takifugu niphobles	Allo	Whole intestine	Shiina *et al*. (2006)
	Whiting	Allo and auto	MI	Smith *et al*. (2007)
	Coral reef fish	Allo and auto	Whole intestine	Smriga *et al*. (2010)
	Japanese costal fish	Allo	Whole intestine	Tanaka *et al*. (2012)
	Common carp	Allo	PI and DI	Sugita & Mizuki (2012)
	Chilean octopus	Allo and auto	Whole intestine	Iehata *et al*. (2015)
DGGE and Clone libraries	Chinese shrimp	Allo and auto	Whole intestine	Liu *et al*. (2011b)
	Mud crab	Allo and auto	Whole intestine	Li *et al*. (2012)
	Rotifer	Allo and auto	Whole intestine	Ishino *et al*. (2012)
Pyrosequencing	Atlantic salmon	Allo	DI	Zarkasi *et al*. (2014)
	Sea bass	Allo and auto	Whole intestine	Carda-Diéguez *et al*. (2013)
	Paddlefish and big-head carp	Allo and auto	S/PI, MI and DI	Li *et al*. (2014a,b)
Fish microbiome	Different species	Allo	S, PC and MI	Sanchez *et al*. (2012)

S, stomach; PC, pyloric caeca; PI, proximal intestine; MI, mid-intestine; DI, distal intestine.
[1] larvae.

to protect the animal, will represent challenges to the microbiota.

Information on GI pH of early life stages of marine fish is available (Falk-Petersen 2005; Yúfera & Darías 2007). In addition, unpublished observations (Å. Krogdahl) made in studies of the GI tract in Atlantic salmon (*Salmo salar* L.) conducted at the Aquaculture Protein Centre over some years indicate that pH in a filled stomach is variable but generally above 4, Fig. 4b. In the pyloric region, mid and DI, all observations made showed values above 7 and mostly above 8. The pH of the chyme seems to be regulated within fairly narrow ranges. The rather high pH observed in the stomach, compared with that in mammals, may be of relevance for microbial survival in the stomach with higher survival during passage of the stomach in fishes. The lack of acidification in the foregut of stomachless fish species makes it even more likely for microbes to survive the passage to the more distal parts of GI tract in these fish compared with fish with stomach. No decrease in pH in the DI was observed (~8.5) as might have been expected if the microbial activity was high. Preliminary observations from cod, in which the DI is separated from the more proximal parts of the gut by a sphincter, indicate lower pH in this distal pouch possibly compared with the mid and pyloric regions. This decrease is likely due to microbial activity.

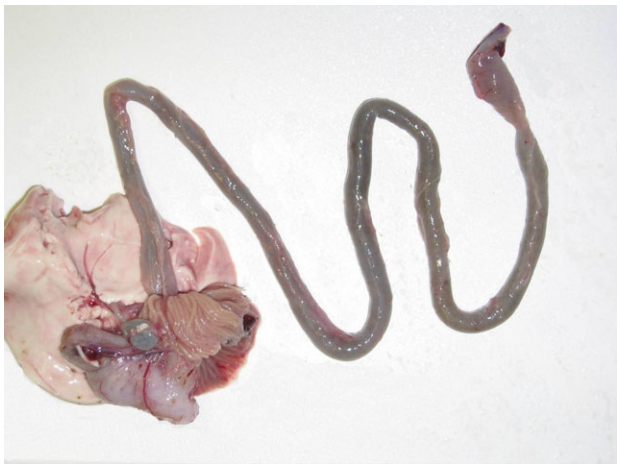

Figure 3 The gastrointestinal tract of Atlantic cod. Note the many pyloric ceaca which may number several hundred in this species. The distal intestine is a pouch closed by sphincters in both ends.

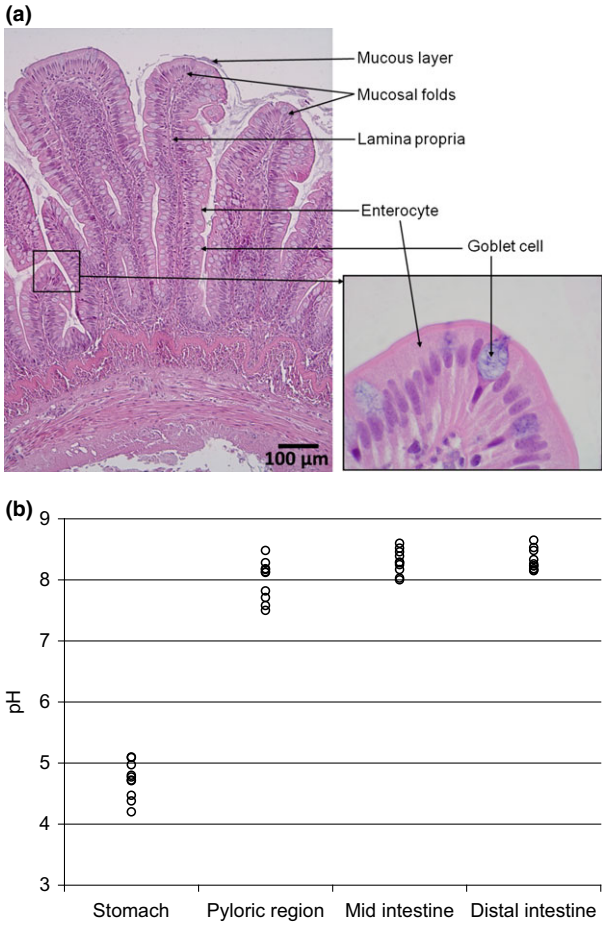

Figure 4 (a) Structure of the GI tract wall. A histological presentation, stained with haematoxylin and eosin, of the wall of the mid-intestine in Atlantic salmon. A layer of mucus, secreted by the goblet cells, covers the mucosal folds. Cells are dying continuously and released from the top of the folds into the chyme, mixing with unabsorbed food material as well as components of endogenous secreta. Photograph: M. Penn. (b) pH in chyme of Atlantic salmon in sea water (H. Holm & Å. Krogdahl, unpublished data). The data originate from three feeding experiments, each testing three diets varying in protein content or amino acid supplementation. Each circle represents the mean pH of observations in several fish fed the same diet. Only fish with content in the gut segments were used. No significant effects of diet on pH were observed within experiment.

Mucosa-associated lymphoid tissue (MALT) in teleost fish is subdivided into gut-associated lymphoid tissue (GALT), skin-associated lymphoid tissue (SALT) and gill-associated lymphoid tissue (GIALT) (Salinas *et al.* 2011). GALT which represents an essential part of an organism's adaptive defence system is considered to protect the host against pathogens not only by fighting the intruding bacteria but also by modulating the composition of the microbiota. Microbiota stability in animals, including fish, has been observed (Rawls *et al.* 2006). Microbial communities transplanted from mice to gnotobiotic zebra fish altered quantitatively in the direction of the *normal* biota of the zebra fish species and vice versa. Antibodies, lysozyme and other antimicrobial components in mucus secreted from the wall of the GI tract may play a key role in this apparant stability of the intestinal microbiota. The function of GALT depends on diet composition, such as its content of oligosaccharides, and the nutritional status regarding essential nutrients, such as selenium (Sweetman *et al.* 2010). In addition, GALT must develop mechanisms to discriminate between pathogenic and commensal micro-organisms (Pérez *et al.* 2010; Suzuki *et al.* 2007).

Passage rate and residence time

It is logical that passage rate and residence time in the various sections along the GI tract may influence the microbial gut community and subsequent host–microbial interactions. Stomach evacuation rate and passage time through the intestine have been observed to vary with temperature, meal size, particle size, feed composition, previous nutritional history, fish size and stress (reviewed by Fänge & Grove 1979; Bromley 1994). Diet is also known to affect passage time (Storebakken *et al.* 1999) and hence may affect microbial growth. No information has been reported on the relationship between growth of the microbiota, gut passage rate and residence time.

In conclusion, the anatomy and function of the GI tract and the digestive process, typical for a fish species,

represent important conditions for the development and growth of the microbiota. Variation in external environmental conditions also greatly impacts the conditions for microbial growth. However, our knowledge of these relationships is very limited in particular for fish.

Molecular biological methods for characterization of the gut microbiota in fish

Several different molecular methods are today available for detecting micro-organisms in a given sample and monitoring the change in microbial communities, without culture-dependent techniques (Zhou *et al.* 2014). In most cases, a combinatorial approach may be necessary for determining the microbial composition (e.g. clone library construction and PCR-based profiling techniques) and is a prerequisite to determine the 'true' microbial communities in the fish gut. An area of research that may shed light on the physiological role of bacteria in the fish gut is to investigate the gene expression of the bacteria and not only determine the presence/absence of the bacteria, that is studying mRNAs and not only DNA.

In the early studies investigating the gut microbiota of fish, conventional culture-based methods, which are time-consuming and selective, were used (for review, see Cahill 1990; Ringø *et al.* 1995). Conventional culture-based techniques, even if several different media are used, do not present a '*true*' picture of the bacterial diversity. Therefore, to present more reliable information about the gut microbiota of fish, molecular methods are necessary. Culture-independent methodologies are useful tools in furthering our understanding of complex ecosystems and have highlighted the limitations associated with culture-dependent techniques. Denaturing gradient gel electrophoresis (DGGE), terminal-restriction fragment length polymorphism (T-RFLP), automated rRNA intergenic spacer analysis (ARISA), single-strand conformation polymorphism (SSCP), 16S rRNA gene clone libraries and the newly developed 16S rRNA tag pyrosequencing method are examples of such culture-independent techniques that have been used to profile bacterial populations in a wide variety of ecosystems (Lee *et al.* 1996; McBain *et al.* 2003; Sakamoto *et al.* 2004; Yannarell & Triplett 2004; Van der Gucht *et al.* 2005), including gut habitats such as the rumen (Edwards *et al.* 2005; Yu & Morrison 2004) and hindgut (Green *et al.* 2006; Simpson *et al.* 1999; Suchodolski *et al.* 2004). In all of these techniques, extracted community DNA is amplified using the polymerase chain reaction (PCR), utilizing the primers specific for conserved regions of 16S rRNA. Examples of the published papers using culture-independent methods in studies evaluating the gut microbiota of aquatic animals are presented in Table 3.

Molecular-based methods to describe the microbial communities in a certain sample can be divided into two groups: (i) the PCR-based techniques which amplify certain fragments of DNA or cDNA using user-defined primers, and (ii) the PCR-independent methods which detect bacteria without any gene- or cDNA amplification. Generally, the PCR-independent methods are less specific and sensitive than PCR-based techniques, and they are less suitable for profiling bacterial communities. They are, however, important tools to visualize bacteria in a spatial scale. Conventional PCR-based methods are qualitative methods when applied to environmental samples, due to the inherent biasing in PCR amplification (Suzuki & Giovannoni 1996; von Wintzingerode *et al.* 1997; Polz & Cavanaugh 1998). When amplifying the 16S rRNA gene, which is by far the most common target gene in studies of gut microbiota, the copy number heterogeneity will affect the diversity in the resulting amplicon (Klappenbach *et al.* 2000). This bias is particularly important to consider whether searching for novel bacteria in environmental samples and extensive sequencing may be required to detect less abundant species. Furthermore, many of the PCR-dependent techniques amplify only a short region of a particular gene, and a precise taxonomic affiliation is often difficult. Nevertheless, amplification of selected gene(s) is necessary as at least a supplementary approach to traditional culture-dependent methods in order to better describe complex bacterial communities, and to better monitor the changes that occur in these communities across temporal and spatial scales when influenced by biotic or abiotic factors. We will here first describe some of the PCR-independent techniques available, and then the methods which are based on the PCR technique.

PCR-independent techniques

In situ *hybridization* *In situ* hybridization involves annealing of a probe to nucleic acids within the bacteria. The specificity is defined by the probe sequence (Amann *et al.* 1995), which usually is in the size of 15–30 nucleotides. A successful annealing of the probe will yield a visualization of the bacteria in the spatial space. It requires prior knowledge about the target sequence(s). *In situ* hybridization is a powerful but challenging technique which requires optimization of several of the involved steps (Amann & Ludwig 2000; Moter & Göbel 2000). The most common

procedure today is to label the probe with a fluorophore, called fluorescent *in situ* hybridization (FISH). This allows for the simultaneous detection of different micro-organisms, using a set of fluorophores with different excitation and emission maxima. The probe can be either RNA- or DNA-oligonucleotides, and the target can be RNA or DNA. If using DNA as the target, both dead and viable bacteria will be detected, while RNA as the target will only reveal viable bacteria. Labelling can be performed directly with a fluorescently labelled probe, which is the fastest, cheapest and easiest way. To increase the labelling sensitivity, which may be relatively low using direct labelling, the probe can be labelled indirectly by enzymatic signal amplification (Yamaguchi *et al*. 1996). The latter approach can increase the signal intensity by up to ten-fold (Schöhuber *et al*. 1997), but the number of positively labelled bacteria may decrease, probably due to insufficient penetration of the high-molecular weight enzymes. The lack of automatization makes *in situ* hybridization unsuitable to conduct high-throughput analyses, but it is a valuable tool in detecting bacteria in the spatial space. FISH has been used successfully to understand the intestinal microbiota of several fish species (Asfie *et al*. 2003; Holben *et al*. 2002; Spanggaard *et al*. 2000; Tanaka *et al*. 2004).

Immunohistochemistry Instead of using oligonucleotides for the detection of micro-organisms, bacteria can be labelled with antibodies which can subsequently be visualized by the use of secondary antibodies. This method has some similarities with *in situ* hybridization; the samples are fixed prior to labelling, the target bacteria can be visualized in the spatial space, and a lack of automatization makes it unsuitable for high-throughput analyses. However, whereas probes for *in situ* hybridization are user-defined and anneal to complementary nucleotides, antibodies are usually raised against whole bacteria and will not necessarily have the desired specificity. Immunohistochemistry is highly suitable to follow the infection route of bacterial strains to which a specific antibody has been raised (Løvoll *et al*. 2009), and antibodies can be used both in light microscopy and electron microscopy studies, depending on the desired degree of magnification. The challenge is to raise a monoclonal antibody with high specificity and with no cross-reactivity against other closely related bacteria (Rengpipat *et al*. 2008), which is a time-consuming and expensive process. In addition, bacteria cultured *in vitro* and used for immunization may have a slightly different morphology *in vivo*, considering that bacteria are affected by the environment in which they grow. This may result in changes in the antigen morphology between *in vitro* and *in vivo* growth (Jung *et al*. 2008). At last, immunohistochemistry does not yield a very high sensitivity. The advantage of using a monoclonal antibody is that a highly specific antibody can differentiate even between different strains, and it requires less optimization compared with *in situ* hybridization.

Transcript analysis with aid of affinity capture New methods are continuously being developed to more accurately determine the composition of microbial communities. One of these is the *transcript analysis with aid of affinity capture* (TRAC) method, which is a multiplexed and sensitive method for relative quantification of bacteria. By solution hybridization of biotinylated nucleic acids and fluorophore-labelled oligonucleotides in combination with capillary electrophoresis, it is possible to make a relative quantification of selected transcripts (Kataja *et al*. 2006; Satokari *et al*. 2005). Technically, biotinylated nucleic acid probes anneal according to their specificities to a pool of mRNA, and the probes are then captured by streptavidin-coated beads, and then separated through capillary electrophoresis. The probes can vary in length from 18 to 41 nucleotides (Rautio *et al*. 2006; Satokari *et al*. 2005) up to several hundred nucleotides (Kataja *et al*. 2006). The size difference between the different probes ensures that the fragments are well separated. This method does not rely on PCR amplification, although amplification of the probe will increase the sensitivity (Kataja *et al*. 2006), and can offer reliable and high-throughput analyses of bacterial communities. Instead of constructing primers annealing to user-defined target(s), probes of different sizes with the same fluorophore are constructed which anneal to specific targets. After capture, the probes are eluted and separated, and the peak intensity can be measured quantitatively. The TRAC method is a profiling technique without the need for PCR amplification and may offer a more reliable estimate of the bacterial composition in a given sample than PCR-based methods.

PCR-based techniques

All PCR-based methods consist of three basic steps: (i) nucleic acid extraction, (ii) amplification of DNA and (iii) analysis (either quantitatively or qualitatively) of PCR products.

Nucleic acid extraction For investigating the presence or absence of bacteria, DNA can be extracted and used as template in either PCR-dependent or PCR-independent methods. There are, however, different DNA extraction methods and these may influence the relative composition

of the DNA pool. He *et al*. (2009) compared the effects of three different DNA extraction methods (lysozyme digestion, CTAB method and bead mill) on the analysis of different micro-ecological environments in a farming pond [pond sludge, feed, intestinal contents and the intestinal wall of grass carp (*Ctenopharyngoden idellus*)] by DGGE of 16S rDNA V3 region. The results revealed obvious effects in the DGGE fingerprints of the different micro-ecological environments by the different DNA extraction methods. Technically, DNA can be relatively easily extracted from bacteria and can subsequently be used as templates in downstream applications. The extraction of RNA, however, is challenging and requires more awareness than DNA extraction. This is because RNases are not easily denaturated, and thus chemicals and equipment have to be RNase-free through the extraction procedure (Jahn *et al*. 2008). The clear reasons for studying the expression of genes, and not purely the genes themselves, is an opportunity to investigate the changes in expression of selected genes due to biotic and abiotic treatments, and also for studying the viable portion of the microbiota.

Clone library constructions Constructing clone libraries of the 16S rRNA gene is probably the most widely used method to gain sequence information from a given sample. The clone library construction consists of several steps that may influence the composition of the clones, for example, the method used for DNA extraction, the primers used for gene amplification, and the conditions used to amplify the gene. As for all PCR-dependent techniques, the user-defined primers determine the amplicon. Usually, primers annealing to highly conserved regions of the 16S rDNA are chosen in order to obtain an amplicon consisting of the highest diversity as possible. It is also important to amplify fragment that gives the best phylogenetic resolution, and thus, primers annealing close to the 5′-end and the 3′-end are preferred (e.g. 8F/27F as the forward primer and 1492R as the reverse primer). The construction of clone libraries is often accompanied by other types of techniques, such as PCR-DGGE, PCR-TGGE or T-RFLP, all of which are typical profiling methods.

PCR-DGGE and PCR-TGGE Combining PCR amplification with separation of the amplicons with either denaturing- or temperature-gradient gel electrophoresis (PCR-DGGE or PCR-TGGE) is a widely used technique to determine the bacterial communities in fish and crustacean (e.g. Hovda *et al*. 2007; Zhou *et al*. 2007; Liu *et al*. 2008; McIntosh *et al*. 2008; Merrifield *et al*. 2009a, 2013; Li *et al*. 2012). In PCR-DGGE, the PCR fragments are separated through a chemical denaturating gradient, while a temperature gradient is used in TGGE (Muyzer & Smalla 1998). Both techniques are based on the separation of PCR products of the same size but with different nucleotide sequences. The different regions in the DNA strand will denature at different time points when migrating through an increasing denaturating agent (chemical or temperature). Depending on the nucleotide composition (specifically the G-C content), the migration behaviour will change. The optimal resolution is achieved when the amplicons are not entirely denaturated, and thus, a so-called GC clamp is added to one of the primers to ensure that there is not a complete denaturation (Myers *et al*. 1985). The bands can be cut from the gel and sequenced; thus, sequence information can be achieved without constructing a clone library prior to the method. Although these methods are much used to describe bacterial communities, they have some limitations that should be noted. The amplicons are relatively small (typically 150–500 bp), and it may be difficult to ascertain a taxonomical affiliation of sequenced bands. Furthermore, the GC clamp may produce primer–dimer formations and overlapping (migration to the same point in the gel) of phylotypes can occur which can lead to an underestimation of community diversity and difficulties in sequencing of bands. Additionally, the sensitivity of staining with traditional staining is relatively low (Nocker *et al*. 2007).

Terminal-restriction fragment length polymorphism Similar to PCR-DGGE and PCR-TGGE, terminal-restriction fragment length polymorphism (T-RFLP) is a profiling technique used to monitor changes in the bacterial community. Here, either the forward or reverse primer is labelled with a fluorophore. A subsequent digestion with restriction enzyme(s) of the amplicon after PCR amplification creates fragments of varying length depending on the restriction site of each sequence. The fragments are then separated using capillary electrophoresis, creating peaks which represents terminal-restriction fragments (TRFs). The separation of the fragments is performed on the basis of differences in fragment length, which is estimated by the comparison to one or several DNA standards run simultaneously. This is a high-resolution technique, which theoretically is able to distinguish sequences differing by only one base pair. With a prior knowledge of the microbial diversity of the sample, for example through clone library sequencing, restriction enzymes can be chosen for creating the highest numbers of TRFs. The clear disadvantage compared with the DGGE

and TGGE techniques is that the fragments cannot be sequenced after separation, and thus, T-RFLP has to be accompanied by sequencing or *in silico* analyses. As the separation is performed by capillary electrophoresis, analyses can be performed much more rapidly than using PCR-DGGE and PCR-TGGE. Although T-RFLP has some limitations, it has proven useful for describing changes in microbial communities (Blackwood *et al*. 2003; Nocker et al. 2007; Dave *et al*. 2011).

Real-time (RT)-PCR RT-PCR is a quantitative PCR-based approach combining traditional PCR amplification with detection of fluorescence signals. It offers the possibility to monitor the abundance of selected gene(s) or transcript(s) in environmental samples, either relatively against a normalization factor (e.g. nucleic acid concentration or housekeeping gene expression), or absolutely by comparing the expression to known quantities of the target gene contained in plasmids. Two different reporter systems are most often used to detect the fluorescence signal, namely the SYBR green assay (Wittwer *et al*. 1997) and the *Taq*Man probe system (Holland *et al*. 1991; Livak *et al*. 1995). SYBR green binds double-stranded DNA, and the former assay thus measures accumulating amounts of SYBR green, and the *Taq*Man probe system utilizes a 5′-end fluorescently labelled probe which emits light when separated from a quencher located at the 3′-end. During the annealing steps, the fluorophore will be released from the probe, and thus, the latter system measures the fluorescence from the released fluorophore. The *Taq*Man probe system is highly specific, but it is more expensive than the SYBR green chemistry. In addition, the SYBR green assay only requires two conserved regions for the two primers to anneal, in contrast to the probe technology which needs a third conserved region for the binding of the probe. Templates for the amplification can be either DNA-extracted from the sample, for purely detecting the specified gene(s), or cDNA-synthesized from the mRNA pool to calculate the gene expression. If using a taxonomic gene, such as 16S rDNA, it may be possible to calculate the relative amount of certain microbes. However, using functional genes as the target(s) makes it possible to link any change in gene expression to functionality. Using bacterial cDNA as template in RT-PCR is still not routinely done, mainly due to the difficulties in isolating high-quality RNA from bacteria (Jahn *et al*. 2008). RT-PCR is, however, a promising method for investigating bacterial processes in environmental samples, but the limitations, such as methods for high-quality RNA isolation and a prior knowledge of the target sequences, should be noticed (Smith & Osborn 2009).

Next-generation sequencing technologies Pyrosequencing is another method that is used for high-throughput sequencing of clone libraries (e.g. Edwards *et al*. 2006; Jones *et al*. 2009). In contrast to traditional sequencing using the Sanger method, pyrosequencing utilizes the released pyrophosphate which is used to produce ATP. ATP is then used by luciferase to convert luciferin to oxyluceferin, and the emitted light from this reaction is measured (Ronaghi *et al*. 1998; Gharizadeh *et al*. 2002). The method has now been scaled up and may determine the composition of hundreds of thousands of sequences simultaneously. It has a great potential for high-throughput sequencing to a considerably lower cost than the Sanger method, but still the sequence length is relatively short (700 bp) and thus, the taxonomic resolution is much weaker than traditional sequencing. In microbiological applications, it has proven useful in analysis of the microbial community in human intestine (Dethlefsen *et al*. 2008), macaque gut (McKenna *et al*. 2008) and tidal flat sediments (Kim *et al*. 2008), in bacterial typing (Jonasson *et al*. 2002), and analysis of single nucleotide polymorphism (Isola *et al*. 2005).

Single-strand conformation polymorphism In the single-strand conformation polymorphism-PCR (SSCP-PCR) technique (Lee *et al*. 1996), denaturated PCR products are separated through either an acrylamide gel or a capillary array sequencer. In non-denaturating conditions, single-stranded DNA folds into tertiary structures based on their nucleotide sequence and the physiochemical environment (e.g. temperature and ionic strength), and these different conformations will separate the PCR products through differences in migration behaviour. If using acrylamide gels, the bands can be cut from the gel and sequenced. This is not possible using a capillary array sequencer. Some major limitations, such as reannealing of PCR products during separation and the unpredicted behaviour of the bands, must be considered if employing this method (Nocker *et al*. 2007).

rRNA intergenic spacer analysis rRNA intergenic spacer analysis (RISA) is a method of microbial community analysis which provides estimates of microbial diversity and community composition without the bias imposed by culture-based approaches or the labour involved with small-subunit rRNA gene clone library construction. RISA was used originally to contrast diversity in soils

(Borneman & Triplett 1997) and more recently to examine microbial diversity in the rhizosphere and marine environments (Acinas *et al*. 1999; Robleto *et al*. 1998). The method involves PCR amplification from total bacterial community DNA of the intergenic region between the small (16S) and large (23S) subunit rRNA genes in the rRNA operon, with oligonucleotide primers targeted to conserved regions in the 16S and 23S genes. The 16S-23S intergenic region, which may encode tRNAs depending on the bacterial species, displays significant heterogeneity in both length and nucleotide sequence. Both types of variation have been extensively used to distinguish bacterial strains and closely related species (Aubel *et al*. 1997; Jensen *et al*. 1993; Maes *et al*. 1997; Navarro *et al*. 1992; Scheinert *et al*. 1996). In RISA, the length heterogeneity of the intergenic spacer is exploited. The PCR product (a mixture of fragments contributed by community members) is electrophoresed in a polyacrylamide gel, and the DNA is visualized by silver staining. The result is a complex banding pattern that provides a community-specific profile, with each DNA band corresponding to at least one organism in the original assemblage. To the author's knowledge, this method has only been used in one fish study (Navarrete *et al*. 2010a).

Effect of dietary lipid

From a microbial point of view, the pyloric caeca is of vital interest as lipid digestion and absorption occur in this organ (Olsen & Ringø 1997). However, due to its complex morphology, only some studies have investigated the microbiota of pyloric caeca in fish (Al-Hisnawi *et al*. 2014; Gildberg & Mikkelsen 1998; Gildberg *et al*. 1997; Lesel & Pointel 1979; Ransom *et al*. 1984; Zhou *et al*. 2009a), and to our knowledge, no investigation so far has evaluated the effect of dietary lipid on microbiota of pyloric caeca, a topic that merits investigations.

Level of dietary lipid

In their study with rainbow trout (*Oncorhynchus mykiss* Walbaum), Lesel *et al*. (1989) fed the fish two different diets, low (50 g kg^{-1}) and high (160 g kg^{-1}) lipid levels. Differences in faecal bacterial composition were observed, as the faecal microbiota of fish fed low lipid level consisted of only *Acinetobacter* spp. and Enterobacteria. In contrast, *Acinetobacter* spp., *Aeromonas* spp., Enterobacteria, *Flavobacterium* spp., *Pseudomonas* spp. and coryneforms were isolated from faeces of fish fed the high-lipid level. However, as only 12 isolates from each dietary group were isolated, no clear conclusion can be drawn.

Earlier diets for cultured salmonids contain high amounts of carbohydrates (approximately 20% of dry weight), but in recent years, there has been a tendency towards decreasing dietary carbohydrate content from about 20% to 10%, with a subsequent increase in the level of dietary lipid from <20 to up to 30%. Based on this tendency, Ringø & Olsen (1999) fed Arctic charr (*Salvelinus alpinus* L.) diets containing high (27%) and low (13%) levels of dietary lipid. In this study, approximately 190 isolates were identified from each dietary group. Dietary manipulation modulated the species composition of carnobacteria, as *Carnobacterium* spp.- and *C. mobile*-like strains were only isolated from DI of fish fed low dietary lipid, while *C. piscicola*-like strains were isolated from proximal intestine. In contrast to these results, *C. divergens*-like isolates were found associated with the mucus layer of proximal and DI of fish fed high dietary lipid.

Different dietary lipid sources

Fish oils were for many years the predominating lipid source in diets for carnivorous fish species. However, the increase in aquaculture led to an increased consumption from 16% of available fish oil in 1988 to 81% in 2002 (Tacon *et al*. 2006). This was close to full exploitation. Although studies with substitutes had been *done* in the past, the prospect of deficiencies spurred extensive work into finding replacements. One obvious choice was vegetable oils (Dosanjh *et al*. 1984; Guillou *et al*. 1995; Hardy *et al*. 1987; Montero *et al*. 2003; Ng *et al*. 2007; Rosenlund *et al*. 2001; Torsteinsen *et al*. 2004). The main reason was that the global production is approximately 100 times higher than that of fish oils [FO] (Bimbo 1990; Tacon *et al*. 2006) with no prospects of limitations. Secondly, they often come at compatible prices compared with FO.

As no information was available about how inclusion of vegetable oils in commercial raw material affects the gut microbiota of fish, Ringø *et al*. (2002) investigated the effect of soybean-, linseed- and marine oils on the hindgut microbiota of Arctic charr. This study showed clear differences in the hindgut microbiota of fish fed different oils (after and prior to challenge with *Aeromonas salmonicida* subsp. *salmonicida*). Carnobacteria were only isolated from the hindgut region of fish fed soybean oil (SBO) and linseed oil before challenge, while *Carnobacterium* spp.- and *C. funditum*-like strains were isolated from fish fed the

same oils after challenge. Furthermore, the ability of carnobacteria to inhibit the growth of *A. salmonicida* ssp. *salmonicida* was highest in strains isolated after challenge. These results might have some interest as Lødemel (2000) and Lødemel *et al.* (2001) demonstrated that survival of Arctic charr after challenge with *A. salmonicida* subsp. *salmonicida* was improved by dietary SBO.

In an unpublished study, E. Ringø, R.E. Olsen & S. Sperstad fed Atlantic salmon and rainbow trout extruded diets consisting of lipid-free protein meal supplemented with either 30% vegetable oil containing either (i) linseed oil, (ii) rapeseed oil, (iii) sunflower oil or (iv) 30% fish oil. As a control diet, the fish were fed a commercial diet.

Nine hundred bacterial strains were isolated from the rearing water, diet and the hindgut of Atlantic salmon. The number of viable aerobic and facultative aerobic autochthonous bacteria associated in the hindgut was reduced from 10^5 bacteria per gram wet weight intestinal tissue (fish fed a commercial diet, prior to experimental start) to approximately 5×10^3 bacteria g^{-1} wet mass intestinal tissue of fish fed dietary vegetable oil, after two months of feeding.

Seven hundred and twenty-six autochthonous bacterial strains were isolated from the digestive tract. Of these, 29 strains died prior to positive identification. A wide range of bacteria were isolated from the hindgut of the five rearing groups (four experimental and one commercial). There was, however, some difference in bacterial composition between the rearing groups (Table 4). In the DI of fish fed the commercial diet, *Psychrobacter* and *Staphylococcus* were the dominant bacterial genera, followed by *Pseudomonas jessenii/fragi*-like and *Psychrobacter submarinus/marincola*-like strains. In addition, *Acinetobacter* spp.- and *Carnobacterium mobile*-like strains were also isolated. Eight strains died prior to positive identification.

When Atlantic salmon was fed the sunflower oil diet, *P. jessenii/fragi*-like strains were dominant followed by *Staphylococcus* spp. However, when feeding the fish with a diet supplemented with rapeseed oil, *P. submarinus/marincola*-like strains and *Staphylococcus* spp. were prevalent. From this rearing group, *Pseudomonas fluorescens*-like and *C. mobile*-like strains were also isolated. In the hind gut of fish fed linseed oil, *Enterococcus pseudoavium*-like strains were the dominant bacterial species, followed by *Acinetobacter* spp. and *Staphylococcus* spp. In contrast to these results, *P. submarinus/marincola*-like strains were dominant in the GI tract of fish fed fish oil. Furthermore, dietary manipulation seemed to modulate the presence of the lactic acid bacteria (LAB), as 13.6% and 9% of the strains isolated in the hindgut of fish fed rapeseed- and fish oil belong to *C. mobile*, respectively. However, very few (~2%) *C. mobile* strains were isolated from the digestive tract of the other dietary groups.

Table 4 Log total viable counts (log TVC) per gram wet mass, number of isolates and changes in log TVC of bacterial species isolated from the digestive tract of seven Atlantic salmon fed; sunflower oil (A), rapeseed oil (B), linseed oil (C) and fish oil (D) and from five fish fed commercial diet (prior to experimental start) (E). After E. Ringø, R.E. Olsen & S. Sperstad (unpublished data)

	A	B	C	D	E
Log TVC	3.47	3.90	3.70	3.60	5.0
No. of isolates	154	154	154	154	110
Gram-negative					
Acinetobacter spp.[1]	n.d	n.d	2.87 (7)	2.59 (5)	3.56 (2)
Pseudomonas fluorescens[1]	1.76 (1)	3.10 (7)	2.41 (5)	2.41 (4)	n.d
Pseudomonas jessenii/fragi[1]	3.30 (7)	n.d	n.d	2.74 (7)	4.10 (5)
Pseudoalteromonas agarivorans[1]	n.d	2.89 (7)	2.66 (4)	n.d	n.d
Psychrobacter spp.[1]	n.d	2.41 (5)	n.d	n.d	4.36 (5)
Psychrobacter submarinus/marincola[1]	2.07 (4)	3.37 (6)	2.11 (2)	3.19 (7)	4.10 (5)
Gram-positive					
Carnobacterium mobile[1]	1.29 (1)	3.04 (5)	n.d	2.56 (6)	3.56 (2)
Enterococcus spp.[1]	2.37 (7)	n.d	n.d	2.46 (5)	n.d
Enterococcus pseudoavium[1]	n.d	2.02 (1)	3.41 (7)	2.31 (4)	n.d
Staphylococcus spp.[1]	2.72 (7)	3.26 (7)	2.79 (6)	2.41 (4)	4.34 (5)
Not identified					4.19
Unknown	1.76	2.56	2.29	2.11	3.86

n.d, not detected.
Unknown – isolates that died prior to positive identification.
The number of fish from which bacteria were isolated is given in brackets.
[1] Partial sequence of 16S rRNA were analysed and edited in BioEdit. An initial BLAST-search in GenBank retrieved the taxonomic groups for which showed highest identities.

Information regarding the antagonistic activity of gut bacteria against fish pathogens has been investigated in several papers during the last decade (Gatesoupe 1999; Ringø *et al*. 2005; Balcázar *et al*. 2007a; Ringø 2008; Pérez-Sánchez *et al*. 2011). Antagonistic activity of intestinal bacteria to inhibit the growth of three fish pathogenic bacteria (*A. salmonicida* subsp. *salmonicida*, *Vibrio anguillarum* and *Vibrio* (*Aliivibrio*) *salmonicida*) was tested by the microtitre plate assay. Of the 692 gut isolates tested, antimicrobial activity against *A. salmonicida* and *V. anguillarum* was only observed in six *C. mobile* strains isolated from Atlantic salmon fed sunflower oil. These six isolates were identified by random-amplified polymorphic DNA polymerase chain reaction as described by Seppola *et al*. (2006).

In an experiment with rainbow trout fed similar diets to that of the previously discussed Atlantic salmon study, 850 bacterial strains were isolated from the rearing water, diet and the hindgut (E. Ringø, R.E. Olsen & S. Sperstad, unpublished data). Of these, 726 autochthonous strains were isolated from DI of fish fed diets supplemented; sunflower-, rapeseed-, linseed- or marine oils and the commercial diet prior to experimental start were characterized. Thirty-four strains, 4.6% of the total bacterial strains isolated, died prior to positive identification.

The number of viable aerobic and facultative aerobic heterotrophic bacteria associated with the DI was reduced from 8×10^4 bacteria per gram wet weight intestinal tissue (rainbow trout fed a commercial diet, prior to experimental start) to approximately 4×10^3 bacteria g^{-1} wet mass intestine in fish fed the experimental diets, after two months of feeding (Table 5). These results were similar to those reported for Atlantic salmon.

The DI microbiota of rainbow trout fed dietary vegetable oils was dominated by *P. submarinus*/*marincola*-like strains and strains belonging to *Staphylococcus linens*/*equorum* (Table 5). Strains belonging to *Acinetobacter*,

Table 5 Log total viable counts (log TVC) per gram wet mass, number of isolates and changes in log TVC of bacterial species isolated from the digestive tract of seven rainbow trout fed; sunflower oil (A), rapeseed oil (B), linseed oil (C) and marine oil (D) and from five fish fed commercial diet (prior to experimental start) (E). After E. Ringø, R.E. Olsen & S. Sperstad (unpublished data)

	A	B	C	D	E
Log TVC	3.90	3.70	3.47	3.60	4.90
No. of isolates	154	154	154	154	110
Gram-negative					
Acinetobacter johnsonii[1]	n.d	1.51 (1)	n.d	2.32 (5)	3.86 (3)
Brachybacterium tyrofermentas	2.62 (6)	1.81 (1)	1.89 (2)	n.d	n.d
Janibacter spp.[1]	n.d	2.00 (2)	n.d	n.d	n.d
Pseudomonas fluorescens[1,2]	n.d	n.d	n.d	n.d	
Pseudomonas jessenii/fragi[1]	2.89 (5)	2.41 (6)	2.50 (5)	2.85 (7)	4.51 (5)
Pseudoalteromonas agarivorans[1,2]	2.41 (3)	n.d	n.d	n.d	n.d
Psychrobacter faecalis[1]	n.d	1.81 (2)	n.d	n.d	
Psychrobacter submarinus/marincola[1]	3.46 (7)	3.25 (7)	3.00 (7)	2.91 (7)	4.16 (4)
Serratia spp.[1]	n.d	n.d	n.d	1.72 (1)	n.d
Shewanella putrefaciens[1]	n.d	n.d	n.d	1.89 (2)	n.d
Gram-positive					
Arthrobacter rhombi[1]	2.56 (4)	2.00 (2)	n.d	n.d	n.d
Bacillus pumilus[1]	n.d	n.d	2.14 (4)	n.d	n.d
Brevibacterium casei[1]	2.41 (4)	n.d	n.d	n.d	n.d
Carnobacterium spp.[1]	n.d	n.d	1.89 (2)	2.46 (5)	3.56 (3)
Carnobacterium mobile[1]	2.89 (5)	n.d	n.d	n.d	n.d
Enterococcus faecalis[1]	n.d	n.d	2.29 (6)	n.d	n.d
Enterococcus gallinarum[1]	2.19 (2)	n.d	n.d	n.d	n.d
Marinilactibacillus psychrotolerans[1]	n.d	2.36 (4)	n.d	n.d	n.d
Staphylococcus spp.[1]	n.d	n.d	n.d	n.d	4.26 (5)
Staphylococcus equorum spp. *linens*[1]	3.19 (7)	3.29 (7)	3.02 (7)	3.24 (7)	n.d
Unknown	2.71	2.47	2.24	2.41	3.56

n.d, not detected.

Unknown – isolates that died prior to positive identification.

The number of fish from which bacteria were isolated is given in brackets.

[1] Partial sequence of 16S rRNA were analysed and edited in BioEdit. An initial BLAST-search in GenBank retrieved the taxonomic groups for which showed highest identities.

[2] *Pseudomonas fluorescens* and *Pseudoalteromonas agarivorans* were also isolated from rearing water.

Arthrobacter, *Brachybacterium*, *Janibacter*, *Pseudomonas*, *Pseudoalteromonas*, *Psychrobacter*, *Brevibacterium*, *Bacillus*, *Brevibacterium*, *Enterococcus*, *Marinilactibacillus psychrotolerans* and carnobacteria were also isolated. However, some changes in microbiota composition seem to occur between the dietary groups. For example, *Enterococcus* strains were not isolated from fish fed dietary rapeseed oil, while *Marinilactibacillus psychrotolerans*-like strains were only isolated from this dietary group. To our knowledge, the new genus and species, *M. psychrotolerans* a halophilic and alkaliphilic marine LAB, was proposed by Ishikawa *et al.* (2003).

A difference in gut microbiota between fish fed vegetable oils and marine oil was observed as *Shewanella putrefaciens* and *Serratia* spp. were only isolated from fish fed marine oil. On the other hand, the clearest differences in gut microbiota were detected between dietary groups fed vegetable oils and marine oil compared with fish fed the commercial diet prior to the start of the experiment, as the hindgut microbiota of fish fed the commercial diet comprised of *Acinetobacter johnsonii*, *Pseudomonas jessenii*, *Psychrobacter submarinus*, *Carnobacterium* spp. and *Staphylococcus* spp.

Fifteen of the 154 strains isolated from the digestive tract of rainbow trout fed sunflower oil were identified as *C. mobile* by 16S rRNA gene sequence analysis, but *C. mobile* was not isolated from the gut of the other rearing groups. On the other hand, 4 strains of *Carnobacterium* spp. were isolated from the gut of two fish fed linseed oil, 11 strains from 5 fish fed marine oil and 5 strains from three fish fed the commercial diet prior the start of the experiment. These results indicate that carnobacteria are present in the alimentary tract of rainbow trout, but generally at relatively low population levels and they seem easily affected by dietary manipulation.

During the last decade, some information has become available about the antagonistic activity of carnobacteria against fish pathogens (Ringø *et al.* 2005). Antagonistic activity against *A. salmonicida* and *V. anguillarum* was observed in 11 of the 15 *C. mobile* strains isolated from rainbow trout fed sunflower oil, but antagonistic activity against the two pathogens was only observed by 4 of the 20 carnobacteria strains isolated from the other rearing groups. An important question rises based on the antagonistic activity of carnobacteria isolated from the distal intestine of Atlantic salmon and rainbow trout. Why was antagonistic activity mostly detected in some *C. mobile* isolated from Atlantic salmon and rainbow trout? This finding has not been elucidated and merits further investigations. The low frequency of antimicrobial activity observed in gut isolates isolated from Atlantic salmon and rainbow trout contradicts the results reported by Makridis *et al.* (2005) in their study on Senegalese sole (*Solea senegalensis*) fed two diets, commercial diet or polychaete (*Hediste diversicolor*), revealing that the numbers of bacterial strains with antibacterial activities towards pathogens increased by feeding the fish polychaete.

Lauzon *et al.* (2008) reported that 13.8% of bacteria isolated from cod rearing (not all gut bacteria) revealed inhibitory activity, but only 3.2% of the bacteria were antagonistic to all three pathogens tested. Similarly, Hjelm *et al.* (2004) identified 8% of turbot larval rearing isolates as inhibitory. Spanggaard *et al.* (2001) reported 4% of isolates (from skin, gills and GIT of rainbow trout) to be inhibitory towards *V. anguillarum*. When evaluating antagonistic activity, one should also pay attention to the method used to determine this property as medium utilized may influence outcome (Lauzon *et al.* 2008) as well as temperature may do (Caipang *et al.* 2010).

When discussing the antagonistic effect of gut bacteria towards pathogens, it is worth to mention that in a probiotic study with Atlantic salmon, Gram *et al.* (2001) used *P. fluorescens* strain AH2 a strain showing strong *in vitro* inhibitory activity towards *A. salmonicida*. However, co-habitant infection by *A. salmonicida* in Atlantic salmon did not result in any effect on furunculosis-related mortality. Based on their results, the authors concluded that a strong *in vitro* growth inhibition cannot be used to predict a possible *in vivo* effect.

Montero *et al.* (2006) studied the effect of vegetable oils on the gut microbiota of gilthead sea bream (*Sparus aurata*) postintraperitoneal challenge with *Vibrio alginolyticus*. As this was not the main emphasis of the study, little information was reported. However, the authors noted that culturable levels of total Gram-positive bacteria and *Vibrio* spp. were present in fish fed a diet containing 60% replacement of fish oil with a blend of rapeseed oil, linseed oil and palm oil (at a 15 : 60 : 25 ratio) in comparison with diets containing 100% fish oil or 100% vegetable oil blend. The authors concluded that this was a result of modified fatty acid composition of the intestinal epithelial cell walls. However, as these preliminary data were presented with little information on the methodology, that is number of replicates and number of isolates identified, the data should be viewed with caution.

In study by Huang (2008), a 1.23% mixture of oil (phospholipid oil: rice bran oil in the ratio 2 : 1) was supplemented into a practical diet for grass carp and the autochthonous gut bacteria was evaluated by 16S rDNA

V3 DGGE after 8-week feeding. The similarity of the bacterial communities between the two dietary groups was 0.73 by the cluster analysis. Uncultured *Proteobacterium*-like organisms were stimulated, while *Clostridium maritimum*-like were depressed by the dietary mixture oil compared with the control.

Electron microscopy Attention has been paid to the importance of scanning electron microscopy (SEM) investigations in gut studies of the microbiota (Ringø & Olsen 1999; Ringø *et al*. 2001a,b, 2002, 2003, 2007a,b; ; Merrifield *et al*. 2009a,b, 2011a,b; Harper *et al*. 2011). Ringø & Olsen (1999) demonstrated a correlation between classical microbial examination and SEM, as both methods showed predominance of coccoid-shaped bacteria in the hindgut region of Arctic charr. In a later study, Ringø *et al*. (2001a) showed substantial associations of both coccoid- and rod-shaped bacterial cells with the apices of, and between, microvilli of the enterocytes in the MI of Arctic charr fed SO. The reason for this difference in colonization pattern of the enterocyte surface has not been elucidated, but Ringø *et al*. (2001a) suggested three possible causes: [1] enterocyte ageing, [2] differentiation, or [3] specific receptors for receptor-mediated endocytosis of bacteria. Compared with that reported by Ringø *et al*. (2001a), clear differences in bacterial colonization of the enterocyte surface in the hindgut were observed when Arctic charr were fed linseed oil (Ringø *et al*. 2002). These differences in bacterial colonization of enterocyte surface between fish fed SO and linseed oil may be related to the different gut microbiota observed between the two dietary groups (Ringø *et al*. 2002). This controversial hypothesis calls for further investigations.

Dietary polyunsaturated fatty acids

Although fatty acids are important in fish metabolism, few studies have evaluated the effect of dietary polyunsaturated fatty acids on the gut microbiota (Ringø 1993a; Ringø *et al*. 1998). In his study on the effect of linoleic acid (18:2n-6) on intestinal microbiota of Arctic charr, Ringø (1993a) was not able to isolate LAB in the intestinal contents, but large numbers of *Aeromonas* spp., *Pseudomonas* spp. and Enterobacteriaceae were isolated when 2.5% linoleic acid was supplemented to a commercial recipe. In contrast to these findings, *Lactobacillus* spp. accounted for approximately 10% of the microbiota when the fish were fed the unsupplemented diet. In a recent study with Arctic charr fed casein-based diets supplemented with different fatty acids [18:2n-6, α-linolenic acid (18:3n-3), or a HUFA mix (20:5n-3 and 22:6n-3)], Ringø *et al*. (1998) showed no suppression of LAB (*Carnobacterium* spp., *Carnobacterium piscicola* and *Lactobacillus plantarum*) in the stomach, PI and DI. However, a significant increase in both total viable counts and population level of LAB was observed in DI and faeces of fish fed 7% 18:3n-3 or 4% HUFA mix. This was due to a large extent to the increased levels of *Carnobacterium* spp. The reason for the increase in LAB in fish fed 7% linolenic acid and HUFA mix has not been elucidated, but the authors suggest that dietary fatty acids influence intestinal membrane composition, function and fluidity which may affect the attachment sites of the gut mucosa. Later, this controversial hypothesis was confirmed by Kankaanpää *et al*. (2001). They demonstrated that culturing of Caco-2-cells with arachidonic acid (20:4n-6) reduced the Caco-2 cell adhesion of LAB, whereas 18:3 (n-3) did not hinder adhesion of *Lactobacillus* GG or *Lactobacillus bulgaricus*, and promoted the adhesion of *Lactobacillus casei* Shirota.

In view of the results observed by Ringø *et al*. (1998), it is interesting to note that the ability of *C. piscicola*-like isolates to inhibit the fish pathogen *A. salmonicida* subsp. *salmonicida* was highest in strains isolated from fish fed linolenic acid or the HUFA mix (E. Ringø, unpublished data). Based on these results, it is recommended that greater attention should be given to the subject of how to increase the level of intestinal carnobacteria with inhibitory effect against fish pathogens by dietary manipulation. The results obtained from fish fed dietary 18:3 (n-3) may lead to the conclusion that it is desirable to increase the level of dietary 18:3 (n-3) in commercial diets in order to obtain a higher population level of intestinal strains of *C. piscicola* able to inhibit growth of *A. salmonicida* subsp. *salmonicida*. However, it is worthwhile to note that feeding the charr an experimental diet with high levels (> 15%) of dietary 18:3 (n-3) increased accumulation of lipid droplets in the enterocytes and caused cell damage which may increase the risk of microbial infections (Olsen *et al*. 1999, 2000).

Effect of diet

The gut microbiota of goldfish (*Carassius auratus*) (Sugita *et al*. 1988a), Atlantic cod (Strøm & Olafsen 1990), Arctic charr (Ringø & Strøm 1994), abalone (*Haliotis discus*) (Tanaka *et al*. 2003), puffer fish (*Takifugu obscurus*) (Yang *et al*. 2007), yellow grouper (*Epinephelus awoora* Temminck & Schlegel, 1942) (Feng *et al*. 2010), gilthead sea bream and goldfish (de Paula Silva *et al*. 2011) and transgenic

common carp (*Cyprinus carpio* L.) (Li *et al.* 2013) have been investigated in an attempt to clarify the effect of different diets on the intestinal microbiota.

In their early study, Sugita *et al.* (1988a) concluded that *Aeromonas hydrophila* and *Bacteroides* type A were predominant in almost all goldfish fed on either the pelleted diets and tubifex worms or pelleted diets and dried daphnia. The authors concluded that the gut microbiota was not influenced by the diets. However, as the fishes were fed the different diets for only a short period of time (22 days), no conclusion can be drawn. In contrast to the conclusion by Sugita *et al.* (1988a), Strøm & Olafsen (1990) demonstrated in a long time experiment (over one year) with Atlantic cod fed a commercial diet that the adherent microbiota was affected by the diet compared to wild fish. In newly caught wild fry, three dominating *Vibrio* strains (designated A, B and C) were detected, whereas *Vibrio* type A was not isolated in fish after one month, and one year of rearing on the commercial diet. *Vibrio* type B was isolated in newly hatched fry, but almost no *Vibrio* type B was noticed in fish fed the commercial diet later in the experiment. An increase in population level of *Pseudomonas* spp. and lactobacilli was also noted during the experiment.

In the 1980s, the commercial catch of the Barents Sea capelin (*Mallotus villosus*) stock was high and it was suggested that the capelin roe might be a good feed alternative to the commercial diets. In a study on Arctic charr fed either a capelin roe diet or a commercial feed, Ringø & Olsen (1994) demonstrated that the gut microbiota was affected. Approximately 55% of the gut bacteria of charr fed on capelin roe were identified as Enterobacteriaceae. In contrast, intestinal Enterobacteriaceae were not isolated from fish fed the commercial feed as in this group; *Aeromonas* spp. and *Pseudomonas* spp. were the predominant genera (about 30% each). In contrast, *Pseudomonas* spp. was not isolated from the gut of fish fed the capelin roe diet. The population level of *Lactobacillus* spp. was not affected by dietary manipulation, but *Leuconostoc* spp. were only isolated from the gut of fish fed capelin roe. Furthermore, *Streptococcus* spp. was only isolated from the gut of charr fed the commercial diet.

Tanaka *et al.* (2003) reported the microbiota of the whole intestine of juvenile abalone fed microalgae and algal pellets. The gut microbiota of abalone fed on microalgae matched the microbiota of sea water but changed when abalone were fed algal pellets; especially alginate – utilizing bacteria including *Vibrio halioticoli*. More recently, Cerezuela *et al.* (2012) investigated the effect of microalgae (*Tetraselmis chuii* and *Phaeodactylum tricornutum*) on the allochthonous bacterial community in gilthead sea beam. The four-week study showed that the experimental diets added *T. chuii* (T) and *P. tricornutum* (P) caused alternations in bacterial diversity, and significant reductions in specific richness, the Shannon's index and range-weighted richness. These results were confirmed by clustering analysis of the DGGE pattern, but fish fed diet T and P showed high similarity (81%). The authors speculate that the modulation of the allochthonous intestinal microbiota results could be related to antimicrobial activity of *T. chuii* towards presumptive *Vibrio* (Makridis *et al.* 2006) and *P. tricornutum,* eicosapentaenoic acid against a range of both Gram-negative and Gram-positive including *Staphylococcus aureus* (Desbois *et al.* 2009).

The gut microbiota of puffer fish fed natural (fresh fish and shrimp) and artificial diets were evaluated by Yang *et al.* (2007). In this study, pooled samples from three fish were evaluated by DGGE. The authors reported that some genera and classes were specific for fish fed natural diet, *Lactococcus*, *Carnobacterium*, *Bacillus*, *Shewanella* and β-Proteobacteria, while Spirochaetales, Ɛ-Proteobacteria and *Trichococcus* were specific for fish fed the artificial diet. In addition, the study displayed that the intestinal microbiota was markedly different from that observed in ovary, liver and skin.

In a more recent study, Feng *et al.* (2010) investigated the autochthonous and allochthonous gut microbiota of yellow grouper fed an extruded sinking diet and natural diet, ice-fresh fish and shrimp. Differences seem to occur between autochthonous bacteria of fish fed the extruded sinking diet as higher bacterial apparent species richness and possibly less abundance existed in this group compared with natural diet-fed fish. Band u1 and u3, and uncultured *Aranicola*, uncultured *Acinetobacter*, *Staphylococcus pasteuri*, *Enterobacter ludwiggi*, *Pantoea agglomerans*, *Enterobacter* sp., *Agrococcus* sp., *Ewingella americana* and uncultured bacterium clone PL3-13 (a Firmicutes) were unique for fish fed extruded sinking diet, while for natural-diet-fed fish, band u6 and u7, and *Enterobacter cloacae*, *Roseibacillus persicicus* and uncultured *Pseudomonas* sp. were unique. In contrast to these results, no difference was demonstrated between the allochthonous gut microbiota of the two feeding groups.

A study on gilthead sea bream investigated the influence of partial substitution of FM with lupin and rapeseed meals on allochthonous bacterial counts and revealed no significant effect in the stomach and intestine between treatments (de Paula Silva *et al.* 2011). Even though some differences were retrieved between the sampling dates, 3, 10, 17, 24 and 30 days of feeding, the Dice similarity

coefficients were generally higher among the gastric replicates by lupin compared with FM- and rapeseed-fed fish.

Vitamin C, methionine, valine, inositol, pantothenic acid, biotin and thiamine

During the last years, some information has accumulated on how vitamin C, methionine, valine, inositol, pantothenic acid, biotin and thiamine modulate the gut microbiota of fish (Dong *et al*. 2013; Feng *et al*. 2011; Ghomi *et al*. 2010; Jiang *et al*. 2009; Liu *et al*. 2011a; Tang *et al*. 2009; Wen *et al*. 2010).

Vitamin C

In their study on kutum (*Rutilus frisii*), Ghomi *et al*. (2010) reported that the inclusion of vitamin C (0, 200 or 400 mg kg^{-1}) to the diet did not affect total counts of bacteria, both allochthonous or autochthonous bacteria and total LAB per gram intestine after 30 and 60 days of feeding. However, feeding the fish 400 mg vitamin C, the authors reported *L. plantarum* and *Lac. lactis*. Liu *et al*. (2011a) investigated the effect of graded levels of dietary vitamin C on the non-adherent gut microbiota juvenile Jian carp (*Cyprinus carpio* var. Jian). Intestinal levels of the *Lactobacillus* and *Bacillus* genera increased with increasing dietary vitamin C. On the other hand, levels of *A. hydrophila* and *Escherichia coli* decreased with increasing levels. Whether these interesting findings improved health effect was not investigated and merits further investigations.

Methionine

As methionine is an indispensable amino acid for carp, Tang *et al*. (2009) addressed to evaluate the effect of methionine on intestinal enzyme activities, immune response and gut microbiota of juvenile Jian carp. Methionine supplementation: levels between 1% and 1.6% significantly increased colony-forming units (CFU) of *Lactobacillus* and *Bacillus* g^{-1} intestinal contents, while *E. coli* and *Aeromonas* sp. was significantly decreased. The mechanism about the effects on microbial populations is unclear, but the authors suggest that it may be related to using methionine as nutrient source.

Valine

Dong *et al*. (2013) reported in their study on juvenile Jian carp on inclusion of valine (5.3 [unsupplemented control], 8.7, 11.8, 14.9 and 20.1 g kg^{-1}) to the diet, only the three highest inclusion levels affect counts of intestinal LAB. However, no information was given whether allochthonous or autochthonous bacteria were isolated. The population levels of *Bacillus* improved with higher levels of valine up to 14.9 g kg^{-1} diet, and then decreased. In contrast to these results, counts of *E. coli* were lowest when the fish were fed 11.8 and 14.9 g valine kg^{-1}. Intestinal *Aeromonas* was significantly higher in fish fed the highest valine level than that of fish fed diets containing ≤18.7 g valine per kg. The mechanism of how dietary valine affects the gut microbiota of fish is unknown, but a previous study of Szymánska *et al*. (2002) reported that valine could be a nitrogen source for bacteria in *Desulfotomaculum ruminis*.

Inositol

Jiang *et al*. (2009) evaluated in a 60-day feeding trial the effect of inositol on intestinal microbiota of juvenile Jian carp. The population level of gut *Lactobacillus* increased from 6.34 (log value) up to 8.06 (log value) with increasing supplementation of dietary inositol, from 163.5 up to 990.3 mg kg^{-1} diet. *A. hydrophila* and *E. coli* decreased with increasing level of dietary inositol up to 232.7 and 687.3 mg kg^{-1} diet, respectively. The authors speculated that the decrease in *E. coli* level in the intestine might be due to production of antibacterial compound(s) produced by lactobacilli; however, no proof was given.

Pantothenic acid

Wen *et al*. (2010) addressed to evaluate immune response, disease resistance against *A. hydrophila* and the allochthonous gut microbiota (*Lactobacillus* spp., *E. coli* and *A. hydrophila*) of juvenile Jian carp fed graded levels of pantothenic acid. *Lactobacillus* spp. increased significantly in response to increased inclusion level of pantothenic acid up to 45.9 mg kg^{-1} and remained constant at levels up to 65.9 mg kg^{-1}. Conversely, the population level of *E. coli* and *A. hydrophila* decreased by increasing inclusion levels. The reason why increasing supplementation level of pantothenic acid displayed this effect on gut microbiota is not known, but in an early study, Sneli *et al*. (1939) reported that pantothenic acid is an essential nutrient for *Lactobacillus*.

Biotin

The effect of biotin on some allochthonous gut bacteria of juvenile Jian carp was reported by Zhao *et al*. (2012b).

Intestinal bacteria belonging to genera *Lactobacillus* and *Bacillus* significantly increased with increasing level of biotin, up to 0.054 and 0.151 mg kg^{-1} diet. The observed effect on *Bacillus* could be related to a previous finding that this bacterium had the inability to form active pyruvate carboxylase from the apoprotein form at biotin-deficient media (Cazzulo *et al*. 1969). As the population level of *E. coli* and *Aeromonas* significantly decreased with increasing supplementation of biotin to the diet, up to 0.151 mg kg^{-1}, the authors speculate that this finding may be related to the production of bacteriocin-like substances produced by lactobacilli. However, as this controversial hypothesis was not confirmed, the topic merits further investigations.

Thiamine

Feng *et al*. (2011) reported the effect of dietary thiamine supplement on three intestinal bacteria, *Lactobacillus*, *E. coli* and *A. hydrophila*. *Lactobacillus* counts increased gradually and highest population level of lactobacilli was obtained when the thiamine level was 0.79 mg kg^{-1}. In contrast to these results, the population level of *E. coli* and *A. hydrophila* was not affected by inclusion of thiamine to the diet. As no supporting mechanisms were put forward by the authors, we recommend that this topic merits further investigations.

As all the studies carried out of juvenile Jian carp evaluating dietary effects on the gut microbiota, only three allochthonous intestinal bacteria,; *Aeromonas*, *E. coli* and *Lactobacillus* were investigated; we recommend that in future studies, both the autochthonous and allochthonous microbiota are evaluated. Furthermore, the ability of gut bacteria, especially *Lactobacillus*, to inhibit *in vitro* growth of some important fish pathogens should be investigated.

Effect of protein sources

As most marine resources used in FM and FO production as exploited to the highest maximum level, simultaneously as the global aquaculture production is increasing, there has been substantial progress in reducing FM in farmed fish diets (Gatlin *et al*. 2007; Hansen & Hemre 2013; Hemre *et al*. 2009 Waagbø *et al*. 2001). During the last decade, effort has focused on evaluating the extent of SBM-induced histological damage (Merrifield *et al*. 2011a), information on SBM meal-induced changes in intestinal mucus composition (Van der Marel *et al*. 2014), and the effect on gut microbiota of fish. To our knowledge, the first studies evaluating the effect of SBM on the gut microbiota of fish were published in 2006 (Heikkinen *et al*. 2006; Ringø *et al*. 2006b). In order to avoid duplication, fish studies on the effect of SBM on intestinal microbiota reviewed by Merrifield *et al*. (2011a) are not discussed in this subsection and readers with special interest are referred to the review and the original papers cited.

Casein versus fish meal and soybean meal

Mansfield *et al*. (2010) evaluated the effect of three diets, included FM and SBM and a synthetic casein-based diet (CN) on the allochthonous DI microbiota of triploid female rainbow trout (~1.6 kg) by three *cpn*60 universal clone libraries, resulting in 1176, 1000 and 1181 sequences from CN, FM and SBM, respectively. When all the sequences were combined, 32 different sequences were noticed. The most frequently observed sequences were identical to *C. maltaromaticum* and accounted for 87.8%, 55% and 97.2% of the clones from CN, FM and SBM, respectively. Overall, highest diversity was noticed from CN (16 different sequences), followed by FM (14) and only four different sequences in the SBM library. It is of interest noticing that one sequence from CN belonged to *Bifidobacterium adolescentis*.

Replacement of FM with SBM

Replacement of fishmeal with SBM (at 30% inclusion) had no significant effect on the levels of total aerobic bacteria, total anaerobic bacteria, presumptive *E. coli*, *Aeromonas*, *Bifidobacterium* or *Clostridium* in the intestine of silver crucian carp (*Carassius auratus gibelio* × *Cyprinus carpio*) (Cai *et al*. (2012). Desai *et al*. (2012) observed that 30% SBM inclusion in rainbow trout diets led to a reduction in Proteobacteria and increase in Firmicutes. Increased *Lactococcus lactis* subsp. *lactis* was observed in the mid-intestine, and a reduction in *Weissella confusa* in the distal intestine, of Atlantic salmon fed a diet containing 20% SBM (Reveco *et al*. 2014).

J.L.G. Vecino & S. Wadsworth (unpublished data) studied a total of 3600 mixed-sex Atlantic salmon *Salmo salar* (5 g; S0 Salmobreed), which were stocked in 9 × 1 m^3 freshwater tanks (400 fish per tank, at 12.3–15.1 °C). Fish were fed for 6 weeks in triplicate tanks either a FM-based diet as a positive control diet, a diet containing 10% SBM (negative control diet), or a diet similar to the negative control diet but with EWOS prebiosal® [a prebiotic product containing a combination of marine and terrestrial complex carbohydrates (Chemoforma Ltd., Augst, Switzer-

land)] added. Digesta samples were stripped from 160 to 170 fish per tank for microbiota analyses at the end of the trial and analysed for total microbial numbers by q-PCR and for microbial community structure by partial sequencing of the 16S rRNA gene as described in Apajalahti *et al.* (2002).

Microbial analyses showed that the inclusion of 10% SBM in the negative control diet increased the total microbial numbers compared with the FM-based diet, while the inclusion of Prebiosal™ in the diet seemed to normalize the increasing numbers of total bacteria to levels similar to those found in the FM diet (Fig. 5).

Figure 5 shows the proportions of all genus level clusters with abundance exceeding 5%. Genus level clusters with abundance lower than 5% were summed into the group 'Others'. *Kluyvera* spp. and other closely related representatives of *Enterobacteriaceae* (named as *Kluyvera* in Fig. 5) comprised approximately one-fourth of the total microbes. The second most abundant cluster (*Peptostreptococcus* in Fig. 5) represented 8% and 21%, in the FM and SBM diets, respectively. Furthermore, there were 3 clusters with abundance of approximately 10% both in the FM and SBM diets, *Sphingomonas* spp., Clostridia cluster I and a cluster representing unidentified micro-organism related to cyanobacteria.

Sphingomonas spp. (family *Sphingomonadaceae*, phylum *Proteobacte*ria) decreased from 1.8×10^6 bacteria in FM-fed salmon to below detection limit in the SBM diet. Prebiosal showed a normalizing effect; and the levels of *Sphingomonas*-like bacteria were around 1.7×10^6, not differing from those found in group fed the FM diet. The genus *Sphingomonas* represents Gram-negative rods with aerobic metabolism. These microbes are not part of the microbiota of the GI tract of warm-blooded animals but are common in the environment. *Sphingomonas* spp. have been frequently misidentified as *Pseudomonas* spp. Therefore, it is possible that previous various studies that have reported *Pseudomonadaceae* from the GI tract microbes in salmonids may actually represent *Sphingomonadaceae*. The genus level cluster *Sphingomonas* spp. is comprised mainly of one single species, namely *Sphingomonas yabuuchiae*.

Another difference was observed in the bacteria representing *Peptostreptococcusis* related to *Clostridium* cluster XIII as reported by Murdoch *et al.* (1997). The amount of these bacteria in the digesta of fish fed SBM diet was almost five times higher than in the FM group (6.4×10^6 versus 1.3×10^6, respectively), while the levels of bacteria resulting from the diet containing Prebiosal were not significantly different to those observed in the FM diet. The bacteria in the genus level cluster *Peptostreptococcus* mainly represented a single species closely related to *Peptostreptococcus anaerobius,* which is the type species of the genus *Peptostreptococcus*. Many species belonging to the genus *Peptostreptococcus* are according to the modern taxonomy positioned to Clostridia clusters XIII and XIV, but the type species and consequently, the bacteria found in the fish samples are members of Clostridia cluster XI. Peptostreptococci are Gram-positive cocci with strictly anaerobic metabolism but also tolerate low levels of oxygen. They get their energy mainly from protein fermentation being only weakly saccharolytic. When considering the ecological niche of these microbes, one should keep in mind that the GI tract of warm-blooded animals also harbours members of Clostridia cluster XI.

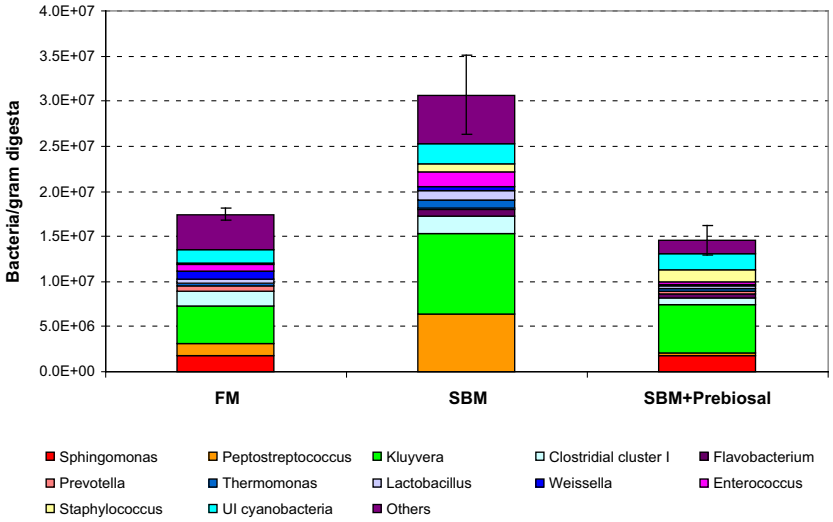

Figure 5 Total number of genus clusters in digesta samples of Atlantic salmon (*Salmo salar* L.) (5 g) fed different diets for 6 weeks (J.L.G. Vecino & S. Wadsworth unpublished data).

The third important diet-related change affected members of *Kluyvera* and other closely related *Enterobacteriaceae*. Abundance of the *Kluyvera* cluster in the digesta from salmon fed FM diet was 4.1×10^6 bacteria per gram digesta compared with 8.8×10^6 and 5.6×10^6 for the samples from the SBM and SBM + prebiosal groups, respectively.

Taxonomically, family *Enterobacteriaceae* comprises many microbial genera that based on 16S rRNA sequence only could be classified to a single genus. The genera that based on 16S sequencing could be considered as one includes *Citrobacter*, *Enterobacter*, *Erwinia*, *Escherichia*, *Klebsiella*, *Kluyvera*, *Proteus*, *Salmonella*, *Shigella* and *Yersinia*. Other methods based on metabolic routes are needed to distinguish these genera from each other. Consequently, 16S sequencing approach cannot prove with total certainty that sequences showing best match to, for example *Kluyvera cryocrescens*, truly represent this species but with a small likelihood, it is also possible that these sequences represent *Citrobacter freundi* or *Erwinia persicina*. However, the fact that the majority of sequences in the *Kluyvera* genus level cluster matched best to *Kluyvera cryocrescens* and the knowledge that unlike most enterobacteria, *Kluyvera cryocrescens* is capable of growing at temperatures as low as 4 °C, a temperature coinciding to conditions for cold-water fish, strongly suggest that the microbes detected in the study truly represent *Kluyvera cryocrescens*. *Kluyvera* are Gram-negative facultative anaerobic rods that are able to grow under both aerobic and anaerobic conditions. Their main metabolic activity is saccharolytic but they can also utilize acetate.

The three major genus level clusters affected by SBM inclusion differed with regard to their response to oxygen concentration and redox potential, *Sphingomonas* being aerobic, *Kluyvera* facultative anaerobic and *Peptostreptococcus* strictly anaerobic, but aerotolerant. When these three clusters were examined in more detail, *Sphingomonas* appeared to be present only when the total level of microbial numbers is low. The presence of *Sphingomonas* was indeed negatively linked to total microbial numbers as shown by principal component analysis (Fig. 6). The negative relationship could indicate that with low microbial fermentation on a FM diet, there was enough oxygen for *Sphingomonas* to prevail and grow but when the microbial fermentation accelerated, due to a new carbohydrate source (e.g. SBM), the fermentation scavenged oxygen leading to anaerobic environment where *Sphingomonas* no longer was successful.

The situation for *Peptostreptococcus*, an obligate anaerobe, was the opposite. In fact, the abundance of

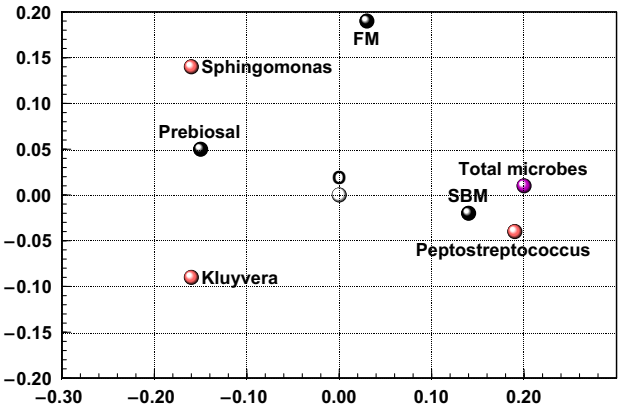

Figure 6 Principal component analysis figure of the data. Points that cluster together share the same data pattern and show a positive correlation to each other's, while points at the opposite sides of the origin (O) show a negative correlation. FM = fish meal, SBM = soybean meal diet; *P* = Prebiosal; *Sphingomonas*, *Kluyvera* and *Peptostreptococcu*s are the relative abundancies of *Sphingomonas*, *Kluyvera* and *Peptostreptococcus*, respectively (J.L.G. Vecino & S. Wadsworth unpublished data).

Sphingomonas was highest when *Peptostreptococcus* was the lowest. It could be hypothesized that maybe only when the facultative aerobes, in this case *Kluyvera*, have used the oxygen and lowered the redox potential to a low enough level, *Peptostreptococcus* were able to compete. High total bacterial numbers and high abundance of *Peptostreptococcus* were associated to SBM inclusion (Fig. 6).

In this triangle, *Kluyvera* was likely to have acted as an important player in the transition phase, shifting the environment from aerobic to anaerobic and lowering the redox potential. The fact that transition role was emphasized by the fact that the absolute numbers of *Kluyvera* were higher in the groups with high total microbial numbers.

Overall, it seems that the inclusion of SBM in the diet increased the total microbial numbers and the abundance of *Peptostreptococcous* and *Kluyvera*. On the other hand, the inclusion of SBM in the diet decreased the abundance of *Sphingomonas* and *Kluyvera* as compared to the FM-fed salmon (Fig. 6). The higher abundance in microbial community found in SBM-fed salmon may also influence or be influenced by nutrient availability/utilization. A previous study demonstrated repeatedly lower macronutrient digestibilities when salmon are fed SBM (Olli *et al.* 1994). Possible changes in the levels of microbial metabolites such as short-chain volatile fatty acids (SCFA) and protein derivatives (indole, skatole, cresol, ammonia, amines/amides) were assessed using gas chromatography in the current study. However, the concentration of microbial

metabolites remained below detection limit in all samples and acetic acid was the only SCFA detected at low concentration (~1 mM) with no differences between the samples, correlated with microbiota.

The inclusion of Prebiosal to the diet containing 10% SBM helped to maintain the number of bacterial species compositions similar to that found in the digesta from the group fed the FM diet. In this trial, no significant differences in growth were noticed between the diets. This is in contrast with five further trials with Atlantic salmon where significant differences in growth were observed.

Huang (2008) compared the effect of dietary inclusion of SBM (1.3% by dry weight) and casein meal (CM; 1.0% by dry weight) on the autochthonous gut microbiota of grass carp. After 8-week feeding, the microbiota from the whole intestine was analysed by 16S rRNA PCR-DGGE. The results demonstrated clear differences between the microbiota of the SBM group and the CM group with similarity between the groups of only 26% ($P < 0.05$) [determined by the unweighted pair group method using the arithmetic mean algorithm (UPGMA)]. Unique bacteria reported from the CM group were identified as follows: uncultured *Lachnospiraceae* bacterium (EU 418523), uncultured *Lactobacillus* (EU418525), uncultured *Clostridium* spp. (EU418527) and uncultured *Proteobacterium* (EU418526). Unique bacteria reported from the SBM group were identified as follows: *Aeromonas* sp. (EU418521), uncultured bacteria (EU418508; EU418512), uncultured actinobacterium (EU418511) and uncultured *Bacillus* spp. (EU418510). The author suggested that the differences in the intestinal autochthonous bacterial communities between the groups may have been due to the different composition of the two protein ingredients.

In a recent study, the effect of partial replacement of SBM (4%) by intestinal casing meal (ICM), prepared from the wastewater of enteric coating and heparin processing, in the diet of cage-cultured, Cyprinidae was addressed on growth performance, feed utilization and allochthonous gut microbiota (Li *et al*. 2014a). The allochthonous bacterial diversity was altered by ICM substitution, however; by feeding ICM, some bacterial species were significantly stimulated, *E. coli* and *Exiguobacterium* in black carp (*Mylopharyngodon piceus*) and species belonging to *Firmicutes*, *Fusobacteria* and *Proteobacteria* in gibel carp (*Carassius gebelio*).

Despite the fact that these studies are beginning to shed light on the effects of dietary SBM on the gut microbiota of salmonids, Atlantic cod and carps, the studies are largely based on cultured isolates. Further culture-independent techniques need to be employed in future studies and investigations need to be extended to other important aquatic species as SBM is one of the commonly used plant proteins incorporated into aquafeeds (Gatlin *et al*. 2007).

Effect of invertebrate meals on allochthonous gut microbiota of mirror carp (Cyprinus carpio)

The effect of dietary invertebrate meals, silkworm (*Bombyx mori*) pupae and polychaete worm (*Nereis virens*), on the intestinal microbial community structure and diversity of mirror carp (*Cyprinus carpio*), was investigated by Z.Y. Wan, S. Davies & D.L. Merrifield (unpublished data). Using V3 16S rRNA PCR-DGGE analysis after Muyzer *et al*. (1993), allochthonous communities from the intestine of three individual fish were investigated. The 16S rRNA DGGE profiles demonstrate clear differences of microbiota between the dietary groups. Fish fed a control diet harboured an average of 14.7 ± 2.0 OTUs, with two species uniquely detected in the control diet-fed fish. Fish fed the silkworm diet (13.4% silkworm meal by dry weight) harboured an average of 15.0 ± 6.1 bacterial OTUs and fish fed the polychaete diet (14.8% polychaete meal by dry weight) harboured an average of 14.0 ± 1.7 bacterial OTUs, while fish fed the silkworm + polychaete worm diet (15.0% invertebrate meal by dry weight) harboured an average of 11.7 ± 2.3 bacterial OTUs. A total of 12 OTUs were identified in one or more of the groups fed the invertebrate meals that were not present in fish fed the control diet. However, the study focused on community structure and species diversity, and none of the species were identified by 16S rRNA sequence analysis.

Rapeseed and cottonseed meal

Dietary rapeseed meal (43.0%) was substituted by cottonseed meal (33.0%) as equal protein content in the practical diet of grass carp cultured in cages, and the variation of intestinal autochthonous bacteria was studied by 16S rDNA V3 DGGE (Huang 2008). The intestinal autochthonous bacterial diversity was modulated by dietary substitution, and the similarity of the bacterial community between the two dietary groups was 0.56, cluster analysis. Uncultured bacterium-like and uncultured beta-*Proteobacterium* clone-like were identified as the unique bacteria in dietary cottonseed meal group, while uncultured bacterium clone 4I6-G5-like and uncultured bacterium clone aab65c09-like were identified as the unique bacteria in the dietary rapeseed meal group.

Wheat middling and corn meal

Six per cent dietary wheat middling was replaced by corn meal in the practical diet of grass carp cultured in cages, and the variation of intestinal autochthonous bacteria was investigated by 16S rDNA V3 DGGE (Huang 2008). The intestinal autochthonous bacterial diversity was affected by this substitution, and the similarity of the bacterial composition between the two dietary groups was 0.71 by cluster analysis.

Dried distiller's grain (DDG)

In a recent study on grass carp, bluntnose black bream (*Megalobrama amblycephala*), gibel carp and black carp fed DDG in cages for 8 weeks, the allochthonous gut microbiota were investigated (He *et al.* 2013). Both Shannon diversity index and Shannon equitability index and the intestinal autochthonous bacterial diversity were affected by dietary DDG.

Other dietary protein sources

In a recent study, Hartviksen *et al.* (2014) investigated the modulation of the allochthonous and autochthonous bacterial gut community of Atlantic salmon following feeding with FM, pea protein concentrate (PPC), soy protein concentrate (SPC), extracted sunflower (ESF), poultry by-product (PBY) and feather meal (FeM) using culture-independent method, total number of 16S DNA copies. In PI, the total allochthonous microbiota was significantly affected by dietary ESF, FeM and PBY compared with the other dietary group, while the autochthonous bacteria levels were unaffected by dietary modulations. Total autochthonous bacteria in DI remained unaffected by dietary treatments. Allochthonous bacteria in PI were dominated by *Corynebacteriaceae*, log values > 7.57, and the log values were significantly higher in fish fed ESF, FeM and PBY. Log values of *Lactobacillaceae* were second most abundant and were significantly higher in fish fed ESF and FeM compared with the other treatments. *Lactobacillaceae* were dominant, log values >5.46, in the autochthonous community in PI, followed by β-*Proteobacteria*, log values about 5.1. The bacterial community, autochthonous, in DI were dominated by *Lactobacillaceae* followed by β-*Proteobacteria* and *Enterobacteriaceae*. The only treatment group with significantly higher population level of *Lactobacillaceae* compared with control was noticed in fish fed PPC. Log values of β-*Proteobacteria* and *Enterobacteriaceae* remained unaffected by dietary treatment.

Fish protein hydrolysates

Information is available on the use of two fish protein hydrolysates (FPH) incorporated into four diets for sea bass larvae and their effect on cultivable intestinal bacteria (Kotzamanis *et al.* 2007). The significant differences noticed were related to the date for TCBS counts, ca. 10^2–10^3 CFU per larva at 17 dph versus $>10^2$ per larva at 26dph. The gut microbiota at 17dph displayed that dose (10% and 19% inclusion) and FPH type (commercial enzymatic FPH or sardine silage) affected the proportion of *Vibrio* spp. Even though only few bacterial species were detected, different intestinal bacteria, *Vibrio* sp. RE1-3, *Enterovibrio norvegicus*, *Marinomonas mediterranea*, *Marinomonas primoryensis*, *Bacillus* sp. and *Pseudoalteromonas* sp., between the treatment groups were noticed. In future studies, other methods should been used and the authors suggested direct counting by epifluorescence microscopy and randomly cloned eubacterial 16S rDNA.

Thymol and carvacrol

Thymol is part of a naturally occurring class of compounds known as biocides, with strong antimicrobial attributes when used alone or with other biocides such as carvacrol. Thymol and carvacrol are reported in oil of thyme and extracted from *Thymus vulgaris* (common thyme). In a recent study by Giannenas *et al.* (2012), dietary supplementation of thymol and carvacrol, 1 g kg^{-1}, on intestinal microbiota were investigated in an 8-week experiment with rainbow trout. Total counts of aerobic gut bacteria were not affected by thymol and carvacrol in contrast to the results of total anaerobes. Population level of gut bacteria belonging to, Enterobacteriaceae and coliforms, were not affected, but the levels of *Lactobacillus* spp., identified by biochemical tests, significantly decreased by supplementation of thymol to the diet. When evaluating whether plant products with high antimicrobial activity modulate the gut microbiota, molecular identification should be included to specifically identify which species are affected.

Effect of krill, chitin, cellulose, raffinose and stachyose

Krill and chitin

The 2nd most abundant biomass (10^{13} metric tons; Jolles & Muzzarelli 1999) in the world is chitin and consists of a β-1,4-linked *N*-acetylglucosamine residues. In their recent

review devoted to the use of krill and chitin in aquaculture, Ringø *et al*. (2012a) discussed the results of six papers (Askarian *et al*. 2012, 2013; Kono *et al*. 1987; Kumar *et al*. 2006; Ringø *et al*. 2006c; Sera & Kimata 1972; Zhou *et al*. 2013a) on the effects of dietary krill and chitin in finfish, Japanese eel and giant freshwater prawn. Readers with special interest in these results are referred to the review of Ringø *et al*. (2012a).

Chitosan is obtained from the partial deacetylation of chitin and is therefore a high-molecular-weight linear composed mainly of 2-amino-2-deoxy-D-glucose units linked through β (1→4) bonds, and the distinction between chitin and chitosan is based on the degree of acetylation. Chitin has acetylation values higher than 50%, while chitosan has lower percentages. As less information is available on chitosan as microbial modulators (Mrazek *et al*. 2010; Terada *et al*. 1995), we recommend that this area merits further investigations. However, such studies should also include effect on innate immune response, gut morphology and its effect on growth performance and resistance in challenge studies.

Cellulose and exogenous cellulase

Cellulose The most abundant biomass (10^{15} metric tons; Wilson & Irwin 1999) in the world is cellulose and consists of a β-1,4-glycosidic linkages. Thus, many cellulose-eating animals require the aid of symbiotic micro-organisms in their GI tract to digest cellulose and make the energy in this compound available to the host (Bergman 1990; Karasov & Martinez del Rio 2007; Mo *et al*. 2004). Information is available on the microbial community in different parts of the GI tract of wood-eating fish (Di Maiuta *et al*. 2013; McDonald *et al*. 2012). However, to our knowledge, only one study has evaluated the effect of cellulose on gut microbiota of fish, Atlantic salmon (Ringø *et al*. 2008). In this study, cultivable autochthonous and allochthonous bacteria were investigated in the distal intestine of fish fed FM and cellulose. Log total viable counts were not significantly affected, but some modulation of bacteria was observed. *Carnobacterium*, both autochthonous and allochthonous and allochthonous *Bacillus*, was only detected in fish fed FM, whereas allochthonous Antarctic seawater bacterium Bsw10170, Arctic seawater bacterium Bsw20461, *Arthrobacter bergeri* and *Staphylococcus equorum* were only reported in fish fed cellulose. Whether these bacteria can contribute to nutrition by production of enzymes, including cellulase, was not investigated, but it is worth noticing that S. *equorum* isolated from Atlantic salmon displayed high cellulase score (Askarian *et al*. 2012).

Exogenous cellulase Several studies have shown that the intestinal microbiota of aquatic animals harbours cellulose-decomposing micro-organisms, including sequences related to *Anoxybacillus*, *Bacillus*, *Carnobacterium*, *Citrobacter*, *Clostridium*, *Leuconostoc*, *Staphylococcus*, *Acinetobacter*, *Phaeobacter*, *Pseudomonas*, *Rhodobacteraceae*, *Vibrio* and *Actinomyces* (Askarian *et al*. 2012; Nelson *et al*. 1999; Ray *et al*. 2012; Tanu *et al*. 2012; Wu *et al*. 2012). To our knowledge, supplementation of these bacteria and their effect on intestinal microbiota is not done. However, in a recent study, Zhou *et al*. (2013b) carried out a 2-month feeding trial with grass carp fed the following; (i) shredded duckweed and wheat flour mixed with exogenous cellulase (3 g kg^{-1}; contains ≥ equal to 1.0 unit mg^{-1} cellulase activity) and (ii) shredded duckweed and similar amount of wheat flour. Pooled contents from the entire intestinal tracts of three fish were investigated by DGGE, and analysis indicated that the allochthonous gut bacterial community was modulated, species and density by supplemental cellulase. Seven DGGE bands were unique for the control fish, whereas four bands were unique for the cellulase treatment, *Sphingomonadaceae* bacterium PB136, *Sphingomonas echinoides*, *Bacillus* sp. CE2 and *Lepothrix* sp. AV011a. Whether these bacteria have cellulolytic activity is unknown and merits investigation even though certain strains of *Bacillus* and *Sphingomonas* are able to produce cellulase in moderate quantities.

Raffinose and stachyose

As no information is available on the effect of SBM carbohydrates on growth, carcass composition, intestinal morphology and gut microbiota, Cai *et al*. (2012) carried out a study on juvenile allogyogenic silver crucian carp (*Carassius auratus gebelio*♀ × *Cyprinus carpio*♂) fed FM and FM substituted with raffinose (6.7 g kg^{-1}) and stachyose (33.9 g kg^{-1}). Evaluation of the gut microbiota (both allochthonous and autochthonous) revealed that the population level of total aerobic bacteria, *E. coli* and *Aeromonas* sp., and total anaerobic bacteria, *Bifidobacterium* sp. and *Clostridium perfringens*, was not modulated by dietary inclusion. However, for further confirmation of these findings, 16S rRNA gene sequencing analysis should have been carried out. Furthermore, the authors should have distinguished the allochthonous and autochthonous microbiota. One interesting observation was noticed in the present

study; supplementation of raffinose and stachyose did not affect intestinal histology.

Other carbohydrate sources

In a recent study, Pedrotti *et al.* (2015) evaluated the dietary effect of different carbohydrate sources, broken rice, dextrin, cassava bagasse, ground corn and wheat bran, on total heterotrophic cultivable autochthonous and amylolytic gut bacteria in DI of tilapia and jundiá (*Rhamdia quelen*). The general findings was no difference in levels of total cultivable bacteria among carbohydrate sources within the same fish species. However, jundiá fed diets containing broken rice revealed higher total bacterial counts than tilapia, and a smaller level of amylolytic bacteria when cassava bagasse or ground corn were included in the diet. DGGE and sequence analysis: all OTUs showed closest relative identity to uncultured bacteria isolated from different sources. Some bands were affected by dietary manipulation. An OTU displaying 95% nucleotide similarity to *Cetobacterium somerae* was revealed in most tilapia groups, except for fish fed wheat bran. Furthermore, three OTUs with low similarity (93–95%) to unidentified bacteria isolated from the HG of Atlantic salmon or intestine of beluga were present only in tilapia and their abundance was suppressed by inclusion of corn. As limited data were presented with regard to bacterial identification by dietary manipulations, the authors suggested that culture-independent quantitative techniques should be incorporated to evaluate the bacterial changes in future studies.

Effect of probiotic supplements

The term *probiotic* is a word derived from Greek and meaning *for life*. During the last 15 years, numerous review papers have been published on the application of probiotics in aquaculture and readers with special interest are referred to the reviews of Ringø & Gatesoupe (1998), Gatesoupe (1999), Ringø & Birkbeck (1999), Gomez-Gil *et al.* (2000), Verschuere *et al.* (2000), Irianto & Austin (2002), Ringø (2004), Burr *et al.* (2005), Gram & Ringø (2005), Ringø *et al.* (2005), Balcázar *et al.* (2006), Farzanfar (2006), Vine *et al.* (2006), Gatesoupe (2007, 2008), Kesarcodi-Watson *et al.* (2008), Tinh *et al.* (2008), Wang *et al.* (2008), Ninawe & Selvin (2009), Qi *et al.* (2009), Zhou *et al.* (2009b), Merrifield *et al.* (2010a), Nayak (2010b), Prado *et al.* (2010), Dimitroglou *et al.* (2011), Lara-Flores (2011), Welker & Lim (2011), Lauzon & Ringø (2012), Al-Marzouk & Saheb (2012), Zhou & Wang (2012), De *et al.* (2014), Mohapatra *et al.* (2013), Newaj-Fyzul *et al.* (2014) and Ringø *et al.* (2014c). Due to the nature of probiotics and the plethora of data currently available, a full discussion of the effects of probiotics on the gut microbiota of fish is beyond the scope of the present review and should be the topic of a separate review. Instead a general summary of key findings will be provided here.

Alterations of the fish gut microbiota have been demonstrated with the probiotic application of *Bacillus* spp. (Bagheri *et al.* 2008; Ghosh *et al.* 2008; Newaj-Fyzul *et al.* 2007), *Vibrio* spp. (Ringø 1999; Ringø *et al.* 1996), LAB (Strøm & Ringø 1993; Gildberg *et al.* 1995; Jöborn *et al.* 1997; Ringø 1999; Bogut *et al.* 2000; Robertson *et al.* 2000; Chang & Liu 2002; Nikoskelainen *et al.* 2003; Panigrahi *et al.* 2004; Aubin *et al.* 2005; Panigrahi *et al.* 2005; Carnevali *et al.* 2006; Kim & Austin 2006; Balcázar *et al.* 2007b; Suzer *et al.* 2008; Vendrell *et al.* 2008; Iehata *et al.* 2009; Lauzon *et al.* 2010a; Løvmo Martinsen *et al.* 2011; Lamari *et al.* 2013), yeasts (Aubin *et al.* 2005; Gatesoupe 2002) and mixed probionts (Gatesoupe 2002; Lauzon *et al.* 2010a,b,c,d; Ramos *et al.* 2013). Probiotic treatments of live feed with *Bacillus* spp. (Gatesoupe 1993; Avella *et al.* 2010), LAB (Gatesoupe 1991, 2002; Villamil *et al.* 2003) and yeasts (Gatesoupe 2002) have also contributed to the control of their microbiota.

The continual application of probiotic cells via dry feed containing from 10^5 to 10^9 colony-forming units g^{-1} has been demonstrated to lead to potentially resident colonization of the intestinal epithelium (Gildberg & Mikkelsen 1998; Gildberg *et al.* 1995, 1997), intestinal mucus (Kim & Austin 2006; Merrifield *et al.* 2010b, 2011b; Newaj-Fyzul *et al.* 2007), transient digesta (Aubin *et al.* 2005; Bagheri *et al.* 2008; Balcázar *et al.* 2007c; Bogut *et al.* 2000; Ferguson *et al.* 2010; Ghosh *et al.* 2008; Gildberg *et al.* 1997; Kim & Austin 2006; Newaj-Fyzul *et al.* 2007; Nikoskelainen *et al.* 2003; Son *et al.* 2009; Vendrell *et al.* 2008), intestine and stomach (Panigrahi *et al.* 2004), and pyloric caeca populations (Gildberg & Mikkelsen 1998). Further, it has been shown that some probionts are able to persist within the digestive tract for up to few weeks post-treatment or after reverting to non-supplemented diets (Balcázar *et al.* 2007b; Kim & Austin 2006; Lauzon *et al.* 2010c,d; Nikoskelainen *et al.* 2003; Panigrahi *et al.* 2005; Robertson *et al.* 2000; Son *et al.* 2009; Villamil *et al.* 2010). Kim & Austin (2006) demonstrated the usefulness of using PCR-DGGE profiles in order to monitor the persistence of *Carnobacterium maltaromaticum* and *C. divergens* within the rainbow trout intestine. After reverting to non-supplemented feeds, the probionts were still detected within the

intestine for at least 3 weeks. Similarly, Balcázar *et al*. (2007b) showed that *Lactococcus lactis* ssp. *lactis*, *Lactobacillus sakei* and *Leuconostoc mesenteroides* displayed an ability to persist within the rainbow trout intestine after reverting to non-supplemented diets. Short-term persistence was relatively high as levels remained at log 4–5 CFU g^{-1} and <log 3 CFU g^{-1} after 1 and 2 weeks, respectively.

Probiotic GI colonization is not restricted to the intestine. For example, Panigrahi *et al*. (2004) fed rainbow trout *Lactobacillus rhamnosus* for 30 days at 10^9 and 10^{11} CFU g^{-1}. *Lb*. *rhamnosus* was recovered from the GI at levels of 10^6–10^8 CFU g^{-1} and 10^5–10^9 CFU g^{-1} intestine and stomach, respectively. After 30 days, *Lb*. *rhamnosus* in fish fed the low level supplementation reached >40% and >70% of the culturable microbiota of the stomach and intestine, respectively. Furthermore, *Lb*. *rhamnosus* levels in fish fed the high level supplementation reached >60% and >80% in the stomach and intestine, respectively. Gildberg & Mikkelsen (1998) demonstrated that *C*. *divergens* (originally isolated from Atlantic salmon and Atlantic cod) displayed better colonization capabilities with regard to Atlantic cod fry pyloric caeca than the intestine.

Several investigations have clearly demonstrated that probiotic GI colonization can alter the indigenous GI microbiota composition as well as total population levels in hosts (Strøm & Ringø 1993; Bogut *et al*. 2000; Aubin *et al*. 2005; Bagheri *et al*. 2008; Ghosh *et al*. 2008; Iehata *et al*. 2009; Avella *et al*. 2010; Lauzon *et al*. 2010a,d). For example, after feeding rainbow trout *Pediococcus acidilactici* or *Saccharomyces cerevisiae* var. *boulardii* for periods of up to 5 months, Aubin *et al*. (2005) enumerated the gut probiotic levels and identified the dominating culturable microbiota by 16S rRNA gene sequence analysis. Changes in the relative and absolute abundance of the indigenous bacteria were observed. After a period of 20-day supplemented feeding, the presence of unique genera such as *Buttiauxella* and *Citrobacter* was confirmed in the control diet-fed fish, but *Serratia* was detected only in the *P*. *acidilactici*-fed fish.

The current literature suggests that the sensitivity of the gut microbiota to probiotic modulation does not appear to be restricted by fish maturation as several investigations have demonstrated the effects of the probiotics on the gut microbiota of fish across a range life stages including larval (Carnevali *et al*. 2006; Lauzon *et al*. 2010b,c,d; Ringø 1999; Ringø *et al*. 1996; Strøm & Ringø 1993; Suzer *et al*. 2008), fry (Aubin *et al*. 2005; Bagheri *et al*. 2008; Bogut *et al*. 2000; Ferguson *et al*. 2010; Ghosh *et al*. 2008; Gildberg & Mikkelsen 1998; Gildberg *et al*. 1995, 1997; Newaj-Fyzul *et al*. 2007; Robertson *et al*. 2000), fingerling (Jöborn *et al*. 1997; Robertson *et al*. 2000) and juvenile stages (Balcázar *et al*. 2007c; Chang & Liu 2002; Iehata *et al*. 2009; Kim & Austin 2006; Lauzon *et al*. 2010a; Nikoskelainen *et al*. 2003; Panigrahi *et al*. 2004, 2005; Vendrell *et al*. 2008). Further, literature regarding the effects of probiotics on the gut microbiota is not only limited to economically important finfish species; studies have also demonstrated probiotic induced modulation of the gut microbiota of ornamental finfish (Ghosh *et al*. 2008) and crustaceans (Gullian *et al*. 2004; Rengpipat *et al*. 2000).

Despite the usefulness of the data accumulated from the present studies, relatively few have utilized culture-independent methods to monitor microbial population changes resulting from probiotic treatments (Ferguson *et al*. 2010). Culture-dependent techniques are often sufficient to track probiotic colonization levels, but probiotic studies should include molecular techniques such as qPCR and NGS. Also, further studies are required to investigate the effects of feeding probiotics to fish throughout various life stages. Long-term trials monitoring the gut microbiota throughout maturation and development of the GI tract would provide a novel insight into the effect of probionts on the gut microbiota. With the introduction of next-generation sequencing-based molecular technologies, the application of omics techniques will contribute to a better understanding of the microbial diversity and functionality in the host (Star *et al*. 2013).

Readers with further interest on the topic, probiotic modulation of the gut microbiota of fish, are referred to the recent review of Merrifield & Carnevali (2014).

Live cells in probiotic products will inevitably lose viability, and the actual products will contain varying proportions of populations of viable-to-non-viable/dead cells. Moreover, there may be further losses of viability of organisms through stomach to the intestine. The phenomenon is known from endothermic animals, and an active debate is ongoing whether or not non-viable forms of beneficial bacterial strains have a role in the conferment of benefits on the host (Adams 2010). This topic requires further evaluation in aquatic animals and merits investigations.

Effect of prebiotics

The modern concept of prebiotics implies the use of selective compounds to favour growth of the protective indigenous gut microbiota. It is well known from endothermic investigations that dietary fibres are fermented by the anaerobic intestinal microbiota, primarily those colonizing

the colon (Gibson 1998; Roberfroid 1993), leading to the production of lactic acid, acetate, propionate, and butyrate and gases (H_2, CO_2 and CH_4) (Roberfroid 1993). The short-chain fatty acids are utilized by the host and appear to promote the health of the intestinal mucosa (Velàzquez *et al*. 1997).

As the first prebiotic study related to aquaculture was carried out by Hanley *et al*. (1995), numerous studies have been carried out on growth performance, carcass composition, immune parameters, intestinal histology and disease resistance against well-known pathogens as well as effect on gut microbiota. Readers with special interest in prebiotics in finfish and shellfish are referred to the review of Burr *et al*. (2005), Gatlin *et al*. (2006), Denev *et al*. (2009), Yousefian & Amiri (2009), Ganguly *et al*. (2010), Merrifield *et al*. (2010a), Ringø *et al*. (2010b), Sweetman *et al*. (2010), Dimitroglou *et al*. (2011), Ganguly *et al*. (2013), Daniels & Hoseinifar (2014), Ringø *et al*. (2014a,c), Torrecillas *et al*. (2014) and Song *et al*. (2014).

Inulin

Although inulin, a polydisperse carbohydrate consisting mainly of β(2→1) fructosyl-fructose links, is not a natural fibre in fish diet, it may have interesting applications in aquaculture. To our knowledge, the first study on the effect of inulin on gut microbiota was carried out by Mahious *et al*. (2006). They investigated the effect of 2% dietary inulin (Raftiline ST) as prebiotic for turbot (*Psetta maxima* L.). The addition of inulin to the diet increased the population level of *Vibrio* sp. SI2411 and *Vibrio* sp. L2C55, while the proportion of other bacteria, not identified, decreased.

During the last decade, some information has become available about fermentation of inulin by fish gut microbiota, notably, *Carnobacterium piscicola* (Ringø *et al*. 1998), *C. mobile* (Ringø & Olsen 1999) and *Carnobacterium* spp. (Ringø & Olsen 1999; Ringø *et al*. 2001a,b). More recently, Ringø *et al*. (2006a) showed that substituting 15% dextrin with 15% inulin resulted in a decrease in total viable counts and alterations of the adherent hindgut microbiota of Arctic charr. This is illustrated by the fact that *C. divergens* was only isolated from the hindgut of fish fed the dextrin diet, while *Carnobacterium maltaromaticum* was only isolated from fish fed the inulin supplemented diet. Furthermore, *Alcaligenes* spp., *Enterobacter* spp., *Microbacterium* spp. and *Micrococcus agilis* were not isolated from the hindgut of fish fed dietary inulin. Moreover, the authors demonstrated that the frequency of gut bacteria, fermentative in O/F medium, able to produce acid from inulin was 43% in bacterial species isolated from fish fed inulin, but only 17% of the bacteria isolated from fish fed dextrin had this ability.

In a later study, Akrami *et al*. (2009) investigated the effects of inulin supplementation (0, 10, 20 and 30 g kg^{-1}) on beluga (*Huso huso*) juveniles (16.14 ± 0.38 g). Their results revealed that LAB levels in the gut were lowest in the control group and in groups fed 20 and 30 g inulin kg^{-1}. Hence, the authors concluded that inulin is not an appropriate dietary supplement for beluga.

On the other hand, Burr *et al*. (2009) evaluate the effect of 1% inulin supplementation in feeds for red drum (*Sciaenops ocellatus*) containing either FM as protein source or FM with 35.5% SBM inclusion. The results of PCR-DGGE analysis revealed that there was a great similarity index and small species richness with some dominant species among the intestinal samples. Moreover, samples from the individual tank biofilters revealed that there was great similarity and high species richness without dominant species to distinct among all the samples. Hence, the authors conclude that there were no differences in the intestinal microbiota between the feeding regimes.

Burr *et al*. (2010) conducted an *in vitro* and an *in vivo* experiment in order to investigate the effect of inulin on hybrid striped bass (*Morone chrysops* × *Morone saxatilis*). In the *in vitro* experiment where fish fed the experimental diet supplemented by 10 g inulin kg^{-1}, the results showed that propionate production was low compared with acetate and butyrate production for all samples. Additionally, there were not significant differences in propionate, acetate and total VFA among the samples after 48 h of incubation. Microbial cluster analysis showed that all samples had a high similarity coefficient (>80%) at 24 and 48 h. The dominant bands were identified as *Clostridium perfringens* and Fusobacteria using the BLAST database. In the *in vivo* experiment, fish fed experimental diets supplemented 10 g of inulin kg^{-1} for 8 weeks. PCR-DGGE microbial analysis revealed that microbial patterns of fish fed inulin had very high similarity index. The main genera identified were as follows: Clostridia, *Bacillus*, *Enterococcus*, *Lysinibacillus* and *Staphylococcus*.

Recently, Mouriño *et al*. (2012) evaluated the effect of dietary inulin (0 and 5 g kg^{-1}) on autochthonous gut microbiota of hybrid surubins (*Pseudoplatystoma* sp.). Inclusion of inulin did not affect total gut bacteria, counts of *Vibrio* and decreased the population level of *Pseudomonas* but increased gut LAB. The authors stated that similar finding was noticed by Burr *et al*. (2008b) when red drums were fed prebiotics. However, in the study of

Mouriño *et al*. (2012), no specifications were given regarding the LAB genera in the gut and only Man-Rogosa–Sharpe agar was used estimate gut LAB.

Transmission electron microscopy (TEM) evaluation of the hindgut of fish fed dextrin or inulin revealed clear differences (Ringø *et al*. 2006a). TEM investigations clearly demonstrated bacterial cells between microvilli in the large intestine of fish fed dietary dextrin. However, by feeding Arctic charr inulin, only few bacteria were observed between the microvilli, but several bacterial-like profiles were observed in the gut lumen. SEM investigations of DI of Arctic charr fed dietary dextrin revealed substantial associations of coccoid and rod-shaped bacterial-like cells at the apical brush border and at the cell surface (Fig. 7). This colonization pattern was completely different as the surface of intestinal cell had no associated bacteria when the fish were fed diet supplemented with inulin (Fig. 8). These differences in bacterial colonization of enterocyte surface between fish fed dextrin or inulin may be related to fact that the gut microbiota isolated from the hind gut was different between the two dietary groups (Ringø *et al*. 2006a). However, this controversial hypothesis calls for further investigation. When evaluating the effect of inulin a plausible question arise, does dietary inulin affect intestinal enterocytes? In a previous study, it was shown by electron microscopy that dietary inulin (15% supplement) had a destructive effect on microvillus organization on both pyloric caeca and hindgut enterocytes of the salmonid fish

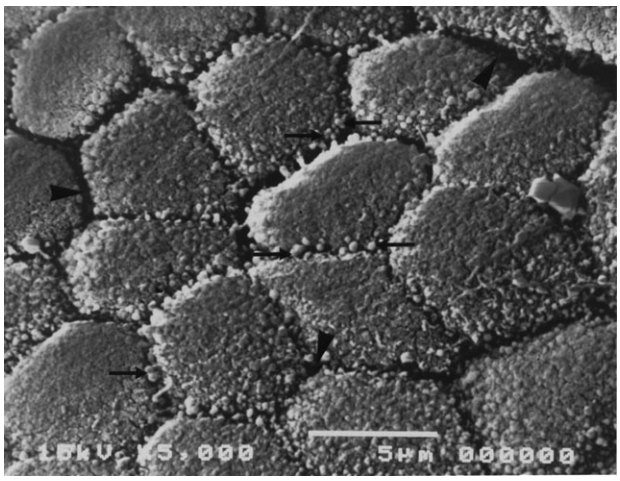

Figure 7 SEM micrograph showing cell apices in the distal intestine of Arctic charr fed dietary dextrin. Associated bacteria (arrows) are located at the apical brush border and the cell surface. Note the spaces (arrowheads) between the cells. After Ringø *et al*. (2006c).

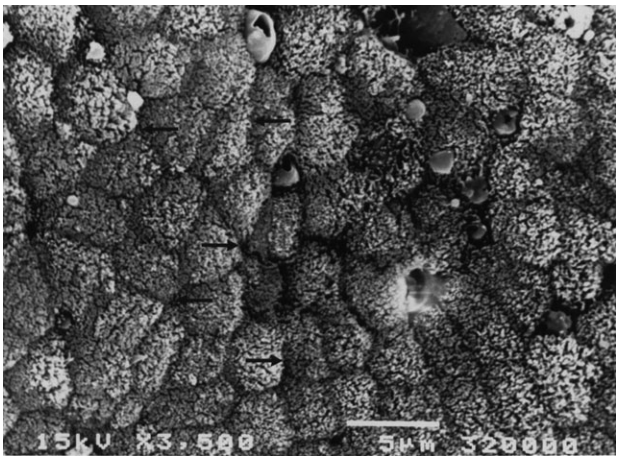

Figure 8 SEM micrograph showing cell apices in the distal intestine of Arctic charr fed dietary inulin. Cell borders (arrows) can be seen but the cells have no associated bacteria discernible at the apical brush border. After Ringø *et al*. (2006c).

Arctic charr compared with enterocytes from fish fed a 15% supplement of dextrin (Olsen *et al*. 2001).

Fructo-oligosaccharides (FOS)

One of the most common prebiotics studied in humans and terrestrial animals is FOS, a general term that includes all non-digestible oligosaccharides composed of fructose and glucose units (Swanson *et al*. 2002a). FOS refers to short and medium chains of β-D-fructans in which fructosyl units are bound by β-(2-1) glycosidic linkages and attached to a terminal glucose unit, a structure similar to inulin. Because of a lack of β-fructosidases, mammalian digestive systems cannot hydrolyse the β-(2-1) glycosidic linkages (Teitelbaum & Walker 2002). However, FOS can be fermented by certain colonic bacteria expressing this enzyme, such as lactobacilli and bifidobacteria species (Hidaka *et al*. 1986; Manning & Gibson 2004; Sghir *et al*. 1998), and will thus selectively support the growth and survival of such bacteria in the GI tract of animals. Despite occasional inconsistent results in terrestrial species, some studies have shown that FOS improved protein digestion and intestinal morphology (Swanson *et al*. 2002b; Teitelbaum & Walker 2002). These modifications might contribute to improved growth, feed efficiency and disease resistance.

In their study on turbot larvae, Mahious *et al*. (2006) investigated the effect of 2% inclusion of Raftilose P95 on growth, gut microbiota and the ability of gut bacteria to utilize Raftilose and Raftiline. Raftilose had a positive effect on growth and some effect was also reported on the

gut bacterial community as *Bacillus* spp. was only isolated when the larvae were fed Raftilose but not in rearing groups fed cellulose powder, Raftiline or lactosucrose. Of the selected gut bacteria isolated from the larvae weaned with Raftilose, *Bacillus* spp. were the only bacteria able to use Raftilose as a single carbon source, but they could not utilize Raftiline.

Burr *et al.* (2008b) evaluated the effects of FOS on the intestinal microbial community of red drum (*Sciaenops ocellatus)*. Intestinal samples were diluted and incubated *in vitro* in one of the liquid media of control diet (commercial diet) and control diet + 2% FOS. After 24 and 48 h of incubation at 25 °C, supernatants were removed for volatile fatty acid (VFA) analysis and DNA-extracted for PCR-DGGE analysis. Acetate, butyrate, propionate and total VFA production remained unaffected in all culture media. DGGE fingerprint analysis demonstrated no differences on the microbial community.

However, oligofructose which is obtained by partial enzymatic hydrolysis of inulin showed different results on beluga juveniles (Hoseinifar *et al.* 2011a). In two 8-week studies with beluga juveniles, the effect of dietary oligofructose (0, 10, 20 and 30 g kg^{-1}) on intestinal microbiota among other parameters such as growth performance, haematological and serum biochemical parameters was studied. LAB levels in the gut were significantly higher in fish fed 20 g oligofructose kg^{-1} and were able to persist for at least 1 week after reverting the prebiotic group back to a control diet.

Short-chain fructo-oligosaccharides

Studies have investigated the effect of short-chain fructo-oligosaccharides (scFOS) on the intestinal microbiota of hybrid tilapia (*Oreochromis niloticus*♀ × *O. aureus*♂) (Lv *et al.* 2007) and white shrimp (*Litopenaeus vannamei*) (Zhou *et al.* 2007). Lv *et al.* (2007) conducted an 8-week feeding trial in order to investigate the effect of scFOS (Profeed®, 95%) on the intestinal microbiota, mortality and growth performance of hybrid tilapia. ScFOS was included at 0, 0.8, or 1.2 g kg^{-1} in practical diet and hand-fed to satiation four times daily to triplicate groups of hybrid tilapia (40 fish/group, initial body weight 5.55 ± 0.02 g) reared in a flow-through system. At the end of the feeding trial, the animals were bulk-weighed and various morphological parameters were assessed. Intestinal samples of three individuals per group were pooled in order to conduct the quantitative analysis of *Vibrio parahaemolyticus, A. hydrophila, Lactobacillus* spp. and *Streptococcus faecalis* by selected agar media. All the intestinal bacteria groups investigated showed a marginal and non-significant increase in hybrid tilapia fed scFOS ($P > 0.10$). The study indicated that dietary scFOS may have beneficial effects on growth, feed conversion and possibly intestinal microbiota of tilapia.

Zhou *et al.* (2007) conducted an 8-week feeding trial to investigate the effects of scFOS (Profeed®, 95%) on the intestinal microbiota, mortality and growth performance in juvenile white shrimp. Five levels of scFOS (0, 0.4, 0.8, 1.2, and 1.6 g kg^{-1}) were incorporated into a diet fed to triplicate groups of white shrimp (30 shrimp/group, IBW 0.172 ± 0.001 g) reared in a recirculation system (110 L aquaria, water temperature 28 °C, salinity 13.5 ± 0.2‰). The shrimp were hand-fed at restricted ration, five times per day. At the end of the feeding trial, the animals were bulk-weighed, and intestinal samples of three animals by experimental unit were pooled in order to analyse *V. parahaemolyticus, A. hydrophila, Lactobacillus* spp., and *S. faecalis* by selected media. Differences in population of *S. faecalis* and *V. parahaemolyticus* were observed between dietary treatments; all groups responded in a quadratic fashion with the highest levels of all bacteria isolated from shrimp fed 0.8 g kg^{-1}. This study indicates that dietary scFOS may have beneficial effects on growth, feed conversion and intestinal microbiota in white shrimp.

Yeast

Burr *et al.* (2008b) evaluated the effects of brewer's yeast and GroBiotic®-A (International Ingredient Corporation, St. Louis, MO, USA) on the intestinal microbial community of red drum (*Sciaenops ocellatus)*. Intestinal samples were diluted and incubated *in vitro* in liquid media of control diet (commercial diet), control diet + 2% (w/w) GroBiotic®-A and control diet + 2% brewer's yeast. After 24 and 48 h of incubation at 25 °C, supernatants were removed for VFA analysis and DNA-extracted for PCR-DGGE analysis. The intestinal content incubated with GroBiotic®-A had significantly higher acetate, butyrate and total VFA concentrations after 24 h of incubation compared with the other treatments. Propionate production remained unaffected in all culture media. DGGE fingerprint analysis demonstrated that GroBiotic®-A and brewer's yeast were able to modulate the microbial community. These findings combined with the findings from Burr *et al.* (2008a) suggest that prebiotics and especially GroBiotic®-A play an important role in red drum nutrition. In a later study, Burr *et al.* (2009) evaluate the effect of 1% GroBiotic®-A supplemen-

tation in feeds for red drum (*Sciaenops ocellatus)* containing either FM as protein source or FM with 35.5% SBM inclusion. The results revealed that there was great similarity index and small species richness with some dominant species among the intestinal samples. Hence, the authors conclude that there were no differences in the intestinal microbiota between the feeding regimes.

Burr *et al*. (2010) conducted two experiments, *in vitro* and an *in vivo* to investigate the effect of GroBiotic®-A on hybrid striped bass (*Morone chrysops* × *Morone saxatilis*). The experimental diet was based on the commercial diet added 0.5% GroBiotic®-A. The intestinal contents were diluted and incubated with the experimental feeds at 25 °C. After 24 and 48 h of incubation at 25 °C, supernatants were removed for VFA analysis and DNA was extracted for PCR-DGGE analysis. The results showed that propionate production was low compared with acetate and butyrate production for all samples. Additionally, there were not significant differences in propionate, acetate and total VFA among the samples after 48 h of incubation. Compared with the control, butyrate production was significantly increased after 48 h but only for samples containing 0.5% GroBiotic®-A. Microbial cluster analysis showed that all samples had a high similarity coefficient (>80%) at 24 and 48 h. The dominant bands were identified as *Clostridium perfringens* and Fusobacteria using the BLAST database. In the *in vivo* experiment, fish fed experimental diets supplemented 10 g GroBiotic®-A kg^{-1} for 8 weeks. PCR-DGGE microbial analysis revealed that microbial patterns of fish fed GroBiotic®-A had very high similarity index. The main genera identified were Clostridia, *Bacillus*, *Enterococcus*, *Lysinibacillus* and *Staphylococcus*. This study showed that GroBiotic®-A can modulate the intestinal microbiota.

A study on juvenile rainbow trout by Azari *et al*. (2011) displayed that inclusion of GroBiotic®-A significantly improved SGR, CF and protein efficient ratio but not FCR. Furthermore, allochthonous intestinal bacterial community and LAB were affected by the GroBiotic®-A addition.

Lochmann *et al*. (2011) investigated the effect of GroBiotic®-A (consisting of a mixture of partially autolysed brewer's yeast, dairy ingredient components and dried fermentation products) (0 and 20 g kg^{-1}) on gut microbiota of fathead minnow (*Pimephales promelas*) in recirculating systems with low or moderate water hardness. Only *Acinetobacter* spp. were enhanced by inclusion of prebiotic in the diet.

Savolainen & Gatlin (2009) evaluated supplementation of dairy-yeast prebiotics at 0 and 20 g kg^{-1} by dry weight in the diet of juvenile goldfish in the presence or absence of phytoplankton and zooplankton. The results showed that dairy yeast tends to improve weight gain and feed efficiency in the presence of phytoplankton and zooplankton. Additionally, dairy-yeast supplementation did not modulate the proximal or distal allochthonous intestinal microbiota and did not improve disease resistance against intraperitoneally administrated *A. hydrophila*.

Hoseinifar *et al*. (2011b) evaluated the effects of dietary inactive yeast (*Saccharomyces cerevisiae* var. *ellipsoideus*) (0, 10 and 20 g kg^{-1}) as prebiotic on intestinal microbiota of beluga juveniles (11.44 ± 0.56 g) for 6 weeks of feeding. Although no substantial effect was revealed on the total autochthonous intestinal heterotrophic bacterial counts, autochthonous LAB levels were significantly elevated in fish fed 20 g yeast kg^{-1}.

Yeast culture and short-chain fructo-oligosaccharides The effects of dietary yeast culture (YC) or scFOS on intestinal autochthonous bacterial communities in juvenile hybrid tilapia were studied by 16S rDNA DGGE (Zhou *et al*. 2009c). Ninety tilapias in tanks (10 fish per tank) were randomly and equally divided into three groups. At the end of an 8-week feeding period of CK (the control treatment), YC (3 g kg^{-1}) or scFOS (1 g kg^{-1}), autochthonous gut bacteria were analysed in intestinal samples of all fish in each tank of a recirculating aquaculture system. The clear differences in the banding patterns indicated the obvious effects of dietary prebiotics on intestinal communities in hybrid tilapia. Higher variation was detected within the dietary YC group. This difference might be due to the effects of certain immune-stimulating agents in YC on the immunity response of hybrid tilapia. It was concluded that dietary prebiotics, YC and scFOS, obviously affected the intestinal bacterial community in hybrid tilapia with different patterns for different kinds. An uncultured bacterium-like, *Thiothrix eikelboomii*-like and an uncultured freshwater bacterium-like were selectively stimulated by dietary scFOS, and *Clostridium* sp.-like and uncultured *Cyanobacterium*-like were selectively stimulated by dietary YC or scFOS.

Yeast and inulin Tapia-Paniagua *et al*. (2011) evaluated the modulation of the intestinal allochthonous microbiota of gilthead sea bream (~80 g) by administration of yeast (*Debaryomyces hansenii*) in combination with inulin. Experimental fish were fed either a commercial diet (control diet), or similar diet supplemented with 1.1% *D. hansenii* strain L2 (10^6 CFU g^{-1}) plus 3% inulin (experimental diet

II) for 4 weeks. After 2 and 4 weeks of feeding, samples of the whole intestine were aseptically removed for allochthonous microbiota analysis using PCR-DGGE and sequence BLAST analysis. The results showed that fish fed the experimental diet had lower species richness and greater similarity index compared with fish fed the control diet for 4 weeks. Genus *Pseudomonas* was dominating the intestinal microbiota in both experimental groups.

Mannan oligosaccharides

According to Spring (2003), the initial interest in using mannan oligosaccharide (MOS) as a feed additive for animal nutrition was adapted from studies at the end of 1980s by Oyofo *et al*. (1989a,b). These studies evaluated the ability of mannose to inhibit the adherence/colonization of microbial pathogens such as *Salmonella typhimurium* to GI epithelial cells of broilers. Many studies in terrestrial animals have shown that MOS is able to reduce the bacterial load and pathogenic bacteria (Castillo *et al*. 2008; Fernandez *et al*. 2002; Grieshop *et al*. 2004; Yang *et al*. 2008) of the intestine. An essential step in the bacterial infection process is the attachment of pathogens to epithelial cells (Swanson *et al*. 2002c). Carbohydrate-binding proteins such as lectins are found on the exterior of cells and are associated with the antigen recognition and fimbrial adhesins (binding) of bacteria, especially of Gram-negatives (Engering *et al*. 1997). Lectins bind to the epithelial cells of the gut by attaching to oligosaccharide components of glycoconjugate receptors. Type-1 fimbrial adhesins, which are common on numerous potentially pathogenic bacterial species, are specific for mannan residues (Newman 2006; Oyofo *et al*. 1989a; Spring *et al*. 2000). It is suggested that MOS is able to interact with these receptors by acting as receptor analogs for Type-1 fimbriae and thus preventing bacterial colonization of the GI tract (Newman 2006; Oyofo *et al*. 1989b; Spring 2003). Furthermore, later studies with MOS supplementation in terrestrial animals have shown MOS to work in several different ways within the digestive tract. MOS can improve gut function and health, by increasing the villi height, uniformity and integrity (Castillo *et al*. 2008; Hooge 2004; Iji *et al*. 2001). As a result, the feed within the digestive tract is more efficiently digested leading to an increased rate of nutrient absorption (Sims *et al*. 2004; Spais *et al*. 2003). The documented benefits of MOS in terrestrial animals have led to initiatives to evaluate the potential of MOS with regard to finfish aquaculture as the majority of common fish pathogens, such as *Vibrio* spp., *Aeromonas* spp. and *Yersinia ruckerii*, are Gram-negative.

Zhou & Li (2004) evaluated the effect of MOS supplementation in the intestinal microbiota of Jian carp (*Cyprinus carpio* var. Jian) fed diets supplemented with 0.24% MOS. The results showed that the presence of *E. coli* was significantly reduced and *Bifidobacterium* spp. and *Lactobacillus* spp. were increased in MOS-fed fish. It has been suggested that Bifidobacteria and LAB are both beneficial microbial species in the GI tract because they produce bacteriocins, acetate and lactate which decrease the luminal pH and create an unfavourable environment for several pathogens (Gibson *et al*. 2003).

Additionally, Torrecillas *et al*. (2007) showed that MOS supplementation is able to confer improved disease resistance of sea bass (*Dicentranchus labrax*) against enteric *V. alginolyticus* infection when challenged by both cohabitation with infected fish and direct bacterial inoculation of the gut. In the trials with direct gut inoculation with *V. alginolyticus*, the number of the infected fish in the control group was doubled within 48 h compared with the MOS group. Furthermore, the number of the infected fish of the MOS group remained unchanged since the start of the trial. The authors suggested that there was a clear need for further investigation in order to fully understand how MOS interacts with the intestinal microbiota of aquatic animals, which they did a few years later (Torrecillas *et al*. 2011). In this later study, where European sea bass were fed either a control diet (MOS not supplemented) or 4 g MOS kg^{-1} for 8 weeks showed a significant reduction in intestinal bacteria translocation *in vivo* and *ex vivo* exposures. Additionally, intestinal morphology was improved in both anterior and posterior regions; acid mucins production goblet cells and eosinophilic granulocytes densities were higher, and intestinal mucus lysozyme activity was increased in MOS-fed fish. It is suggested that the dietary supplementation of MOS is strongly related with several mechanisms of intestinal protection against the bacterial translocation as well as the improved intestinal morphology.

Another study with juvenile and subadult rainbow trout under commercial rearing conditions (Dimitroglou *et al*. 2009) also demonstrates that dietary MOS has a clear effect on the gut microbiota of rainbow trout. Dietary MOS (0.2% of feed dry weight) significantly reduced the aerobically cultivated bacterial load within the GI tract. Additionally, changes in the relative abundance of the microbiota identified were observed. In the juvenile group of fish, MOS-fed fish displayed a significant reduction in the relative abundance of *Micrococcus* spp., *Aeromonas*/*Vibrio* spp. and a group of unidentified Gram-positive isolates. Coinciding with these changes, a significant increase of

Enterococcus spp. was found. In the subadult group of fish, MOS-fed fish also displayed a significant reduction in *Micrococcus* spp. and Enterobacteria compared with the control group. An increase of *Pseudomonas* spp. was also found in the subadult MOS-fed group. Culture-independent analysis using PCR-DGGE showed that dietary MOS supplementation resulted in reduced bacterial species richness in both juvenile and subadult groups. Non-metric multidimensional scaling (nMDS) analysis also demonstrated a clear effect on the microbiota; MOS caused a distinct spatial shift of bacterial communities away from the control groups.

An *in vitro* experiment and an *in vivo* experiment have been conducted by Burr *et al*. (2010) in order to investigate the effect of MOS on hybrid striped bass. In the *in vitro* experiment, the intestinal contents were diluted and incubated with the experimental feeds at 25 °C. The experimental diet was based on the commercial diet added 1% MOS. After 24 and 48 h of incubation at 25 °C, supernatants were removed for VFA analysis and DNA was extracted for PCR-DGGE analysis. The results showed that propionate production was low compared with acetate and butyrate production for all samples. Additionally, there were not significant differences in propionate, acetate and total VFA among the samples after 48 h of incubation. Compared with the control, produced butyrate significantly increased after 48 h. Microbial cluster analysis showed that all samples had a high similarity coefficient (>80%) at 24 and 48 h. The dominant bands were identified as *Clostridium perfringens* and Fusobacteria using the BLAST database. In the *in vivo* experiment, fish fed experimental diets supplemented 10 g of MOS kg^{-1} for 8 weeks. PCR-DGGE microbial analysis revealed that microbial patterns of fish fed MOS had a very high similarity index. The main genera identified were *Clostridia*, *Bacillus*, *Enterococcus*, *Lysinibacillus* and *Staphylococcus*. This study showed that MOS did not affect the growth performance of the cultivated fish but modulates the intestinal microbiota.

Mansour *et al*. (2012) reported results from a study evaluating dietary inclusion of MOS at 0, 2, 4 g kg^{-1} on growth performance, haematological parameters and intestinal LAB levels of beluga juvenile (46.89 ± 2.57 g). The results of this study showed that at the inclusions level, MOS did not influence growth performance, haematological parameters or LAB levels in the gut. On the other hand, He *et al*. (2011) displayed that *Saccharoculture*, with a high content of MOS, improved SGR, FCR and the autochthonous gut microbiota of gibel carp (*Carassius auratus gibelio*), but non-specific immunity factors [serum lysozyme activity and complement activity (C3 and C4)] were not significantly affected, even though *Saccharoculture* slightly increased to a certain extent the content of C3.

On the other hand, the use of MOS supplementation (0, 2 and 4 g kg^{-1}) in gilthead sea bream nutrition using smaller size of fish (~105 g) showed alterations of circulating leucocytes proportions as well as increased total leucocyte number within 2 weeks of feeding (Dimitroglou *et al*. 2011). Despite that, blood serum lysozyme and alternative complement haemolytic activity remained unaffected. Microbial analysis revealed that MOS supplementation reduced the aerobic culturable intestinal microbial load without altering the relative abundance of the identified bacterial species in such short period of time.

A longer study, by Dimitroglou *et al*. (2010), using dietary MOS supplementation (0, 2 and 4 g kg^{-1}), fish weight ~24 g and experimental duration up to 9 weeks, using diets either FM as protein source, or partially replacement of FM by SBM, showed that body proximate composition and growth parameters, such as mean final weight, SGR, FCR, PER, remained unaffected. Histological evaluation of the anterior and the posterior intestinal tract of sea bream revealed that posterior intestine underlies greater changes. More specific, posterior villi absorptive surface was significantly improved by adding MOS to the FM diets. Electron microscopy revealed that dietary MOS had a pronounced effect at the ultrastructural level, as microvilli density was increased in both FM-based and SBM-included diets. Similarly, microvilli length was increased in both examined areas of the FM-based diets but only in the posterior region for fish fed diets with SMB included. Microbial analysis using PCR-DGGE demonstrated that the effect of dietary MOS on the allochthonous microbial populations was more distinctive in FM-based diets, that is increased species diversity, richness and reduced similarity between the different FM groups, compared with SBM-fed fish, where species diversity and richness remained unaffected and group similarity was higher. It is believed that the large numbers of oligosaccharides that SBM includes play an important role in intestinal microbial community and it is possible to subdue the effect of MOS supplementation (Gibson *et al*. 2004). Nevertheless, like previous studies using other prebiotics (i.e. inulin), a close relation between intestinal histology and intestinal microbiology should be considered evident.

Daniels *et al*. (2010) evaluated the effect of MOS supplementation, enriched *Artemia*, on gut microbiota of European lobster (*Homarus gammarus*) by DGGE fingerprints and culturable bacterial levels. The DGGE profile of the MOS group was relatively dissimilar to the control group

(34.6% dissimilar). Most of the selected DGGE bands were most closely related to Vibrionaceae. On the other hand, culture-based analysis from selective media displayed that dietary MOS did not appear to affect levels of *Vibrio*, but total viable counts were significantly lower at stages I and III when fed lobster were fed MOS enriched with *Artemia*. Furthermore, histological examination revealed that MOS significantly increased in microvilli length and density.

Gluco-oligosaccharides

Two experiments, *an in vitro* and an *in vivo*, have been conducted by Burr *et al*. (2010) in order to investigate the effect of gluco-oligosaccharides (GLOS) on hybrid striped bass (*Morone chrysops* × *Morone saxatilis*). In the *in vitro* experiment, intestinal contents were diluted and incubated with the experimental feeds at 25 °C. The experimental feed was based on the commercial feed with the addition of 0.5% GOS. After 24 and 48 h of incubation at 25 °C, supernatants were removed for VFA analysis and DNA was extracted for PCR-DGGE analysis. The results showed that propionate production was low compared with acetate and butyrate production for all samples. Additionally, there were not significant differences in propionate, acetate and total VFA among the samples after 48 h of incubation. Compared with the control, produced butyrate significantly increased after 48 h but only for samples containing 0.5% GLOS. Microbial cluster analysis showed that all samples had a high similarity coefficient (>80%) at 24 and 48 h. The dominant bands were identified as *Clostridium perfringens* and Fusobacteria using the BLAST database. In the *in vivo* experiment, fish-fed experimental diets supplemented 10 g GOS kg^{-1} for 8 weeks. PCR-DGGE microbial analysis revealed that microbial similarity coefficient of fish fed GLOS was <80% compared with the microbial community of the control diet-fed fish. The main genera identified were *Clostridia*, *Bacillus*, *Enterococcus*, *Lysinibacillus* and *Staphylococcus*. This study showed that GLOS did not affect the growth performance of the cultivated fish but modulates the intestinal microbiota.

Arabinoxylan-oligosaccharides

Information on the prebiotic potential of arabinoxylan-oligosaccharides (AXOS) has been studied in endothermic animals (Geraylou *et al*. 2012), but less information is available about its effect on the gut microbiota of fish, Siberian sturgeon (*Acipenser baerii*) (Geraylou *et al*. 2012, 2013a,b). The general finding from these studies is that AXOS modulate the gut microbiota including LAB.

DVAQUA®

DVAQUA® is a feed ingredient produced by fermenting selected liquid and cereal grain raw ingredients with baker's yeast (*Saccharomyces cerevisiae*) and drying the entire culture media without destroying the yeast factors, B vitamins and other fermentation products (retrieved 16.08.2012: http://www.diamondv.com/languages/en/tech-aqua/\). It therefore consists of yeast cell walls (β-glucans and mannan oligosaccharides), that is immunostimulating compounds (Ringø *et al*. 2012b).

In a study with hybrid tilapia, the fish were fed six iso-nitrogenous and iso-caloric experimental diets in cages (He *et al*. 2009). The diets contained six levels of DVAQUA: 0, 0.125, 0.25, 0.5, 1.0 and 2.0 g kg^{-1} diet. The purpose of this study was to evaluate the effects of dietary yeast culture on growth performance, intestinal autochthonous bacterial community using 16S rDNA and DGGE, as well as non-specific immunity of hybrid tilapia. Supplementation of dietary yeast culture showed no effects on growth performance, diet conversion and survival rate of the hybrid tilapia. However, the autochthonous gut bacteria community was affected. Various potentially beneficial bacteria were stimulated by dietary yeast culture at different feeding periods, while potential harmful species such as *E. coli* serotype O20: H42-like, uncultured bacilli bacterium clone MS030A1_F02-like and *P. fluorescens* strain YC0357-like were depressed. Non-specific immunity of hybrid tilapia, such as serum lysozyme activity, serum C3 and C4 activities, head kidney macrophage phagocytic activity and head kidney macrophage respiratory burst activity, was improved by dietary yeast culture.

He *et al*. (2010) evaluated the effect of florfenicol and DAVQUA, inclusion level 5 g per kg, on growth performance, non-specific immunity and gut bacterial community in hybrid tilapia in a 16-week feeding trial. In fish fed DAVQUA, improved non-specific immunity and increased intestinal bacterial count and bacterial diversity were observed. Dietary DAVQUA increased the abundance of *Pseudomonas migulae*, *Brevundimnas vesicularis*, uncultured α-*Proteobacterium* and *Clostridium* sp. but decreased the abundance of *Leifsonia* sp., *Pseudomonas plecoglossicida* and *Pseudomonas migulae* compared with fish fed the control diet. The band intensity of *Lactococcus lactis* subsp. *lactis* was unaffected by dietary treatment.

Readers with special interest on probiotics and prebiotics are referred to the recent Wiley-Blackwell book entitled '*Aquaculture Nutrition: Gut Health, Probiotics and Prebiotics*' edited by Merrifield & Ringø. Synbiotic refers to nutritional supplements combining a mixture of probiotics and prebiotics in a form of synergism (Gibson & Roberfroid 1995). To avoid duplication, readers with special interest on the effect of synbiotics on gut microbiota are referred to the recent review of Ringø & Song (2016).

Effect of acidifiers, acidic calcium sulphate, sodium butyrate, poly-β-hydroxybutyrate and potassium diformate

Acidifiers

Dietary acidifiers have been reported as beneficial in aquaculture where they confer benefits such as improved feed utilization, growth and resistance to bacterial pathogens (Lückstädt 2007). Short-chain fatty acid (SCFA) as a group contain one to seven carbon atoms and exist as straight or branched chain compounds, which include formic, acetic, propionic, butyric, isobutyric, valeric, isovaleric, 2-methylbutyric, hexanoic and heptanoic acids. All are produced, along with small amounts of other organic compounds, such as methane, carbon dioxide, lactate and alcohol by microbial fermentation of carbohydrate substrates in the GI tract of herbivorous animals. Acetic, propionic and butyric acids are the predominant forms of SCFA and are produced mainly from the fermentation of plant materials, including celluloses, fibre, starches and sugars.

There has been an increasing interest on the use of acidifiers in aquaculture due to the removal of antibiotic growth promoters by the European Union in 2006. However, the available publications have reported divergent results from their use (Lückstädt 2006, 2008). This confusion is compounded by inadequate differentiation in the literature between the chemical form of the supplement, either the acid or a salt of the respective acid. A distinction is needed because the salts do not result in a net gain of a proton when dissociated in the acidic environment of the stomach and thus have no capacity for reduction in the stomach pH required to increase the residence time of ingested material which is often one of the cited goals of SCFA supplementation in terrestrial animal literature (Baruah 2008). However, the literature supports a greater antibacterial action in the hindgut for SCFA salts (Lückstädt 2008). The bactericidal effect of an acid depends on its structure with the short-chain fatty acids being more effective than those with a higher molecular weight and therefore less capacity for diffusion through cellular membranes. These organic acids, in contrast to inorganic acids, are able to penetrate the bacterial cell wall, in their undissociated form, where they then dissociate. The proton (H^+) reduces the intracellular pH, thus forcing the bacteria to use its own energy to retain osmotic balance (Salmond *et al*. 1984). The anion (A−) hinders the synthesis of DNA and RNA, preventing the multiplication of the micro-organisms and altering the synthesis of proteins. This action mechanism makes them more effective against Gram-negative bacteria with a more accessible cellular membrane (*E. coli*, *Salmonella* spp. *Klebsiella* spp., *Yersinia* spp., and *Campylobacter* spp.). Therefore, the Gram-positive wall of LAB (*Lactobacillus* spp., *Enterococcus faecium*) and the slightly more acid medium conferred by the organic acids will favour the development of the latter. The above actions reduce numbers of pathogenic bacteria in the intestine of mono-gastric animals such as pigs, poultry, rabbits and broiler chicks (Hofacre 1997; Ozduven *et al*. 2009) as well as inhibition of the growth of some potential pathogenic bacteria such as *Salmonella* spp. and *Vibrio* spp. in *Artemia franciscana* (Defoirdt *et al*. 2007).

Historically direct additions of SCFAs in cultured fish diets were principally in the form of fish silages which can be preserved by the addition of formic acid alone or in combination with propionic acid or sulphuric acid (Asgard & Austreng 1985a,b; Gildberg & Raa 1977) with recent publications focussing on the use of SCFA to increase mineral bio-availability in the GI tract (Baruah *et al*. 2007).

Acidic calcium sulphate

In a recent study by Anuta *et al*. (2011), the authors investigated the effect of different inclusion level of acidic calcium sulphate (ACS; Vitoxal; 0.4%, 1.2% and 2%) on allochthonous gut microbiota of Pacific white shrimp (*Litopenaeus vannamei*) by DGGE analysis. Inclusion of ACS to the basal diet modulated the gut bacterial community based on Dice similarity coefficient, but no bands were sequenced and thus no species were identified. The authors put forward the hypothesis that beneficial gut bacteria proliferate in ACS-fed shrimps such that pathogenic bacteria may be excluded from the enteric environment. However, this hypothesis merits further investigations. In addition, as some stress indicators and immune response were also improved in shrimp fed ACS-supplemented diets, the authors indicate that further studies are needed to clarify the specific physiological action.

Sodium butyrate

Modulation of the gut microbiota by sodium butyrate has been reported in broiler chickens (Czerwinski *et al.* 2012), early-weaned pigs (Castillo *et al.* 2006) as well as for fish (Liu *et al.* 2014; Owen *et al.* 2006). In the study of Owen *et al.* (2006), the authors investigated the effect of the sodium butyrate on the allochthonous microbiota of the hindgut of African catfish (*Clarias gariepinus* Burchell). Diets contained either FM as the protein sources of partial replacement with SBM were supplemented with 0.2% and 2% sodium butyrate supplementation. After 15 days of feeding on the experimental diets, the culturable allochthonous microbiota was investigated. Total viable counts of aerobic and facultative colonies for both treatments ranged between 10^9 and 10^{10} CFU g^{-1}; despite marginal variation between samples, no significant differences were observed. A total of 250 isolates were identified based on standard biochemical tests. The microbiota of the fish fed the FM-based control diet was dominated by *Enterococcus* spp. and Enterobacteriaceae. *Staphylococcus* and *Micrococcus* were also identified as minor components of the gut microbiota. The microbiota was clearly affected by 0.2% sodium butyrate supplementation; *Enterococcus* levels increased, while Enterobacteriaceae levels decreased compared with the control. This coincided with a marginal insignificant improvement of SGR and FCR. Greater bacterial diversity was observed in the SBM-fed fish as *Enterococcus, Micrococcus and Staphylococcus* were the dominant groups but *Pseudomonas*, Enterobacteria (Enterobacteriaceae) and *Acinetobacter* were also identified. Dietary supplementation of 0.2% and 2.0% sodium butyrate did not seem to affect levels of *Enterococcus*, which remained the dominant group. However, *Micrococcus and Staphylococcus* were no longer dominant and were displaced by Enterobacteria (Enterobacteriaceae). Unfortunately, further identification of Enterobacteriaceae, which was one of the most dominant groups in virtually all treatments, was not conducted. Species level identification may have provided a useful insight as to specifically which species were affected by sodium butyrate.

In a recent study, Liu *et al.* (2014) evaluated the effect of sodium butyrate microencapsulation on growth, immunity response, gut morphology and gut microbiota of common carp. The effects of prefed oxidized soybean oil (SBO)-induced stress (pre-oxidized stress) and dietary microencapsulated sodium butyrate type (MSB1.5 or MSB3.0; 1.5 h or 3 h sustained release) on production, intestinal mucosa, immunity and bacteria in juvenile common carp were investigated. Modulation of gut adhesive bacteria within each segment investigated was not obvious when common carp fed diets with MSB, as similarity coefficients of >0.79 were observed.

Poly-β-hydroxybutyrate

The use of poly-β-hydroxybutyrate (PHB) as a dietary component for aquatic animals is not inspired by a direct action by which it affects the host or the gut microbiota. The rationale can be found in the release of metabolites in the GI tract with the aim of improving the health status of the host or increasing the protection of the host against infections. PHB is a compound that is synthesized by a variety of micro-organisms mainly under conditions of nutrient limitation and carbon excess (Jendrossek & Handrick 2002). The chemical structure makes it an interesting compound for application in aquaculture settings. It is insoluble in water and consists of an aliphatic C3-polyester backbone with a methyl group situated at the β-position of the molecule.

Poly-β-hydroxybutyrate is produced intracellularly in the form of granules in the cytoplasm (Lee 1996). When it is released in case of cell lysis, it can be metabolized by other bacteria. These excrete extracellular PHB depolymerases converting PHB into water-soluble monomers, namely β-hydroxybutyric acid (Tokiwa & Calabia 2004). β-hydroxybutyric acid is known to exhibit some antimicrobial, insecticidal and antiviral activities (Tokiwa & Ugwu 2007). As such, the idea grew to apply PHB as a means to control infectious diseases in aquaculture systems (Defoirdt *et al.* 2009). Indeed, it was found that treatment with PHB resulted in the protection of brine shrimp (*Artemia fransciscana*) against vibriosis (Defoirdt *et al.* 2007). It led, even more, to a growth increase of juvenile European sea bass (*Dicentrarchus labrax*) (De Schryver *et al.* 2010) and Siberian sturgeon fingerlings (*Acipenser baerii*) (Najdegerami *et al.* 2012), and it increased larval survival during the culture of freshwater prawn (*Macrobrachium rosenbergii*) (Nhan *et al.* 2010). It thus seems that PHB has more to it than just an anti-infective activity.

During GI passage, PHB comes in close contact with the microbiota inhabiting the gut. It is likely that PHB induces modifications at the microbial level resulting directly or indirectly in effects at the host level. It was and still is thus important to gain insight into the interactions between PHB and the intestinal microbial community as was attempted in several studies.

In a first case, higher modifications could be observed in the intestinal microbial community composition of juvenile European sea bass according to the PHB content in the diet (De Schryver *et al*. 2010). This showed that although the effects of PHB were observed at the level of the host (i.e. increased growth in this specific case), dose-dependent processes were also progressing at the microbial level.

Najdegerami *et al*. (2012) observed in a trial on Siberian sturgeon fingerlings that modifications resulting from the presence of PHB in the intestinal tract represented a selection in the gut microbiota. The main proliferating bacteria were phylogenetically closely related to *Bacillus* spp. and *Ruminococcaceae* spp. In addition, Nhan *et al*. (2010) reported that PHB decreased the number of *Vibrio* spp. in the intestinal tract of freshwater prawn larvae.

Bacteria which are enhanced by PHB may represent niche populations able to metabolize PHB during GI passage. The capacity to produce extracellular PHB depolymerases is a widespread phenomenon amongst bacteria and has been shown under aerobic, anaerobic and thermophilic conditions (Tokiwa & Ugwu 2007). PHB depolymerising bacteria had, however, never been isolated from intestinal environments. Having access to such bacteria and applying these as probiotics for aquatic animals could contribute considerably to the beneficial effects related to PHB application. Liu *et al*. (2010) succeeded in confirming this theory. They isolated for the first time PHB-degrading strains from the intestinal environment of aquatic animals being fed with PHB in the diet. *Acidovorax* sp. was isolated from Siberian sturgeon, and *Acinetobacter* sp. and *Ochrobactrum* sp. were isolated from European sea bass and freshwater prawn, respectively. The application of these PHB-degrading strains in combination with PHB could increase the protection of *Artemia franciscana* against vibriosis as compared to the application of PHB alone.

The bacteria stimulated by the incorporation of PHB in the diet may, however, not only be PHB degraders. It was shown that PHB could also protect *A. franciscana* from vibriosis under gnotobiotic conditions with only the pathogen being present (Defoirdt *et al*. 2007). This indicated that the host digestive enzymes also act on PHB, possibly altering the intestinal environmental conditions (for example, pH decrease). Bacteria belonging to the selected groups thus not necessarily are able to metabolize PHB, but may be species that are better adapted to the new environment. Of course, it is highly likely that the selected populations represent both active and passive responders to the presence of PHB in the diet. The application of the pyrosequencing technology could contribute considerably in obtaining an overview of how PHB modifies the intestinal microbial community structure and composition.

Going further than observational analysis, PHB has also been used as a model compound to illustrate new approaches on how to study the effect of dietary components on the gut microbiota in aquatic animals. It is important to go further than the descriptive analysis of the gut microbiota based on plating or molecular fingerprinting analysis and try to evolve towards giving an explanatory function to microbial community changes. This can imply non-conventional approaches to describe the gut microbiota based on molecular techniques as was illustrated in the work of De Schryver *et al*. (2011). Here, juvenile sea bass were fed with PHB and the DGGE fingerprinting patterns from the intestinal microbial community were converted into numerical data and interpreted to describe the dynamics, the composition, the evenness and the functionality of the gut microbiota according to the PHB treatment the fish were subjected to. The aim of such investigations is to assess how dietary components modulate the intestinal microbiota, how this can be directed and how this can contribute to a positive outcome of the host.

Potassium diformate

The aim of the study of Zhou *et al*. (2009d) was to investigate the effect of potassium diformate (PDF) and two antibiotics on growth performance, feed conversion and gut microbiota of hybrid tilapia. PDF is the first substance approved as non-antibiotic growth promoter by the Europe Union and is an alternative substance for growth promoters of tilapia. Flavomycin and quinocetone, or a combination of them are used as antibiotic growth promoters in aqua-feed in China. Pooled gut contents sampled from four replicate tanks were analysed for bacterial community by 16S rDNA PCR and denaturing gradient gel electrophoresis (DGGE). Dietary PDF or antibiotics modulated the gut microbiota with different patterns for dosages of PDF or different kinds of growth promoters. Supplementation of PDF stimulated colonization of some intestinal bacteria such as uncultured *Actinobacterium* (EU249319), *Mycobacterium peregrinum* (EU249312), uncultured *alpha Proteobacterium* (EU249320; EU249306) and uncultured *Verrucomicrobiae* bacterium (EU249308). However, uncultured *Rhizobiales* bacterium (EU249305), uncultured *actinobacterium* (EU249313) and uncultured Eubacterium (EU249300) were less detected in the gut. Furthermore,

dietary antibiotics affected the tilapia's growth performance possibly through depressing most of the intestinal bacteria.

Effect of metals

Iron

To our knowledge, only one study has evaluated the effect of iron on the microbiota of fish (Gatesoupe *et al.* 1997). In this study with seabass larvae (*Dicentrarchus labrax*), three diets were as follows: iron-limited (omission of ferrous sulphate), addition of 1 g of ferrous sulphate kg^{-1} (compound diet) and *Artemia* as control. Bacterial count in larvae fed diet included with ferrous sulphate was 10^9 CFU g^{-1} larvae, while the bacterial count of larvae fed the iron-limited or *Artemia* was approximately 3×10^8 CFU g^{-1} larvae. The microbiota differs between the treatments groups, and in larvae fed *Artemia*, a more diverse microbiota consisting of *Vibrio* spp. I, II and III, *Acinetobacter*, *Moraxella* and uncharacterized bacteria were detected. In contrast, *Vibrio* spp. I and III, and *Enterobacteriaceae* were reported in larvae fed the iron-limited, while *Vibrio* spp. I and III were detected in larvae fed the compound diet. The effect of iron on gut microbiota merits further investigations as, for example, the iron content in feed typically used in Japan ranged between 112 and 2785 mg kg^{-1} (Watanabe 2009). As a natural consequence, large amounts of iron are supplied to the intestinal tract; it is unlikely that siderophores produced by some gut bacteria could inhibit adherence and colonization of fish pathogens in the GI tract. The topic, modulatory effect of iron on gut microbiota, is highly relevant for further investigations especially related to the recent study of Sugita *et al.* (2012) evaluating the diversity of siderophores-producing bacteria isolated from the digestive tract of Japanese fish species.

Copper

Some studies have revealed that Cu^{2+}-exchanged montmorillonite (Cu-MMT) had strong antibacterial ability towards *A. hydrophila*, *P. fluorescens*, *V. parahaemolyticus* and *E. coli* (Hu & Xia 2005, 2006; Hu *et al.* 2005) as well as its effect on gut microbiota of Nile tilapia (Hu *et al.* 2007). Supplementation of Cu-MMT reduced the total intestinal aerobic bacterial counts and decreased the counts of *Aeromonas*, *Vibrio*, *Pseudomonas*, *Flavobacterium*, *Acinetobacter*, *Alcaligenes* and Enterobacteriaceae compared with control diet-fed tilapia (Hu *et al.* 2007). The authors suggested that modulation caused by Cu-MMT might be related to bacterial accumulation on surface and its effect on bacterial adhesion to Caco-2 cells.

Metal nanoparticles

Merrifield *et al.* (2013) revealed that nanoparticles (NPs; silver, Ag and copper, Cu) with antimicrobial properties modulated the intestinal microbial community structure in zebra fish; *Cetobacterium somerae* strains and two unidentified bacterial clones from the Firmicutes phylum were suppressed by Cu-NP exposure but were present in Ag-NP and control. The loss of *C. somerae* in fish fed Cu-NP resulted in greater bacterial species richness and diversity.

Chromic oxide

Chromic oxide (Cr_2O_3) has been one of the most widely used indicators for the determination of nutrient digestibility in fish (Olsen & Ringø 1997; Ringø & Olsen 1994), and it was assumed that the compound was inert. However, some investigations have revealed that by feeding Arctic charr a diet containing Cr_2O_3, population level of lactobacilli in gut and faecal samples remained stable, while counts of Gram-negative bacteria genera declined (Ringø 1993b,c, 1994). Similar observations were made when Gram-negative gut isolates and LAB were grown *in vivo* on tryptic soy agar added 5% glucose (TSAg) plates with or without supplement of Cr_2O_3. The reason for the decline in Gram-negatives in the GI tract has not been elucidated, but Ringø (1993b) put forward two hypothesis; (a) chromic oxide affects the attachment sites in the gut mucosa/epithelium or (b) oxidase-positive bacteria may be more sensitive to Cr_2O_3. G. Gislason & E. Ringø (unpublished data) revealed that supplementation of Cr_2O_3 to the diet increased the intestinal activity of the enzyme cholytaurin hydrolase, possibly through the selection of lactobacilli and streptococci as demonstrated by Ringø (1993b).

Phosphorus

Xie *et al.* (2011) carried out a nine-week feeding trial with juvenile Jian carp where the effect of dietary phosphorus was investigated on intestinal *Aeromonas*, *E. coli* and *Lactobacillus*. *Aeromonas* and *E. coli* decreased with increasing inclusion level of phosphorus up to 3.6 and 5.5 g kg^{-1}, respectively. Contrary, the population level of *Lactobacillus* increased when increasing level of phosphorus was included; maximum level was reported when 9.2 g phosphorus was included kg^{-1}. The authors suggested that the

effect on intestinal *Aeromonas* and *E. coli* may be due to the production of antibacterial substances produced by lactobacilli. However, this controversial hypothesis merits further investigations.

The immune system and effect of immunostimulants

In order to fully appreciate the effects of dietary components on the gut microbiota and the corresponding influences on fish health, it is vital to consider the relationship of dietary components and gut microbiota on the efficient functioning of the immune system. The majority of our understanding of how the gut immune system works is derived from extensive studies in human and other mammalian systems. In healthy subjects, GALT running the length of the gut is integral to maintaining both innate and adaptive immune responses that allow the gut to tolerate food/commensal organisms while being able to readily respond to pathogens.

With the current progression in the studies of the teleost fish immune system, it is becoming apparent that teleosts share similarities with more developed mammalian systems (Magnadottir 2008; Rombout *et al*. 2011). The innate immune system is a non-specific response consisting of natural physical barriers such as the gut lining layer, mucus, commensal organisms; cellular mechanisms utilizing macrophages and neutrophils involved in clearance of pathogenic material by phagocytosis and oxygen-dependent and oxygen-independent mechanisms of killing, reviewed in Foey & Picchietti (2014), whereas the killing of extracellular pathogens is mediated by natural killer cells and eosinophils (Plouffe *et al*. 2005). These non-specific innate defences are reinforced by the release of soluble factors such as complement (Nonaka & Smith 2000), lysozyme (Saurabh & Sahoo 2008), onchorhyncins (Fernandes *et al*. 2003, 2004), agglutinins and iron-binding siderophores (e.g. transferrin). The barrier function of the gut mucosa is integral to defence against pathogens and maintaining tolerance to food-derived nutrients that must be absorbed for the fish to thrive. The inter-relationship between epithelial cells, mucus, antimicrobial products and commensal organisms resident in the gut is vital for the health and well-being of the fish.

Teleost fish also utilize cells characteristic of an antigen-specific adaptive immune response that is suggestive of immunological memory. The utilization of monoclonal antibodies available and functional analyses have allowed for the reporting of both helper and cytotoxic T cells as well as differential subpopulations of B cells (Miller *et al*. 1998; Scapigliati *et al*. 1999). In addition, the increasing frequency by which cytokine homologues are being reported in fish is suggestive of both efficient innate and adaptive immune systems. Innate system cytokines include IL-1β (Hong *et al*. 2004), IL-18 (Zou *et al*. 2004) and TNFα (Laing *et al*. 2001), whereas homologues of adaptive system cytokines such as the regulatory cytokines, IL-2, IL-10 and TGFβ have also been identified (Bird *et al*. 2005; Daniels & Secombes 1999; Zou *et al*. 2003). Thus, it is possible that the immune system of teleost fish may closely resemble the human immune system. The inter-relationships between dietary components and commensal organisms in the gut effectively prime regulatory mechanisms resulting in mucosal tolerance which allows the subject to tolerate food and obtain the nutrients from it without mounting a harmful immune response to what effectively is foreign material ingested by the fish. Under stressful situations such as predation, temperature and pH changes, this tolerance can be broken, resulting in inappropriate immune activation, gut dysfunction and a failure to thrive. The appropriate dietary intake and function of commensal organisms that reinforce the gut barrier immune system will be integral in preventing the breakdown of mucosal tolerance.

The use of probiotic bacteria has been demonstrated to prevent binding of pathogenic organisms to gut epithelial cells, compete with pathogenic organisms for available nutrients, provide fermentation products that facilitate epithelial cell growth/survival and serve to modulate the mucosal immune system in teleost fish (Foey & Picchietti 2014). A recent study examining the effects of probiotic bacteria *Lb. rhamnosus*, *Ent. faecium* and *Bacillus subtilis* fed to the rainbow trout reported an augmentation of superoxide production, complement activity and gene expression of IL-1β, TNFα and the regulatory cytokine, TGFβ (Panigrahi *et al*. 2007). These results are suggestive of probiotic feeds effectively boosting the innate immune system and regulatory adaptive immune system. The production of fermentation products such as SCFAs (butyrate) also modulates barrier function, decreasing epithelial barrier permeability by supporting epithelial cell growth and inducing the expression of tight junction proteins as well as suppressing pro-inflammatory cytokines in favour of anti-inflammatory and regulatory cytokines (Van Nuenen *et al*. 2005). Thus, probiotic bacteria have an important role in maintenance of an efficient immune system and epithelial barrier that allows the fish to tolerate gut luminal contents while extracting required nutrients essential for growth

(Picchietti *et al*. 2007; Salinas *et al*. 2011). In addition, to probiotic bacteria, nutrients play an important role in maintaining an efficient immune system. Just how dietary nutrients achieve this and how they interact with both resident commensal organisms of the gut and the immune system of the gut require more research; a combinatorial approach to FM replacement by more defined alternative feeds utilizing dietary micronutrients and probiotics may represent an effective approach to maintaining fish health and productivity.

Effect of immunostimulants on gut microbiota

Immunostimulants seem to be valuable for the control of fish diseases (e.g. Maqsood *et al*. 2011; Raa 1996; Ringø *et al*. 2012b; Sakai 1999; Soltanian *et al*. 2009; Vadstein 1997). However, the ability of immunostimulants to modulate the gut microbiota in a healthier way and decrease the infection pressure by improving the gut function is less available (Ringø *et al*. 2012b). Few studies have investigated the effect of immunostimulants on the gut microbiota of fish (Gildberg & Mikkelsen 1998; Hoseinifar *et al*. 2011b; Merrifield *et al*. 2011c; Rawling *et al*. 2009; Skjermo *et al*. 2006).

In their recent review devoted to immunostimulants and nucleotides, Ringø *et al*. (2012b) discussed the results on the effect of immunostimulants on Atlantic cod fry (Gildberg & Mikkelsen 1998), Atlantic cod larvae (Skjermo *et al*. 2006) and juvenile beluga (*Huso huso*) (Hoseinifar *et al*. 2011b). However, as Ringø *et al*. (2012b) did not present information from the studies of Rawling *et al*. (2009) and Merrifield *et al*. (2011c), they will be discussed.

Rawling *et al*. (2009) revealed that dietary inclusion of Sangrovit® in the diets of tilapia had no significant effects on the total number of viable autochthonous or allochthonous bacteria in PI. However, a significant reduction in allochthonous LAB was observed when the fish were fed an inclusion level of Sangrovit® at 75 and 100 mg kg^{-1}. Unfortunately, the microbiota composing was not identified and should be a topic for further investigation. The population levels of culturable autochthonous LAB were too low to enumerate. In a study with juvenile tilapia, Merrifield *et al*. (2011c) investigated the effect of Ergosan (5 g kg^{-1}) on the allochthonous and autochthonous gut bacterial community and revealed that numbers of OTUs, species diversity and richness were unaffected by dietary treatments within the allochthonous and autochthonous communities. Furthermore, the authors reported trends towards elevated survival and body protein content, and a lower microvilli density in posterior intestine, but the values were not significantly affected by Ergosan.

Based on the fact that less information is available about the effect of immunostimulants on the '*good*' gut microbiota in fish with antagonistic activity against fish pathogenic bacteria, this should be a topic of further research as the GI tract is a potential port of entry for pathogenic bacteria (Birkbeck & Ringø 2005; Harikrishnan & Balasundaram 2005; Ringø *et al*. 2004, 2007a,b; Salinas *et al*. 2008; Sugita *et al*. 2008).

Readers with further interest on the issue are referred to the recent review of Vadstein *et al*. (2012).

Effect of antibiotics

The most commonly used antibiotics in fish farming in the 1970s and 1980s were oxolinic acid, oxytetracycline (OTC), furazolidone, potential sulphonamides (sulphadiazine and trimethoprim) and amoxycillin. However, the indiscriminate use of those chemicals in disease control in many sections of the aquaculture industry has led to selective pressure of antibiotic resistance in bacteria, a property that may be readily transferred to other bacteria (Amàbile-Cuevas *et al*. 1995; Aoki *et al*. 1985; Cabello 2006; Ringø *et al*. 2014c; Romero *et al*. 2012; Sørum 2006; Towner 1995). Readers with special interest in the use of antibiotics are referred to the reviews of Cabello (2006), Sørum (2006) and Romero *et al*. (2012).

Use of antibiotics to control pathogenic bacteria can also reduce the numbers of non-pathogenic bacteria in the gut, and numerous studies are available on the effect of antibiotics on intestinal microbiota of fish (Austin & Al-Zahrani 1988; Bakke-McKellep *et al*. 2007; Cantas *et al*. 2011; Fukumoto *et al*. 1987; Hansen *et al*. 1992; He *et al*. 2010, 2012; Kerry *et al*. 1997; Lesel *et al*. 1989; Liu *et al*. 2012; Navarrete *et al*. 2008; Naviner *et al*. 2007; Sugita *et al*. 1988b, 1989; Takemura & Kusuda 1988; Tamminen *et al*. 2011).

In their study with rainbow trout, Austin & Al-Zahrani (1988) used erythromycin, oxolinic acid (OA), OTC, penicillin G and sulphafurazole to study the effect of antimicrobial compounds on aerobic heterotrophic gut microbiota (Table 6). A general increase in bacterial population level in the GI tract was observed during a 10-day treatment when the fish were administered OA, OTC and sulphafurazole which are commonly used for the treatment of Gram-negative pathogens. After the treatment, however, there seemed to be a steady decrease during the following two–week of period. Conversely, erythromycin and penicillin G, which are used to treat some diseases caused by Gram-positive

Table 6 Composition (%) of the bacterial population in the digestive tract of rainbow trout during the administration of antimicrobial compounds via medicated food. After Austin & Al-Zahrani (1988)

Treatment regime		Erythromycin			Oxolinic acid			Oxytetracycline			Penicillin G			Sulphafurazole		
Sampling day	Control	1	5	10	1	5	10	1	5	10	1	5	10	1	5	10
Taxon																
Gram-negative																
Acinetobacter spp.	8	5	0	0	52	40	36	44	40	32	0	10	5	0	0	10
Aeromonas spp.	16	35	20	0	8	0	8	4	0	0	70	30	25	30	20	15
Alcaligenes spp.	0	0	5	5	0	4	0	4	0	0	10	5	0	10	0	0
Enterobacteriaceae	20	15	25	40	16	8	4	12	8	4	5	0	0	0	5	10
Flavobacterium spp.	4	0	10	10	0	8	12	0	12	18	0	15	0	0	0	10
Methylomonas spp.	0	0	0	0	0	0	0	0	0	0	0	0	0	5	0	0
Pseudomonas spp.	12	25	5	15	0	4	0	12	0	0	0	10	0	5	10	10
Gram-positive																
Bacillus spp.	8	10	15	0	0	0	0	8	8	12	10	5	40	40	40	35
Coryneforms	12	0	10	25	12	8	8	8	12	20	5	10	0	0	5	5
Micrococcus spp.	12	5	5	0	4	20	24	4	12	2	0	0	0	0	15	0
Staphylococcus spp.	8	5	5	20	8	8	8	4	8	12	0	15	30	10	5	5

bacteria, caused a rapid reduction in bacterial numbers within the GI tract. Moreover, a dramatic effect was found for penicillin G as the oesophagus and stomach appeared to be totally devoid of bacteria through the treatment regime. Austin & Al-Zahrani (1988) also examined the bacterial composition in the GI tract during administration, focusing on some groups of Gram-negative bacteria (*Pseudomonas, Acinetobacter, Aeromonas* and *Enterobacteriaceae*) and Gram-positive bacteria (*Bacillus, Micrococcus, Staphylococcus* and coryneforms). Under OTC and oxolinic acid treatment, *Acinetobacter* became the most frequent bacterial group; in contrast under sulphafurazolone treatment, *Bacillus* was the most prevalent. Furthermore, the use of antimicrobial compounds led to an enhanced level of resistance among the microbiota in the GI tract. For example, use of erythromycin resulted in total resistance to the drug within 10 days. Moreover, there was an enhanced level of resistance to OA and sulphafurazole.

In the 1980s, Japanese scientists evaluated the effect of OTC and OA on faecal microbiota of goldfish. Fukumoto *et al*. (1987) reported that oral administration of OTC at dosage of 50 mg kg^{-1} body weight for seven days did not significantly alter the faecal microbiota of goldfish. Sugita *et al*. (1988b) came to similar conclusion in their study on the effect of OTC (50 mg kg^{-1}) on the faecal microbiota of goldfish. In the microbiota, several bacterial groups were observed, *A. hydrophila*, *Aeromonas punctata*, *Plesiomonas shigelloides*, *Pseudomonas* spp. and *Bacteroides* type A, but their predominance was not significantly affected by OTC administration.

In a later study, Sugita *et al*. (1989) investigated the effect of OA on faecal microbiota of goldfish. OA was orally administrated to three goldfish at 20 mg kg^{-1} body weight for 7 days. As *A. hydrophila* and *Bacteroides* type A were the dominant groups in the faeces of all fish, the authors suggested that the faecal microbiota was not affected markedly by the oral administration of OA. The conclusion of Fukumoto *et al*. (1987) and Sugita *et al*. (1988b, 1989) from studies of goldfish is in accordance with the results of Takemura & Kusuda (1988) that oral josamycin administration did not significantly affected the intestinal microbiota of yellowtail (*Seriola quinqueradiata*).

In their study on the effect of oral administration of gentamicin and flumequin in two different diets, low- and high-lipid diets fed to rainbow trout, Lesel *et al*. (1989) observed that antibiotic supplement reduced the bacterial faecal counts. Significantly lower bacterial population levels were detected on Mac Conkey agar plates, while only a small decrease was observed on tryptic soy agar (TSA) plates. With respect to the effect of antibiotics on faecal microbiota, only a small difference was seen when rainbow trout were fed the lipid-rich or lipid-low diets with supplement of antibiotics. Before and after antibiotic treatment, four or five taxa were identified in faeces, but only one to three when fish were fed medicated diet. The following bacterial genera were observed in the normal diet; *Aeromonas*, Enterobacteria, *Pseudomonas* and coryneforms. In contrast, *Flavobacterium* spp. was the only bacterial genus identified when gentamicin and flumequin were included into the low lipid diet. Coryneforms and Enterobacteria were also detected in low fractions, in lipid-rich diet with antibiotics. The results of Lesel *et al*. (1989) might open for the speculation that inclusion of antibiotics reduce the microbiota

composition, but as only 12 of 100 isolates from each dietary group were identified, no clear conclusion can be drawn from this experiment.

The reduction in microbiota diversity by antibiotics was also observed in the study of Lauzon *et al.* (2010b) who examined the culturable microbiota of Atlantic cod rearing systems. Two tanks were followed, but larvae from one of them showed unhealthy behaviour, 55 days posthatch, and were treated with interspectin-L WS (40 ppm in rearing water). This treatment resulted in a more homogenous microbiota when analysed one day later, where *Psychroserpens burtonensis*; 13 of 20 analysed strains dominated compared with only 5% of the analysed strains in control larvae, the untreated tank. Other studies indicate that florfenicol treatment may reduce the diversity of the intestinal microbiota in tilapia (He *et al.* 2010, 2012). These authors described the use of florfenicol as a growth promoter in the feed and the effect on the microbiota composition assessed by TTGE analysis and qPCR. Their results indicated that florfenicol included in the diet (0.02 g kg^{-1}) reduced bacterial diversity estimated using Shannon index and decreased the estimated intestinal bacterial count. Florfenicol treatment affected the relative abundance of some particular groups such as Actinobacteria and Proteobacteria, as estimated by qPCR using rpoB.

The intestinal microbiota of various larval fish have been described in numerous investigations (Hansen & Olafsen 1999; Ringø & Birkbeck 1999; Vadstein *et al.* 2012). However, less is known about the effect of chemotherapy on the indigenous microbiota of larvae (Hansen *et al.* 1992). The antibiotics used by Hansen *et al.* (1992), penicillin and streptomycin were those originally recommended to minimize bacterial growth on herring (*Clupea harengus*) eggs (Blaxter & Hunter 1982). The intestinal microbiota of herring larvae in sand-filtered sea water (group STB) was all Gram-negative, oxidase-positive, motile and non-pigmented rods, most probably members of the genus *Pseudomonas* or *Alteromonas*. Bacterial isolates from larvae incubated in sand-filtered sea water containing penicillin and streptomycin (group STA) were all Gram-negative, oxidase-positive, yellow-pigmented and non-motile rods. Based on biochemical and physiological characteristics, the authors suggest that these isolates consisted almost exclusively of three or four *Flavobacterium* species. With respect of antibiotic susceptibility, isolates from the STB group were susceptible to penicillin, ampicillin, streptomycin, chloramphenicol and sulfamethoxazole-trimethoprim. In contrast, STA isolates were resistant to all antibiotics tested, except for O/129 and novobiocin. Screening of STA isolates did not reveal plasmids; thus, antibiotic resistance was most likely not plasmid coded. The results of Hansen *et al.* (1992) strongly suggest that the intestinal microbiota of herring larvae was affected by addition of antibiotics to the incubation water. In an analogous study of Atlantic halibut larvae, Verner-Jeffreys *et al.* (2004) investigated how the addition of OA, OTC and amoxycillin to the inflow water affected the larval gut microbiota. The addition of antibiotics at 110 and 180 day-degrees posthatched greatly reduced bacterial numbers in the larval gut 220 day-degrees posthatched. The microbiota of control larvae was heterogeneous from batch to batch and consisted high species richness corresponding to 6 phenons based on clustering into BIOLOG GN. In contrast, few culturable bacteria were recovered from larvae exposed to antibiotics (<10 CFU/larvae) and none of them were assigned to any phenon.

Kerry *et al.* (1997) investigated whether the administration of OTC via medicated feed to Atlantic salmon smolts resulted in a selection for an increase in the frequency of resistance of the intestinal microbiota. Prior to medication, the mean frequency of resistance was $4.7 \pm 4.8\%$ but after being fed the medicated feed for 5 days, the mean frequency of resistance decreased marginally to $4.1 \pm 2.1\%$. On the day after the end of medication, the frequency of resistance was found to be $1.7 \pm 1.3\%$. The mean frequency of resistance of the intestinal microbiota 6, 11, and 16 days after the end of OTC administration was found to be $5.9 \pm 2.1\%$, $9.9 \pm 0.7\%$ and $0.1 \pm 0.1\%$, respectively. Based on the nonparametric method of Mann–Whitney, the authors concluded that none of the data sets were sufficiently different from those collected prior to experimental start to justify the hypothesis that selection for increased resistance had occurred.

As mentioned above, studies have focused mainly on describing the frequency of antibiotic resistance during and following the use of antibiotics (Kerry *et al.* 1997), antibiotic susceptibility of fish pathogens (Akinbowale *et al.* 2007; Depaola *et al.* 1995; Giraud *et al.* 2006; Schmidt *et al.* 2000), and description of molecular determinants of antibiotic resistance (Adams *et al.* 1998; Izumi & Aranishi 2004; Miranda *et al.* 2003; Schmidt *et al.* 2000). However, the effects of chemotherapeutic agents on the bacterial ecology of the guts of fishes have received limited attention. Navarrete *et al.* (2008) reported that bacterial diversity of gut microbiota of salmon was reduced by OTC treatment. Microbiota from untreated fishes was more diverse, and their main components were *Pseudomonas*, *Acinetobacter*, *Bacillus*, *Flavobacterium*, *Psychrobacter* and *Brevundimonas*.

In contrast, the microbiota of the OTC treated group displayed a lower diversity and was only composed of *Aeromonas*, clustering with *A. sobria* and *A. salmonicida*. In these isolates, the presence of class A family Tet tetracycline resistance genes (*tet*A, *tet*B, *tet*C, tetD, *tet*E and *tet*H) was assessed by PCR and HaeIII digestion of amplicons (Jacobs & Chenia 2007; Schnabel & Jones 1999). The *tet*E determinant was detected most frequently among the isolates (78%), while 22% of the isolates possessed tetD/H determinants. This promotion of *Aeromonas* spp. prevalence supports the current concern that antibiotic treatment can eradicate micro-organisms from the normal microbiota and facilitate the proliferation of opportunistic pathogens by minimizing competition and dispersion-resistant determinants. The occurrence of OTC-resistant bacteria in salmon farming has been shown before (Jacobs & Chenia 2007; Miranda & Zemelman 2002). Also, mobile resistance determinants have been demonstrated in several genera (Adams *et al*. 1998; Miranda & Rojas 2007; Miranda & Zemelman 2002; Schmidt *et al*. 2001). The presence of bacteria harbouring resistance determinants could be related to the widespread use of antibiotics in aquaculture (Cabello 2006). Some authors have even suggested that common components of the microbiota could disperse resistance genes via horizontal gene transfer because of the high density and proximity of resident bacteria in the GI tract microenvironment (Salyers *et al*. 2004; Summers 2002).

Some authors suggest that a successful culture environment in a hatchery depends on preservation of a diverse microbial community that includes innocuous and beneficial bacteria (Schulze *et al*. 2006). It has been suggested that high diversity in the fish intestinal microbiota and environment, including innocuous and favourable bacteria, is beneficial for the health of fish. Antibiotic treatments can eradicate susceptible micro-organisms, which can then promote the colonization by resistant opportunistic bacteria (Moffitt & Mobin 2006; Navarrete *et al*. 2008; Raveh *et al*. 2006; Vollaard & Clasener 1994).

Bakke-McKellep *et al*. (2007) investigated the effect of oxytetracycline on the autochthonous and allochthonous microbiota of Atlantic salmon fed FM, SBM or inulin. OTC inclusion caused a greater reduction in bacteria from digesta (allochthonous) than mucosa (adherent or autochthonous). Microbiota composition was different depending on the diet. The bacteria *Nocardia corynebacteroides* and *Rathayibacter tritici* were exclusively reported in the FM-fed fish; *Vibrio* spp., *Arthrobacter agillis*, *Brachybacterium* spp., *Kocuria carniphila* and *Rhodococcus* spp. were isolated only from the SBM-fed fish. In some cases, the total counts of viable bacteria were lower than the detection level when OTC was added to the diets. Therefore, no adherent bacteria were identified from fish fed the FM + OTC diet or from the distal intestinal digesta of fish fed the SBM + OTC. In fish fed SBM signals of enteritis were observed, however, OTC inclusion did not affect histological scores and a slight decreasing in the inflammation signals was observed in the fish fed SBM-OTC. Bacterial involvement in the pathogenesis cannot be excluded despite efforts to rule this out in the current study. Although some antibiotics were effective in reducing bacterial numbers, in accordance with previous investigations in various fish species (Austin & Al-Zahrani 1988), no influence on the inflammatory response was observed. Several explanations have been suggested as follows: (i) the microbiota had no influence on SBM-induced enteritis; (ii) the activity of OTC was not sufficiently reduce numbers of potentially key adherent bacteria; and (iii) the absence of microbiota in the digesta of the SBM + OTC-fed salmon may have a similar detrimental effect on the intestine as the SBM.

The application of natural and innocuous compounds has potential in aquaculture as an alternative to antibiotics. The essential oils (EOs) have been proposed because many plants contain phenolic compounds, and these EOs comprise the majority of plant antimicrobial components. Navarrete *et al*. (2008) evaluated the effect of diet supplementation with *Thymus vulgaris* essential oil (TVEO) on the allochthonous microbial composition of rainbow trout. DNA was extracted directly from the intestinal contents, and the 16S rRNA genes were amplified by PCR. The bacterial composition was analysed using temporal temperature-gradient electrophoresis (TTGE). No significant changes ($P > 0.05$) were detected in the TTGE profiles of TVEO-treated trout compared with the controls. The Dice similarity index revealed a high stability (Cs > 70%) of the intestinal microbiota in both groups during the five-week period. Sequence analyses of the TTGE bands revealed the same bacterial composition in both groups, with most bacteria belonging to the *Proteobacteria* and *Firmicutes* phyla. Furthermore, *in vitro* antibacterial activity of TVEO was assessed against 22 gut isolates and fish pathogens. Based on their results, the authors suggested that essential oils could be used as alternatives for managing bacterial populations and avoiding bacterial resistance.

Currently, molecular approaches and massive sequencing methods have become available; hence these could be important tools to elucidate the diversity of antibiotic-resistance genes present in the fish gut. The resistome concept

has been used to describe the diversity of antibiotic resistance that exists naturally in a particular environment (Fernández-Alarcón *et al*. 2010). However, the resistome of aquaculture environments has been poorly described and it will require more studies using molecular approaches. These approaches should allow the diversity of antibiotic-resistance genes in the gut to be analysed, even when no antibiotics are used and also permit the effects of antibiotics on bacterial populations to be evaluated.

Future perspectives

Mucus is secreted from goblet cells, specialized epithelial cells, and in endothermic animals, it is known that through the intestine mucus forms two distinct layers, the firmly adherent mucus layer and the loosely adherent mucus layer (Atuma *et al*. 2001). These layers vary in thickness throughout the intestine (Inglis *et al*. 2012), and evidence indicates that bacteria readily colonize the loosely adherent mucus layer, but not the adherent mucus layer (Johansson *et al*. 2008). In aquatic animals, such information is not available and this merits further investigations, as mucus-associated bacteria are important in host health.

During the 1970s–1990s, numerous studies evaluated the intestinal microbiota of fish using traditional culture technique (Cahill 1990; Hansen & Olafsen 1999; Horsley 1977; Ringø & Birkbeck 1999; Ringø *et al*. 1995), and the statement, *the intestinal microbiota in fish appears to be simpler than that of endothermic animals* was put forward (Ringø *et al*. 1995). However, this statement can be questioned based on the numerous recent studies using molecular methods, particularly as metagenomic studies using next-generation sequencing platforms reveal 100s–1000s of bacterial OTUs intestine of fish. By traditional bacteriological techniques, the major handicap to successful analysis of the gut microbiota is the inability to cultivate all of the members of the bacterial community under laboratory conditions. In a study of the intestinal microbiota of Arctic charr, Ringø *et al*. (2001a) suggested that only 3% of the bacterial community associated with the distal intestine are culturable. A similar underestimation of bacterial numbers has been observed in natural environment, soil, where growth on agar plates only accounted for 2–4% of the total bacterial population counted using microscopy (Olsen & Bakken 1987). This problem has long been known from studies of faecal microbiota of human (Tannock *et al*. 2000) and has been solved to some extent in microbial ecology by the use of analytical approaches that are DNA-based and hence are referred to as molecular methods (Vaughan *et al*. 2000). Nucleic acid-based techniques now permit the analysis of even the non-culturable members of the bacterial community. PCR coupled with DGGE provides a useful technique for comparisons of faecal or intestinal microbiota.

Recent publications indicate that this approach is efficient to study the GI bacterial community structure of fishes (e.g. Griffiths *et al*. 2001; Hovda *et al*. 2007; Jensen *et al*. 2004; Romero & Navarrete 2006). However, the number of studies is still limited. Analysis of bacterial communities has been commonly performed by analysis of PCR-TTGE/DDGE. DGGE of PCR-amplified DNA fragments offers a rapid means for the study of complex bacterial populations in environmental samples, either at a gross taxonomic level (Muyzer *et al*. 1993) or at more refined, for example genus, levels (Garbeva *et al*. 2003). The gene of choice has been the 16S rDNA gene due to its mosaic structure, ubiquity, and the growing database. However, the disadvantages are the heterogeneity of the different rrs operon and poor discrimination between closely related bacteria (Magne *et al*. 2006). These inconveniences had led to the proposal of other genes that can be more resolute to analyse bacterial communities, like the spacer region (ITS) between the 16S and 23S rRNA genes and the rpoB gene. Another concern is related to metabolic state of the bacteria detected using DNA-based approaches, whereas studies based on RNA extraction and detection of ribosomal RNA sequences could give a more comprehensive vision of the active bacterial population within the fish gut.

Adhesion capacity and/or colonization are of importance when evaluating the intestinal microbiota of aquatic animals. Even though fish microbiologists have gained some knowledge about adherence of bacteria in the GI tract of fish during the last two decades, it is a long way to go compared with the information available from non-aquaculture studies. For example, in a study using crude mucus from small intestine of a 23-day-old healthy piglet, Macías-Rodríguez *et al*. (2009) demonstrated that adhesion of the potential probiotic *Lactobacillus fermentum* originally isolated from faeces of a piglet involved two adhesion-associated proteins with a relative molecular weight of 29 and 32 kDa that are attached non-covalently to the cell surface. In a study with *Lb. rhamnosus* a piliated bacterium, von Ossowski *et al*. (2010) reported that 2 pilin subunits (SpaB and SpaC) in the SpaCBA pilus fibre are involved in binding to intestinal mucus. Moreover, Huang *et al*. (2013) evaluated the relationship between adhesive ability of probiotic bacteria and acid residues of soluble in the human colonic mucin (sHCM). Based on

their results using a Biacore binding assay, the authors concluded that there was a strong relationship between probiotic adhesion and acid residues of sHCM. Furthermore, the recognition of intestinal receptors by bacterial fimbriae and the attachment of *E. coli* to the brush border of epithelial cells are considered as the primary requisites for pathogenesis. In a recent study, González-Ortiz *et al.* (2014) reported that wheat bran (WB), casein glycomacropeptide (CGMP) and locust bean (LB) significantly reduced the number of enterotoxigenic *E. coli* (ETEC K88) attached to the intestinal mucus. Based on their results, the authors suggested that WB, CGMP and LB are good candidates to be added in diets of weaned piglets to prevent ETEC K88-induced diarrhoea. To our knowledge, no aquatic studies have been carried out on cell surface components of marine probiotic bacteria responsible for mucosal adhesion and prevention of pathogen colonization, and we recommend that these topics merit investigations.

A SCFA transport mechanism has been reported in tilapia (Titus & Ahearn 1988) and may be common to other fish species, thus future research on the interactions of SCFA and gut microbiota should firstly examine whether the host animal has an existing SCFA producing bacterial population. Subsequently investigations can determine whether this population can be increased via the provision of SCFA due to the 'positive feedback' mechanism of the lower pH favouring SCFA producing Gram-positive bacteria. Additionally, while the mode of action against Gram-negative bacterial pathogens is accepted, the antibacterial action of SCFA against ubiquitous aquatic Gram-negative pathogens such as *A. salmonicida* subsp. *salmonicida* has yet to be confirmed.

Alternative feed ingredients, pea and lupin, have already proved their potential as feed ingredients for salmonids (Zhang *et al.* 2012). Even though knowledge of the impact of pea on gut microbiota of human and golden Syrian hamsters (Dominika *et al.* 2011; Marinangeli *et al.* 2011) and lupin on gilthead sea bream and goldfish (de Paula Silva *et al.* 2011) is available, more information is needed on aquatic animals.

During the last decade, numerous studies have investigated inclusion of antinutritional factors (ANFs) and genetically modified plants on general biological effects, growth, gut histology and immunology (Francis *et al.* 2001; Gatlin *et al.* 2007; Krogdahl *et al.* 2010; Sissener *et al.* 2011). However *per se*, no information is available about their effect on gut microbiota. This topic merits further investigations; as the gut microbiota may modify ANFs, and hence their interactions and biological effects. Furthermore, the intestinal disorder reported in Atlantic salmon fed soy bean protein concentrate at high seawater temperatures may be due to modulation of the intestinal microbiota and merits further investigations.

The intestinal microbiota is undoubtedly an important factor in determining the health status of endothermic animals (Power *et al.* 2014). Compared to the considerable increase in the studies of the effect commensal microbes exert in the mammalian gut from 1996 up to 2009 (Sekirov *et al.* 2010), less studies have been carried out on the role played by the GI microbiota in aquatic animals health and disease. Therefore, the topic '*dietary effect on gut microbiota of aquatic animals*' is probably a never-ending story.

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