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THE FISH GUT MICROBIOME
CONTRIBUTIONS TO ITS CHARACTERIZATION AND POTENTIAL APPLICATIONS

Dissertation
for obtaining a doctorate degree
at the University of Natural Resources and Life Sciences
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Submitted by
Mahdi GHANBARI

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DEDICATION

To my parents for instilling in me the importance of hard work and higher education.

To my wife for her endless support and encouragement.

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List of Publications

Publications related to this thesis

1. **Ghanbari**, M., Shahraki, H., Kneifel, W. & Domig, K.J. (2015). Community membership and structure of mucosa and luminal-associated intestinal microbiota in snow trout (*Schizothorax zarudnyi*) as revealed by pyrosequencing. FEMS Microbiology Ecology. Under Review.

2. **Ghanbari**, M., Kneifel, W. & Domig, K.J. (2015). A new view of fish gut microbiome: advances using next-generation sequencing. Aquaculture, 448, 464-475.

3. **Ghanbari**, M., Jami, M., Domig, K.J. & Kneifel, W. (2013). Seafood biopreservation by lactic acid bacteria – A review. LWT - Food Science and Technology, 54 (2), 315-324.

4. **Ghanbari**, M., Jami, M., Kneifel, W. & Domig, K.J. (2013). Antimicrobial activity and partial characterization of bacteriocins produced by lactobacilli isolated from Sturgeon fish. Journal of Food Control, 32 (2), 379-385.

Other publications

5. Jami, M., Lai, Q., Shahriari Moghadam, M., **Ghanbari, M.,** Kneifel, W. & Domig, K. (2015). *Celeribacter persicus* sp. nov., a polycyclic aromatic hydrocarbon-degrading bacterium isolated from the mangrove sediments at Nayband Bay in the Persian Gulf, Iran. International Journal of Systematic and Evolutionary Microbiology. Under review

6. Jami, M., **Ghanbari, M**., Domig, K.J. & Kneifel, W. (2015). Phylogenetic diversity and biological activity of culturable Actinobacteria isolated from fresh water fish. Microbiological Research**,** 175, 6-15.

7. Jami, M., **Ghanbari, M**., Zunabovic, M., Domig, K.J. & Kneifel, W. (2014). *Listeria monocytogenes* in seafood: a continuing challenge for food safety. Comprehensive Reviews in Food Science and Food Safety**,** 13 (5), 798-813.

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1. **Ghanbari, M.,** Shahraki, H, Kneifel, W. & Domig, K. J. (2015). Mucosa-Associated Microbiota in the Snow trout Intestine (Schizothorax zarudnyi, Nikolskii, 1897) Differ from Lumen-associated Microbiota, as Revealed by 454 Pyrosequencing. FEMS 2015, June 7-11, Maastricht, The Netherlands

2. **Ghanbari, M.,** Domig, K. J. & Kneifel, W. (2014). Barcoded Pyrosequencing-Based Diversity Analysis of The Intestinal Microbiome in Snow Trout (*Schizothorax zarudnyi*) – Oral Presentaion. ÖGHMP 2014, June 2-5, Bad Ischl, Austria. **ÖGHMP Travel Award.**

3. **Ghanbari, M.**, Domig, K. J. & Kneifel, W. (2014). High-Throughput Sequencing and Metagenomics: Moving Forward in the Culture-Independent Analysis of Fish Microbial Ecology – Oral Presentaion. AquaCyprus 2014, May 15-17, Girne, Northern Cyprus.

4. **Ghanbari, M.**, Jami, M., Domig, K. J. & Kneifel, W. (2012). Seafood Biopreservation Using Lactic Acid Bacteria – Oral Presentation. AQUA 2012-Global Aquaculture-Securing our Future, Sep 1-5, 2012, Prague, Czech Republic.

5. Jami, M., **Ghanbari, M.,** Domig, K. J. & Kneifel, W. (2012). Antimicrobial Potential Produced By Lactobacillus Casei AP8 Isolated From The Microbiota of Sturgeon Fish. AQUA 2012 – Global Aquaculture - Securing our Future, Sep 1-5. Prague, Czech Republic.

Table of contents

CHAPTER 1

INTRODUCTION

1.1 Aquaculture; Trend and developments

Aquaculture is the rearing of fish, shrimps, other crustaceans and mollusks for human consumption. It has been practised for centuries: the earliest known publication dates from 500 BC. In many of the developing and developed regions of the world, the demand for fish has been growing. Given population growth, expanding urbanization, and rising incomes in the developing world, this trend is expected to continue. Because levels of capture fish production have stagnated over the past decades (FAO, 2014a), the world will thus be more dependent on aquaculture in the coming decades (Msangi & Batka, 2015). For the first time in history, more fish for human consumption have originated from farms than from wild capture, having reached almost parity in 2012 according to the latest global report from the Food and Agriculture Organization of the United Nations (FAO, 2014a). Given the importance of animal-based proteins for the provision of nutrients for human health (especially the types of micronutrients found in fish-based proteins, like omega-3 fatty acids), it is clear that fish comprise an essential component of a food-secure future. Considering both the limits to expanding rangelands for livestock and the ecological constraints to increasing capture fishery production, aquaculture represents the next and perhaps even the last-remaining frontier of large-scale animal protein production (Msangi & Batka, 2015).

Global fish production has grown steadily in the last five decades (Figure 1), with food fish supply increasing at an average annual rate of 3.2 percent, outpacing world population growth at 1.6 percent (FAO, 2014a). The term “food fish” includes finfishes, crustaceans, molluscs, amphibians, freshwater turtles and other aquatic animals (such as sea cucumbers, sea urchins, sea squirts and edible jellyfish) produced for the intended use as food for human consumption. World food fish aquaculture production expanded at an average annual rate of 6.2 percent in the period 2000–2012, more slowly than in the periods 1980–1990 (10.8 percent) and 1990–2000 (9.5 percent). World food fish aquaculture production more than doubled from 32.4 million tonnes in 2000 to 66.6 million tonnes in 2012 (FAO, 2014a). World per capita apparent fish consumption increased from an average of 9.9 kg in the 1960s to 19.2 kg in 2012 (FAO, 2014a) (Table 1). This impressive development has been driven by a combination of population growth, rising incomes and urbanization,

and facilitated by the strong expansion of fish production and more efficient distribution channels.

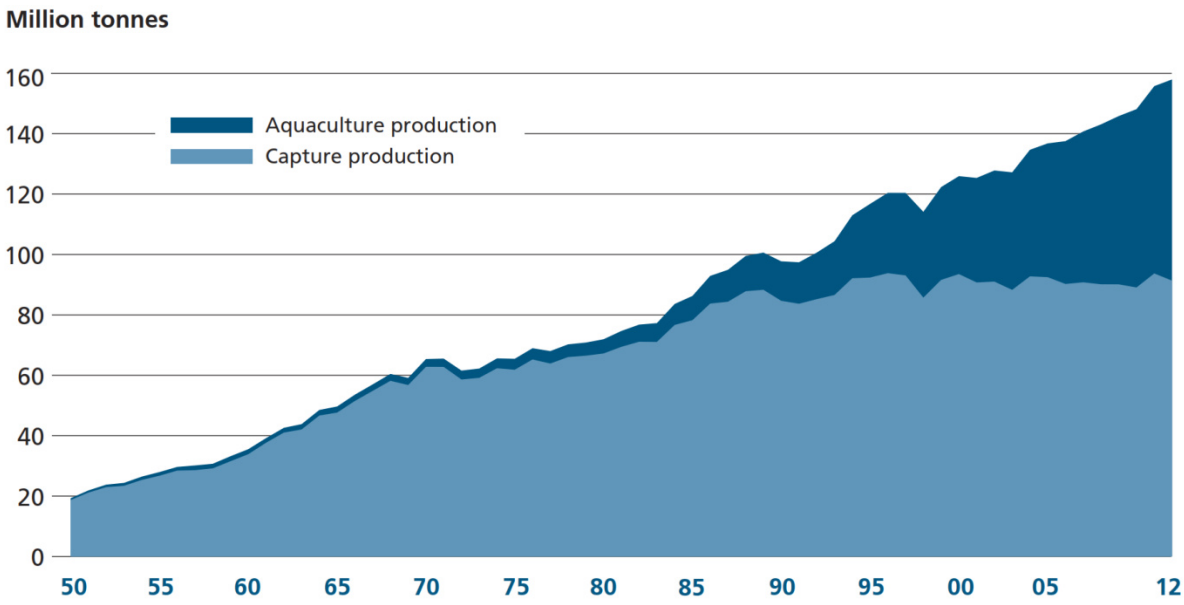

Figure 1. World capture fisheries and aquaculture production (FAO, 2014a).

Despite the surge in annual per capita apparent fish consumption in developing regions (from 5.2 kg in 1961 to 17.8 kg in 2010) and low-income food-deficit countries (LIFDCs) (from 4.9 to 10.9 kg), developed regions still have higher levels of consumption, although the gap is narrowing (Msangi & Batka, 2015). A sizeable and growing share of fish consumed in developed countries consists of imports, owing to steady demand and declining domestic fishery production. In developing countries, fish consumption tends to be based on locally and seasonally available products, with supply driving the fish chain. However, fuelled by rising domestic income and wealth, consumers in emerging economies are experiencing a diversification of the types of fish available owing to an increase in fishery imports (FAO, 2014b, Msangi & Batka, 2015).

Table 1. World fisheries and aquaculture production and utilization (FAO, 2014a).

	2007	2008	2009	2010	2011	2012
			(Million tonnes)			
PRODUCTION						
Capture						
Inland	10.1	10.3	10.5	11.3	11.1	11.6
Marine	80.7	79.9	79.6	77.8	82.6	79.7
Total capture	**90.8**	**90.1**	**90.1**	**89.1**	**93.7**	**91.3**
Aquaculture						
Inland	29.9	32.4	34.3	36.8	38.7	41.9
Marine	20.0	20.5	21.4	22.3	23.3	24.7
Total aquaculture	**49.9**	**52.9**	**55.7**	**59.0**	**62.0**	**66.6**
TOTAL WORLD FISHERIES	**140.7**	**143.1**	**145.8**	**148.1**	**155.7**	**158.0**
UTILIZATION[1]						
Human consumption	117.3	120.9	123.7	128.2	131.2	136.2
Non-food uses	23.4	22.2	22.1	19.9	24.5	21.7
Population (*billions*)	6.7	6.8	6.8	6.9	7.0	7.1
Per capita food fish supply (*kg*)	17.6	17.9	18.1	18.5	18.7	19.2

1.2 Aquaculture trends and developments in the Middle East

As in the rest of the world, aquaculture's contribution to the Middle East's total production has also grown. This trend is stronger in the Middle East than in many other parts of the world. Indeed, in 2001, out of a total of 2.4 million tonnes in the Middle East, 78.6% was from capture fisheries while only 21.4 % was from aquaculture. In 2011, out of a total of 3.4 million tonnes, 56% were from capture fisheries while 44% contributed by aquaculture (FAO, 2014b) (Fig. 2).

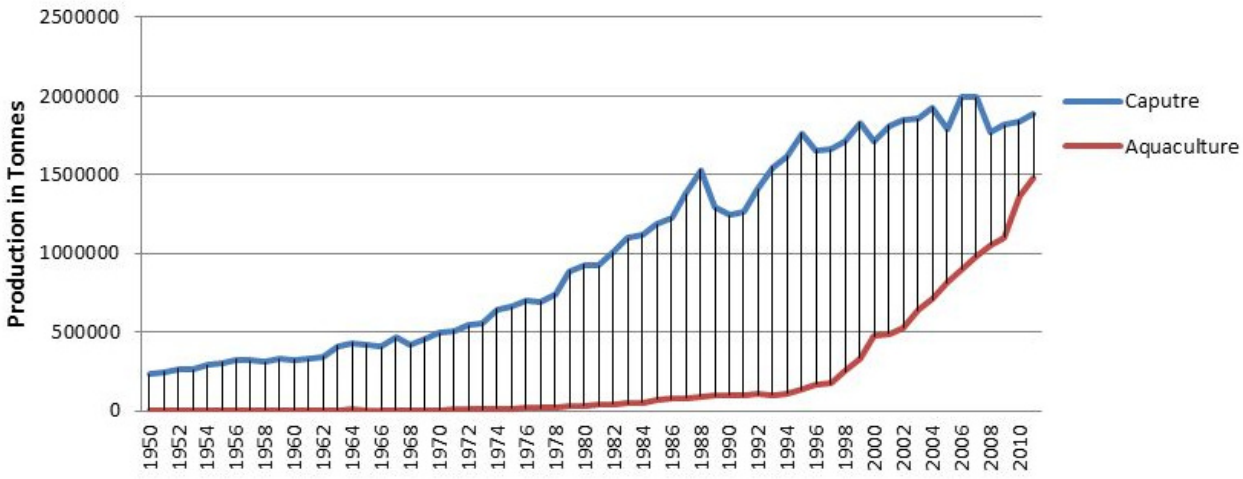

Figure 2. Middle East capture fisheries and aquaculture production (volume in tonnes) (FAO, 2014b).

The main aquaculture producers in the region are Egypt, Saudi Arabia and Iran. In 2011, 72% of all production in Egypt was from aquaculture, while Saudi Arabia and The Islamic Republic of Iran produced 41% and 33% from aquaculture respectively (FAO, 2014b). Fisheries and aquaculture production in the Middle East are relatively small and remain underdeveloped. Production from the region amounts to only 2% of the total world production. Major producers are Egypt, Iran and Turkey, with Egypt being the largest producer as well as the largest importer (FAO, 2014b) (Figure 3).

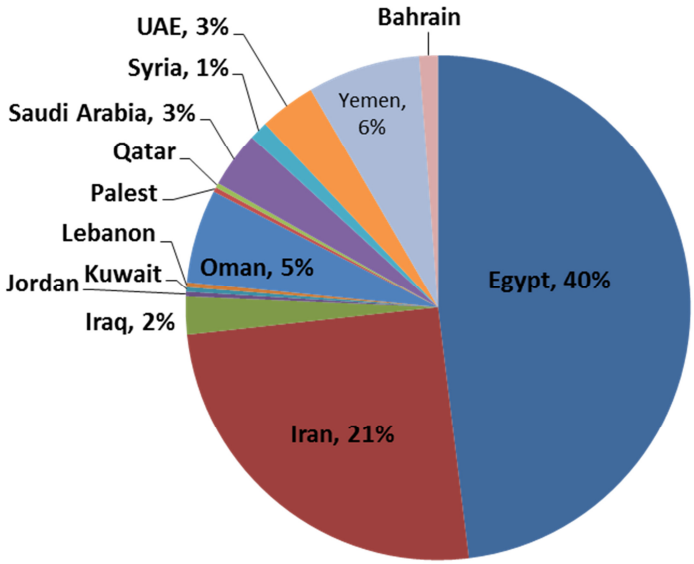

Figure 3. Middle East capture fisheries and aquaculture production by country (FAO, 2014b).

1.3 Fisheries and aquaculture production in Iran

The Islamic Republic of Iran is located in the Middle East between latitudes of 25° 00' and 39° 47'N and longitude of 44° 02' and 63° 02' E. The total area of the country is 1,648,195 km^2 which includes 1,636 million km^2 land area and 12,000 km^2 of water surface. The coast line stretches for 2,700 km to the south in the Persian Gulf and Oman Sea and in the north along the Caspian Sea. Weather conditions differ greatly across Iran allowing a range of different types of aquaculture to be practiced. The Islamic Republic of Iran, however, has only given serious attention to this activity during the last three decades where investment in aquaculture development began in the early 1980s along the Caspian Sea coasts regions and in some of the south-western provinces (Kalbassi *et al.*, 2013).

The main types of aquaculture activities in Iran are: warm-water fish culture of Chinese carps (48.43% in 2009), cold-water culture of rainbow trout (35.52% in 2009), shrimp culture (2.47% in 2009) and culture-based fisheries and juvenile production for stock enhancement (13.5% in 2009); For instance, sturgeon fish *(Acipenser guldenstaedti*, *A. persicus*, *A. stellatus*, *A. nudiventris* and *Huso huso*), Caspian kutum (Rutilus kutum) and Snow trout (*Schizothorax zarudnyi*), are artificially propagated in the governmental hatcheries and their fingerlings stocked into the Caspian Sea and Hamun international wetland for the rehabilitation of their respective fisheries (Kalbassi *et al.*, 2013).

The contribution of aquaculture to fishery production in Iran has progressively increased during the last decades (Figure 4). Aquaculture production increased rapidly from 3,219 tonnes in 1978 to 370,876 tonnes in 2013, representing approximately 34.6 percent of the total fish production (Iranian Fisheries Organization, 2015) (Figure 4). From the food security and job opportunity aspect, the fisheries and aquaculture industry in Iran are in a favourable position and governmental investment in this sector is substantial. Per capita fish consumption increased from 1 kg/year in 1980 to 8.5 kg by 2013.

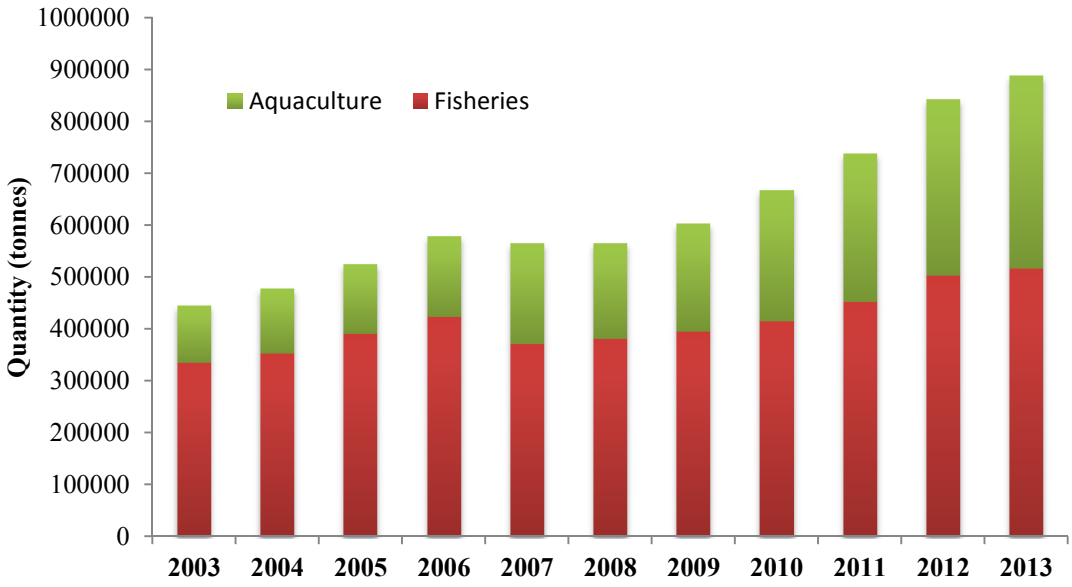

Figure 4. Total fisheries and aquaculture production in Iran, 2003-2013 (Iranian Fisheries Organization, 2015).

A fish consumption campaign, initiated by the Iranian Fisheries Organization, has been in place since 1998, particularly in inland cities which seems to have had a positive effect on people's diet. The value of fisheries commodities exported in 2013 was USD 304,426 million (Figure 5). The role of fisheries and aquaculture as a contributor to the Iranian economy is rather small, but with good potential for increases in production. It seems likely that its contribution to the country's economy will develop in the future. Iran's fisheries development plans aim to increase fish production, improve the welfare of fishers and farmers, promote exports, increase fish consumption and provide greater food safety and food security (IFO, 2008, Kalbassi *et al.*, 2013).

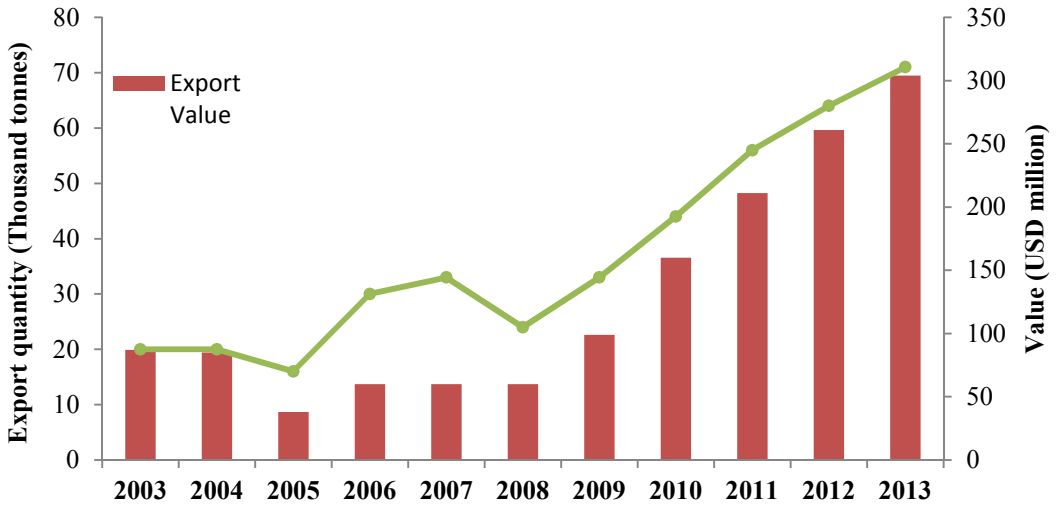

Figure 5. Total value of fisheries commodities exported in 2003-2013 (Iranian Fisheries Organization, 2015).

1.4 Lightly preserved seafood

Lightly preserved seafood constitutes a broad group of chilled stored ready-to-eat (RTE) foods characterised by having pH > 5.0 and < 6 % NaCl in the water phase of the product (Mejlholm & Dalgaard, 2007). Due to the considerable economic importance of these products there has been an increased international competition in the production of lightly preserved seafood (Mejlholm & Dalgaard, 2007, Ghanbari *et al.*, 2013). Different processing and preservation methods are used for its manufacture, mainly to extend the shelf life and/or to obtain desirable sensory characteristics (e.g. smoke flavour) of the products. The use of different methods for production results in lightly preserved seafood with varied characteristics and storage conditions. In Iran salted fish and cold-smoked fish are regarded as traditional products that are prepared from cultivated fish caught from fish farms (commonly fertilized by cow manure) and from the Caspian Sea (Basti *et al.*, 2006, Ghanbari *et al.*, 2008). These products are the most consumed seafood in Northern part of Iran (Basti *et al.*, 2006, Tavakoli *et al.*, 2012) and are prepared by mixed salting alone and/or followed by cold smoking methods. The smoked fish is usually kept in plastic bags outside the refrigerator in fish markets in Iran and is traditionally consumed raw or undercooked (Ghanbari *et al.*, 2008).

In the last years, the traditional processes like salting, smoking and canning applied to seafood have decreased in favor of milder technologies involving lower

salt content, lower cooking temperature and vacuum (VP) or modified atmosphere packing (MAP) (Pilet & Leroi, 2011). The treatments are usually not sufficient to destroy microorganisms and in some cases psychrotolerant pathogenic and spoiling bacteria can develop during the extended shelf-life of these products (Ghanbari *et al.*, 2013). As several of these products are eaten raw, it is therefore essential that adequate preservation technologies are applied to maintain its safety and quality (Pilet & Leroi, 2011). To harmonize consumer demands with the necessary safety standards, traditional means of controlling microbial spoilage and safety hazards in seafood are being replaced by alternative solutions gaining more and more attention and summarized under the so-called "biopreservation technology". It consists of inoculating food with microorganisms or their metabolites, selected for their antibacterial properties and may be an efficient way of extending shelf life and ensuring food safety through the inhibition of spoilage as well as pathogenic bacteria without altering the nutritional quality of raw materials and food products (Cortesi et al., 2009, Campos et al., 2012).

1.5 Aims and objectives of the thesis

This thesis is sub-structured into two major areas that are briefly addressed below and more in-depth supplemented with corresponding papers published.

1.5.1 Fish gut-associated bacteria as protective cultures

The first aim of this research was to find answers to the question ´**Are fish gut associated bacteria an effective tools to inhibit the growth of food-borne pathogens, food spoilage bacteria and contaminants in aquaculture**?´.

Subsequently, the second chapter concentrates on a **comprehensive discussion about the biopreservation strategy as an alternative way for seafood preservation, its relevant issues and on its applicability to fish and fishery products**.

In the third chapter, **the significance of fish gut-associated bacteria for their bio-protective potential was elucidated by characterizing their antimicrobial properties and their ability to inhibit the growth of food-borne pathogens, food spoilage bacteria and contaminants in aquaculture.**

1.5.2 Snow trout gut microbiome: applying next generation sequencing to conservation problems

So far, our current information on the microbiota of fishes has been largely derived from culture-based approaches, which often possess dubious sensitivity and only comprise a limited fraction of microbial communities (Austin, 2002). More recently and in line with other studies of microbial biodiversity, emphasis has been placed on the next generation sequencing (NGS) techniques that provide more rapid and new high resolution insight into the structure and diversity of fish-associated microbial communities within the digestive tracts of fresh water and marine fish. These methods have substantially refined and re-defined the knowledge of the microbial diversity in both external surfaces as well as within the digestive tract of freshwater and marine fish (Namba *et al.*, 2007, Wu *et al.*, 2010, Lan & Love, 2012, Wu *et al.*, 2012a, Larsen *et al.*, 2013).

While in the first phase of this thesis a limited range of fish intestinal microbiota and their properties were targeted and studied, in the second phase such an NGS approach was adapted to provide more in-depth knowledge of the community membership, structure, functional and the relationship between the constituent members of the resident microbes. . Accordingly, chapter 4 highlights the **potential of next-generation sequencing platforms for the analysis of the fish gut microbiota**. Corresponding results in this field are presented and addressed in relation to the perspectives and future research direction of fish gut microbial ecology.

For the analysis of fish gut-associated microbiome the Snow trout (*Schizothorax zarudnyi*, Nikolskii, 1897) was selected as a practically relevant example. Snow trout, a cold water riverine and short migratory fish, is one of the most commercially relevant and important native fish in Hamoun Lake in Iran distributed in the South Eastern region of Asia. It belongs to the family Cyprinidae and sub-family Schizothoracinae, which are widely distributed in the Himalayan and sub-Himalayan area and much of the rest of Asia. Unfortunately, the species population is threading due to a combination of overfishing, invading species and environmental degradation. Many efforts have been made to control this, but no technology has been developed yet for commercial culture, particularly due to the problem of not being able to develop appropriate feed and the high mortality rate

(>70%). As a part of this process, it has been suggested that it is essential to obtain greater understanding of the indigenous snow trout behavior. Nothing is known about the nature of the intestinal microflora in snow trout and its effects on fish health and physiology, a fact that seems to be a very important as well as critical point. Therefore, it was aimed to apply one of next generation sequencing technologies, the pyrosequencing technique, to better understand the ecology of bacterial communities associated with the gut microbiota of Snow trout. In addition, some insight into behavioral differences, fish physiology, and fish nutrition could be facilitated. Finally, chapter 5 provides some **results of the experiments on taxonomic and in silico functional profiling of the gut microbiome in Snow trout** (*Schizothorax zarudnyi*, Nikolskii, 1897), **based on a pyrosequencing approach and on bioinformatics**.

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CHAPTER 2

Seafood biopreservation by lactic acid bacteria – a review

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Seafood biopreservation by lactic acid bacteria – A review

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ABSTRACT

Biopreservation is a powerful and natural tool to extend shelf life and to enhance the safety of foods by applying naturally occurring microorganisms and/or their inherent antibacterial compounds of defined quality and at certain quantities. In this context, lactic acid bacteria (LAB) possess a major potential for use in biopreservation because most LAB are generally recognized as safe, and they naturally dominate the microflora of many foods. The antagonistic and inhibitory properties of LAB are due to different factors such as the competition for nutrients and the production of one or more antimicrobially active metabolites such as organic acids (prevailingly lactic and acetic acid), hydrogen peroxide, and antimicrobial peptides (bacteriocins). This review addresses various aspects related to the biological preservation of seafood and seafood products by LAB and their metabolites.

1. Introduction

1.1. Trends and developments

The growing interest of consumers in nutritional aspects and the parallel attention paid on food quality issues have contributed to the increasing consumption of fish and fish products. Usually, this product category is considered as of high nutritional value and highly recommended by nutritionists. However, fish and seafood products are also known to be susceptible to spoilage due to microbiological and biochemical degradation (Dalgaard, Madsen, Samieian, & Emborg, 2006; Mejlholm et al., 2008). Accordingly, the development of effective processing treatments to extend the shelf life of fresh fish products has become a must. In addition, the consumers increasingly demand for high-quality but minimally processed seafood (Campos, Castro, Aubourg, & Velázquez, 2012). In this context, lower levels of salt, fat, acid and sugar and/or the complete or partial removal of chemically synthesized additives have become essential.

In the last years, traditional processes like salting, smoking and canning applied to fish and seafood have decreased in favour of so-called mild technologies involving the application of lower salt concentrations, lower heating temperature and packaging under vacuum (VP)* or under modified atmosphere (MAP; Dalgaard et al., 2006; Emborg, Laursen, Rathjen, & Dalgaard, 2002). However, the drawback of these trends is that safety hurdles are weakened and foodborne illness outbreaks may increase (Cortesi, Panebianco, Giuffrida, & Anastasio, 2009; Mejlholm et al., 2008). Therefore new methodologies are sought to ensure food safety and to extend the shelf-life of foods.

Hitherto, approaches to reduce the risk of food poisoning outbreaks have relied on the search for the addition of efficient chemical preservatives or on the application of more drastic physical treatments such as heating, refrigeration, application of high hydrostatic pressure (HHP), ionizing radiation, pulsed-light, ozone, ultrasound technologies etc. In spite of some possible advantages, such treatments possess several drawbacks and limitations when applied to seafood products. Among these, the toxicity of some commonly used chemical preservatives (e.g., nitrite) (Cleveland, Montville, Nes, & Chikindas, 2001) and the alteration of sensory and nutritional properties of seafood may be exemplarily mentioned. Due to the delicate nature of seafood, physical treatments may induce considerable quality losses (e.g., freezing

Abbreviations: VP, vacuum packed; MAP, modified atmosphere packed; HHP, high hydrostatic pressure; LAB, lactic acid bacteria; LPFP, lightly preserved fish product; SPFP, semi-preserved fish product; GRAS, generally recognized as safe; QPS, qualified presumption of safety; LMM, low-molecular-mass; HMM, high-molecular-mass; HSP, heat shock protein; CSS, cold smoked salmon.

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damage, discolouration in case of HHP and ionizing radiation) (Devlieghere, Vermeiren, & Debevere, 2004; Zhou, Xu, & Liu, 2010).

Among alternative food preservation strategies, particular attention has been paid to biopreservation techniques, which extend the shelf-life and enhance the hygienic quality, thereby minimizing the negative impact on the nutritional and sensory properties. Biological preservation usually refers to the use of a natural or controlled microflora and/or its antimicrobial metabolites (Garcia, Rodriguez, Rodriguez, & Martinez, 2010; Nilsson et al., 2005). Lactic acid bacteria (LAB) are interesting candidates, which can be used for this approach. In fact, they often are naturally present in food products and may act as powerful competitors to contaminating spoilage microorganisms, by producing a wide range of antimicrobial metabolites such as organic acids, diacetyl, acetoin, hydrogen peroxide, reuterin, reutericyclin, antifungal peptides, and bacteriocins. Hence, the last two decades have seen pronounced advancements in using LAB and their metabolites for natural food preservation (Cleveland et al., 2001; Gálvez, Abriouel, López, & Omar, 2007; Nes, 2011; Nilsson et al., 2005).

1.2. Bacterial hazards associated with fish and seafood products

In general, fish and seafood including related products are a risky group of foodstuffs. The diverse nutrient composition of seafood provides an ideal environment for growth and propagation of spoilage microorganisms and common food-borne pathogens (Dalgaard et al., 2006; Emborg et al., 2002). Table 1 presents an overview on the major bacterial hazards associated with aquatic food products.

Pathogenic bacteria found in seafood can be categorized into three general groups (Calo-Mata et al., 2008; Mejlholm et al., 2008): (1) Bacteria (indigenous bacteria) that belong to the natural microflora of fish, such as *Clostridium botulinum*, pathogenic *Vibrio* spp., *Aeromonas hydrophila*; (2) Enteric bacteria (non-indigenous bacteria) that are present due to faecal and/or environmental contamination, such as *Salmonella* spp., *Shigella* spp., pathogenic *Escherichia coli*, *Staphylococcus aureus*; and (3) bacterial contaminants during processing, storage, or preparation for consumption, (such as *Bacillus cereus*, *Listeria monocytogenes*, *Staph. aureus*, *Clostridium perfringens, Cl. botulinum, Salmonella* spp.).

The presence of indigenous microorganisms in fresh cultured products is usually not a safety concern since they are mainly present at low levels that do not cause a disease, and in case of adequate cooking, food safety hazards are insignificant in those products. Therefore, the real hazard concerns are more related to products where growth of those bacteria is feasible during storage and which are eaten raw or insufficiently cooked (Mejlholm et al., 2008). In this context it has to be mentioned that the development of official guidelines to minimize faecal contamination of shellfish and harvesting waters has strongly reduced the incidence of enteric bacteria in seafood, though these bacteria can still be isolated from various seafood in many countries, indicating the steady potential for transmission to humans (Table 1).

2. Lactic acid bacteria in fish and seafood products

2.1. Lactic acid bacteria as natural contaminants

Usually, LAB are not considered as genuine micro-flora of the aquatic environment, but certain genera, including *Carnobacterium, Enterococcus, Lactobacillus* and *Lactococcus,* have been found associated in fresh and sea water fresh fish (Table 2).

LAB have also been isolated from processed aquatic food products such as lightly preserved fish products (LPFP) and semi-preserved fish products (SPFP). The LPFP category includes fish products preserved by low levels of salt (<6% [w/w] NaCl in the aqueous phase) and, for some products, the addition of preservatives (sorbate, benzoate, NO_2, or smoke) plays some role. The pH of these products is relatively high (>5.0), and they often are packaged under vacuum and need to be stored and distributed at low cooling temperatures (<5 °C). This is a group of high-value delicacy products (cold-smoked, pickled ["gravad"], or marinated fish, brined shellfish) that are typically consumed as ready-to-eat products, without any heat treatment (Mejlholm & Dalgaard, 2007; Mejlholm et al., 2008). LAB dominating vacuum-packaged cold-smoked fish products include the genera of *Lactobacillus*, *Lactococcus, Leuconostoc* and *Carnobacterium* (Gancel, Dzierszinski, & Tailliez, 1997). Many studies have shown that carnobacteria are quite common in chilled fresh and lightly preserved seafood, but at higher storage temperatures (15–25 °C),

Table 1
Bacterial hazards in fish and seafood products – a survey.

Bacteria	Product identified	References
Aeromonas spp.	Fish, shellfish	Fernandes, Flick, & Thomas, 1998; Isonhood & Drake, 2002
Clostridium botulinum Type E	Spores on surface, in intestine, on gills (trout, herring, salmon); vacuum packaged smoked fish products, cans, fermented fish, salted fish	Haagsma, 1991; Hatheway, 1995; Sramova & Benes, 1998; Johnson, 2000
Cl. perfringens	Cod, tuna salad, boiled salmon	Hewitt et al., 1986; Khatib et al., 1994; Aschfalk & Muller, 2002
Escherichia coli	Fresh fish, tuna paste, salted salmon roe, processed seafood	Ayulo, Machado, & Scussel, 1994; Calo-Mata et al., 2008; Asai et al., 1999; Mitsuda et al., 1998; Semanchek & Golden, 1998; Pierard et al., 1999;
Listeria monocytogenes	Ubiquitous, 3–10% human carriers; rarely in seawater or seawater fish, more frequently in freshwater and aquaculture fish, cold smoked products, salted fish products, hot smoked products, raw fish, prawns, mussels, oysters	Hoffman, Gall, Norton, & Wiedmann, 2003; Thimothe, Nightingale, Gall, Scott, & Wiedmann, 2004; Alves, De Martinis, Destro, Vogel, & Gram, 2005; Gudmundsdöttir et al., 2005; Miettinen & Wirtanen, 2005; Beaufort et al., 2007; Calo-Mata et al., 2008; Zunabovic, Domig, & Kneifel, 2011
Salmonella spp.	In intestine (tilapia and carp); prawns, mollusks, alaska pollack; eel and catfish, smoked eel, smoked halibut, dried anchovy	Heinitz, Ruble, Wagner & Tatini, 2000; Ling, Goh, Wang, Neo & Chua, 2002; Olgunoğlu, 2012
Staphylococcus aureus	Contamination from infected persons, fresh fish and fish fillets (*Cynoscion leiarchus*), smoked fish	Ayulo et al., 1994; Eklund, Peterson, Poysky, Paranjpye, & Pelroy, 2004
Vibrio parahaemolyticus	Shellfish, crustaceans on the skin, gills, intestine, fish-balls, fried mackerel (*Scomber scombrus*), tuna (*Thunnus thynnus*), and sardines (*Sardina pilchardus*),	Baffone, Pianei, Bruscolini, Barbieri, & Cierio, 2000; Calo-Mata et al., 2008; Daniels et al., 2000; IDSC, 1997
V. cholera Serovar O1 and O139	Prawns, shellfish, squid, seafood, uncooked fish marinade seviche (*Cilus gilberti*)	Kam, Leung, Ho, Ho, & Saw, 1995; Calo-Mata et al., 2008

Table 2
Reports on lactic acid bacteria isolated from fish.

Lactic acid bacteria	Product identified	References
Lactobacillus spp.	Arctic charr Atlantic cod Atlantic salmon Brown trout Herring Sturgeon fish Various fish	Kvasnikov, Kovalenko, & Materinskaya, 1977; Schrøder, Clausen, Sandberg, & Raa, 1980; Strøm & Olafsen, 1990; Olsen, Aagnes, & Mathiesen, 1994; Ringø, Bendiksen, Gausen, Sundsfjord, & Olsen, 1998; Ringø & Gatesoupe, 1998; Westerdahl, Joborn, Olsson, Kjelleberg, & Conway, 1998; Gonzalez, Enicinas, Garcıa Lopez, & Otero, 2000; Ringo, 2004; Bucio, Hartemink, Schrama, Verreth, & Rombouts, 2006; Ghanbari, Rezaei, Jami, & Nazari, 2009.
Carnobacterium spp.	Arctic charr Rainbow trout Brown trout Various fish	Ringø & Gatesoupe, 1998; González et al., 2000; Jöborn, Dorsch, Olsson, Westerdahl, & Kjelleberg, 1999; Gonzalez et al., 2000; Ringo, 2004
Aerococcus spp.	Atlantic salmon	Westerdahl et al., 1998; Ringo, 2004
Enterococcus spp.	Common carp Brown trout	Kvasnikov et al., 1977; Cai, Suyanandana, Saman, & Benno, 1999; Gonzalez et al., 2000; Ringo, 2004; Campos, Rodríguez, Calo-Mata, Prado & Barros-Velazquez, 2006
Lactococcus spp.	Common carp Brown trout	Cai et al., 1999; Gonzalez et al., 2000; Campos et al., 2006
Leuconostoc spp.	Arctic charr	Ringo, 2004
Pediococcus ssp.	Common carp, Rohu	Cai et al., 1999; Halami, Chandrashekar, & Joseph, 1999
Streptococcus spp.	Arctic charr Atlantic salmon Carp, Eel, European Eel, Japanese Goldfish Rainbow trout Various salmonids Turbot Yellowtail	Ringø & Olsen, 1999; Ringø et al., 2000; Ringo, 2004
Vagococcus spp.	Brown trout	Gonzalez et al., 2000
Weissella hellencia	Flounder	Byun, Park, Benno, & Oh, 1997

other species including *Enterococcus* spp. could dominate the microbial spoilage community of seafood (Dalgaard et al., 2003; Emborg et al., 2002).

Fish products with a high salt content (>6% NaCl in aqueous phase) or with a pH below 5.0 and to which preservatives (benzoate, sorbate, nitrate) are added are defined as "semi-preserved" (Mejlholm et al., 2008). Typically, the European products (e.g., salted and/or marinated herring, anchovies, caviar) are distributed at cooled temperatures (<10 °C). In marinated or dried fish, salted and fermented fish, the lactic acid microflora can be quite diverse, since the presence of lactobacilli and pediococci has been reported. Table 3 summarizes the most relevant species isolated from different ready-to-eat seafood products.

2.2. Biopreservation using lactic acid bacteria

Biopreservation of fish and seafood products is an alternative to meet safety standards and to control microbial deterioration without negative impact on the sensory quality of the product. The selection of LAB possessing the GRAS (generally recognized as safe) status (US Food and Drug Administration) as protective cultures is generally agreed as beneficial for extending the shelf-life of seafood products (Calo-Mata et al., 2008; Leroi, 2010). Likewise, they also fulfil the QPS (qualified presumption of safety) requirements (EFSA, 2007). Seafood-borne LAB are often able to grow even at refrigerated temperatures and are compatible to the seafood environment (modified-atmosphere packaging, low pH, high salt concentrations, presence of additives like, e.g., lactic acid or acetic acid). Importantly, their growth can also suppress more potent spoilage germs by means of antagonistic and inhibitory activities (either through the competition for nutrients or the production of one or more antimicrobially active metabolites; Nilsson, 1997; Nilsson, Gram, & Huss, 1999; Nilsson et al., 2005; Nes, 2011). Hence LAB usually meet the necessary requirements for biopreservation of seafood products, which are dealt with below.

2.2.1. Requirements of biopreservation cultures

When using live microbial antagonists in seafood biopreservation, there are a number of criteria and requirements, which must be taken into account (Calo-Mata et al., 2008; Leroi, 2010). Primarily, consumer protection is the most important aspect, in particular in terms of ready-to-eat seafood, but also for other types of this food category since cross-contamination, both at the retail and consumer level, is possible. Furthermore, the extent to which such protective cultures or their metabolites may affect the sensory attributes of the seafood product should be taken into consideration. Hence, protective cultures should not cause any detrimental effects on the target food. Since certain LAB may also contribute to spoilage or at least to some degradation of food ingredients in a number of foods, it is essential to consider their effect on chemical, physical and sensory quality parameters (Castellano, Gonzalez, Carduza, & Vignolo, 2010). Another important requirement for a successful application of protective cultures is their ability to produce sufficiently active antagonistic metabolites against a broad spectrum of relevant food-borne pathogen and/or spoilage bacteria and fungi. In addition, the capability of surviving adverse conditions encountered during technological treatments and maintaining inhibitory activities during storage are of great significance. In general, the most relevant requirements of biopreservative agents can be summarized according to Fig. 1.

2.2.2. Antimicrobial components from lactic acid bacteria

Table 4 presents a survey of the diversity of antimicrobials produced by LAB. The most relevant antimicrobial substance produced by LAB is lactic acid and the concomitant reduction of pH (Stiles, 1996). Lactic acid is also produced by other genera (e.g. *Brochothrix*). The antimicrobial effect of organic acids in food ecosystem lies in the reduction of pH, as well as the nature of the un-dissociated form of the organic acid, which inhibits the growth of unwanted microorganisms (Gould, 1991). In addition, LAB produce various other antimicrobial compounds, which can be classified as low-molecular-mass (LMM) compounds such as hydrogen peroxide (H_2O_2), carbon dioxide (CO_2), diacetyl (2,3-butanedione), and high-molecular-mass (HMM) compounds like bacteriocins

Table 3
Reports on lactic acid bacteria isolated from ready-to-eat seafood products.

Product type	Lactic acid bacteria	References
Brine shrimp	*Aerococcus viridans*	Dalgaard & Jørgensen, 2000; Dalgaard et al., 2003
	Carnobacterium divergens	
	C. maltaromaticum,	Mejlholm, Bøknæs, & Dalgaard, 2005
	C. spp.	Dalgaard & Jørgensen, 2000; Dalgaard et al., 2003
	Enterococcus faecalis	Dalgaard & Jørgensen, 2000; Dalgaard et al., 2003; Mejlholm & Dalgaard, 2007
	Ent. gallinarum	Dalgaard & Jørgensen, 2000; Dalgaard et al., 2003
	Ent. malodoratus	Mejlholm & Dalgaard, 2007
	Lactobacillus curvatus	Dalgaard & Jørgensen, 2000; Dalgaard et al., 2003
	Lb. spp.	From & Huss, 1990
	Lb. sakei	Mejlholm & Dalgaard, 2007
	Lactococcus garvieae	Dalgaard & Jørgensen, 2000; Dalgaard et al., 2003
	Streptococcus sp.	From & Huss, 1990
Cold smoked fish	*C. divergens*	Leroi, Joffraud, Chevalier, & Cardinal, 1998
	C. piscicola/maltaromaticum	Paludan-Müller, Dalgaard, Huss, & Gram 1998; Leroi et al., 1998; Gonzalez-Rodriguez, Sanz, Santos, Otero, & Garcia-Lopez, 2002; Olofsson, Ahrné, & Molin, 2007
	Ent. faecalis	Gonzalez-Rodriguez et al., 2002
	Ent. spp.	Lyhs, Björkroth, Hyytiä, & Korkeala, 1998
	Lb. alimentarius	Leroi et al., 1998
	Lb. casei ssp. *tolerans*	Gonzalez-Rodriguez et al., 2002
	Lb. coryneformis	
	Lb. curvatus	Truelstrup, Hansen, & Huss, 1998; Lyhs, Björkroth, & Korkeala, 1999; Jorgensen, Dalgaard, & Huss, 2000; Gonzalez-Rodriguez et al., 2002
	Lb. delbrueckii ssp. *delbrueckii*	Gonzalez-Rodriguez et al., 2002
	Lb. farciminis	Leroi et al., 1998
	Lb. homohiochii	Gonzalez-Rodriguez et al., 2002
	Lb. plantarum	Gancel et al., 1997; Hansen & Huss, 1998; Lyhs et al., 1999; Gonzalez-Rodriguez et al., 2002
	Lb. pentosus	Gancel et al., 1997
	Lb. sakei	Leroi et al., 1998; Truelstrup Hansen & Huss, 1998; Lyhs et al., 1999; Jorgensen et al., 2000; Gonzalez-Rodriguez et al., 2002
	Lc. spp.	Paludan-Müller et al., 1998
	Leuconostoc carnosum	Hansen & Huss, 1998
	Leuc. citreum	Lyhs et al., 1999
	Leuc. gelidum	Hansen & Huss, 1998
	Leuc. mesenteroides	Hansen & Huss, 1998; Lyhs et al., 1999
	Weissella kandleri	Gonzalez-Rodriguez et al., 2002
Fermented fish	*Lb. acidophilus*	Tanasupawat, Shida, Okada & Komagata, 2000
	Lb. brevis	Lee, Jun, Ha, & Paik, 2000
	Lb. pentosus	Paludan-Müller et al., 1998; Tanasupawat, Okada & Komagata, 1998
	Lb. plantarum	Tanasupawat et al., 1998
	Lc. lactis	Lee et al., 2000
	Lc. lactis ssp. *lactis*	Paludan-Müller et al., 1998
	Leuc. citreum	
	Pediococcus pentosaceus	
Salted, marinated or dried fish	*C.* spp.	Basby, Jeppesen, & Huss, 1998
	Ent. faecalis, Ent. faecium	Thapa, Pal, & Tamang, 2006
	Lb. alimentarius, Lb. buchneri	Lyhs, Korkeala, & Björkroth, 2002
	Lb. delbrueckii ssp. *lactis*	
	Lb. plantarum	
	Lc. lactis	Thapa et al., 2006
	Leuc. mesenteroides	
	Ped. pentosaceus, W. confusa	
Seafood salad	*C. piscicola*	Andrighetto et al., 2009
	Ent. spp., *Ent. faecalis,*	
	Lb. curvatus	
	Lb. malfermentans	
	Lb. paraplantarum	
	Lb. sanfranciscensis	
	Lc. lactis	
	Leuc. mesenteroides	
	Leuc. pseudomesenteroides	
	Ped. spp.	
	Str. parauberis	
	Vagococcus spp.	
	W. spp.	
Sugar-salted (Gravad) fish	*C. divergens, C. piscicola*	Lyhs et al., 2002
	Lb. curvatus ssp. *melibiosus*	
	Lb. curvatus ssp. *curvatus*	
	Lb. curvatus, Lb sakei	Leisner, Millan, Huss, & Larsen, 1994
	Lb. sakei	Jeppesen and Huss, 1993; Lyhs et al., 2002
	Leuc. spp.	Leisner et al., 1994
	W. viridescens	

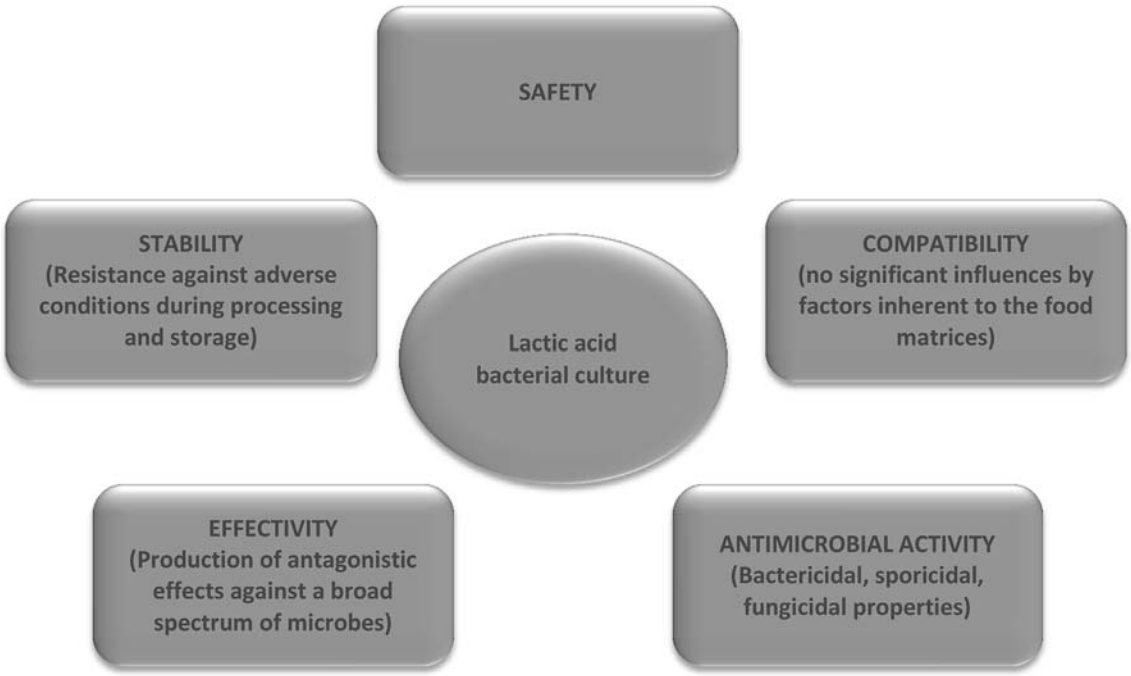

Fig. 1. Principles of potential LAB protective cultures and/or their inhibitory metabolites.

(Nes, 2011). The production of bacteriocins by LAB is very significant for applications in food systems and thus, unsurprisingly, these have been most extensively investigated. Among numerous bacteriocins so far characterized, nisin is best defined, and the only purified bacteriocin preparation approved for use in food products (Cleveland et al., 2001; Gálvez et al., 2007; Garcia et al., 2010; Nes, 2011; Nilsson, 1997; Nilsson et al., 1999).

2.2.3. Understanding LAB adaptation to stressful environments

Seafood biopreservation strategies reinforce the need for having robust LAB since they have to survive steps of food processing, to resist the food environment and to express specific functions under unfavourable conditions. The ability to quickly respond to stress is essential for survival, and it is now well established that LAB, like other bacteria, have evolved defence mechanisms against stress

Table 4
Antimicrobials produced by lactic acid bacteria- a concise survey.

Antimicrobial	Category	Subcategory	Remarks	Examples	References
Bacteriocin	Class I Lantibiotics, lanthionine containing, Class II Non-modified heat stable bacteriocins	Type A	Elongated molecules, molecular mass 2–5 kDa,	Nisin A	Gross & Morell, 1971
		Type B	Globular molecules; molecular mass 1.8–2.1 kDa	Mersacidin	Niu & Neu, 1991
		Subclass IIa	Pediocin like bacteriocins, listericidal, small (<10 kDa), narrow spectrum bacteriocins	Pediocin PA-1/AcH Leucocin A	Henderson, Chopko, & Van Wasserman, 1992; Motlagh et al., 1992; Hasting et al., 1991
		Subclass IIb	Two peptide bacteriocins, small (<10 kDa)	Lactococcin G Lactacin F	Nissen-Meyer, Holo, Håvarstein, Sletten, & Nes, 1992; Alison, Frémaux, & Klaenhammer, 1994
		Subclass IIc	Cyclic bacteriocins, small (<10 kDa)	Entrocin AS-48 Gassericin A Lactocyclicin Q	Samyn et al., 1994 Kawai, Saito, Kitazawa, & Itoh, 1998 Sawa et al., 2009
		Subclass IId	Non-pediocin single linear bacteriocin, small (<10 kDa)	Lactococcin A Lacticin Q	Holo, Nilssen, & Nes, 1991; Fujita, Ichimasa, Zendo, Koga, & Yoneyama, 2007
	Bacteriolysins		Non-bacteriocin lytic proteins, large, heat labile proteins, often murein hydrolases	Entrolysin A	Nilsen, Nes, & Holo, 2003
Organic acid	Lactic acid	Major metabolite of LAB fermentation. Active against putrefactive and Gram-negative bacteria, some fungi			Woolford 1975; Lindgren & Dobrogosz, 1990
	Acetic and propionic acids	More antimicrobially effective than lactic acid. Active against Putrefactive bacteria, clostridia. some yeasts and fungi			Ahamad & Marth 1989; Wong & Chen 1988; Richards, Xing, & King, 1995
Other	Hydrogen peroxide	Active against pathogens and psychotropic spoilage organisms e.g. *Staphylococcus aureus*, *Pseudomonas* sp.			Davidson, Post, Braner, & McCurdy, 1983; Cords & Dychdala, 1993
	Carbon dioxide	Active against Gram positive and specially Gram-negative psychrotrophic bacteria e.g. *Enterobacteriaceae* and *Listeria*			Devlieghere & Debevre, 2000; Ouwehand & Vesterlund, 2004
	Diacetyl	Active against Gram positive and Gram-negative bacteria e.g. *Listeria, Salmonella, Yersinia, E. coli*, and *Aeromonas*			Jay, 1982; Ouwehand & Vesterlund, 2004
	Fatty acids	Active against Gram-positive bacteria and some fungi			Gould, 1991
	Reuterin	Active against broad spectrum of Gram-positive and Gram-negative bacteria, yeast, fungi and protozoa e.g. species of *Salmonella, Shigella, Clostridium, Staphylococcus, Listeria, Candida*, and *Trypanosoma*			Axelsson, Chung, Dobrogosz, & Lindgren, 1989

that allow them to withstand harsh conditions and sudden environmental changes. While genes implicated in stress responses are numerous, in LAB the characterization of their actual role and regulation differs widely between species (Champomier -Vergès, Zagorec, & Fadda, 2010). Indeed, The functional conservation of several stress proteins (for example, HS proteins, Csp etc.) and some of their regulators (e.g. HrcA, CtsR) render even more striking the potentials of LAB for use in biopreservation (van de Guchte et al., 2002).

Cold acclimation of selected LAB constitutes a real advantage in bacterial competition against spoilage and pathogenic psychrotrophic bacteria. These LAB can rapidly adapt to a temperature downshift and can continue to grow at a reduced rate after a temperature downshift to about 20 °C below the optimal growth temperature. Although more important temperature downshifts lead to growth arrest, there are some LAB isolates, which are compatible with this situation (Garnier et al., 2010). To withstand cooling temperature, LAB have developed a cold-shock response, which is based on the synthesis of a number of cold-induced proteins (CIPs). Most proteins include a family of closely related low-molecular weight (~7.5 kDa) proteins termed cold-shock proteins (CSP) (Phadtare, 2004). CSP proteins or corresponding genes, as part of the cold-adaptive response, were detected and/or identified in several strains of LAB such as *Lactobacillus lactis* ssp. *cremoris*, *Streptococcus thermophilus*, *Pediococcus pentosaceus*, *Enterococcus faecalis*, *Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus plantarum* and *Streptococcus pyogenes* (van de Guchte et al., 2002). Similar to other Gram positive bacteria, heat shock response in LAB is characterized by the induction of a set of heat shock proteins (HSP) (Champomier-Vergès et al., 2010). By a proteomic approach using 2D PAGE gels, heat shock treatment of *Lactococcus lactis* strongly induced DnaK, GroEL, and GroES (Auffray, Gansel, Thammavongs, & Boutibonnes, 1992; Whitaker & Batt, 1991). These proteins are well-known chaperones, which hold up folding and maturation of emerging or denatured proteins (Kaufman, 1999).

In LAB three types of starvation conditions (i.e. carbohydrate starvation, phosphate starvation, and nitrogen [amino acids] starvation) have been largely studied, but the majority of the reports relates to carbohydrate starvation. Using 2D-electrophoresis it has been established that starvation induced the synthesis of specific proteins in LAB. In *Lb. acidophilus*, 16 proteins are induced in stationary phase (Hartke, Bouche, Gansel, Boutibonnes, & Auffray, 1994).

2.3. Application in seafood and seafood products

Since some psychrotrophic pathogenic and spoilage bacteria can survive and grow in lightly preserved fish products, research has been conducted on this subject. Next to the raw bivalve molluscs, LPFP and SPFP constitute the most dangerous group of fish products. Almost any of the pathogenic organisms listed in Table 1 may be transferred via these products. Particularly, the presence and growth of *L. monocytogenes* is of major concern. *L. monocytogenes* has frequently been isolated from LPFP like cold smoked salmon (CSS). This organism is a frequent contaminant in the raw material: it survives the salting and cold-smoking (<30 °C) process and it is able to grow in the final product at chilling temperature (Table 1). In this regard, the use of lactic acid bacteria isolated from seafood and/or their bacteriocins have proved successful in preventing or delaying the growth of this pathogen under conditions mimicking those existing in lightly preserved fish products (Table 5).

Based on the reports on the application of LAB protective culture and/or their bacteriocins in seafood products, the recognized strengths of biopreservative agents can be summarized as follows: (1) promoting extended shelf life of seafood during storage time, (2) contributing to decreased risk for transmission of foodborne pathogens in LPFP and SPFP, (3) amelioration of economic losses due to seafood spoilage, (4) allowing reduced application of chemical preservatives and drastic physical treatments such as heating, refrigeration, etc. also causing better preservation nutritional quality of food, (5) good option for the industry due to cost effective technology, and (6) a proper response to consumer demands for minimally processed, safe, preservative-free foods (Gálvez et al., 2007).

However, there are also some drawbacks that must be considered. The effectiveness of protective cultures and/or their inhibitory compounds in food can be limited by a range of factors such as the narrow activity spectrum of some agents, the spontaneous loss of bacteriocinogenicity (genetic instability), limited diffusion behaviour in solid matrices, inactivation through proteolytic enzymes or binding to food ingredients such as lipids, poor adaptation of the culture to (refrigerated) food environments, low production levels and the emergence of bacteriocin-resistant bacteria. In addition, most LAB protective cultures are not capable of surviving commercial heat treatment processes and therefore usually are added by dipping or spraying only after heat treatment. This increases the costs and bears the problem of product supplementation with viable microbial cells (Devlieghere et al., 2004).

3. Regulatory issues in biopreservation of seafood and seafood products

At present, three major challenges severely restrict the application of seafood biopreservatives: (1) issues relating to microbial safety, (2) regulatory aspects, and (3) the formulation of microorganism.

In general, there are stringent regulatory requirements for the use of naturally-occurring antimicrobial cultures and substances, such as bacteriocins, in food preservation. As one of the key regulatory aspects, protective cultures selected for biopreservation need to take into account their impact on the nutritional and sensory features of perishable products. Presumably, this is one of the main reasons for which in spite of more studies in other food sectors, the information on the application of LAB or bacteriocins to seafood is limited. In case of protective cultures, safety aspects (e.g., production of histamine) of the bioprotective bacteria should be considered. However, it is important to note that in seafood the production of histamine connected to bioprotective LAB has not been reported so far. Furthermore, promising strains of LAB selected for seafood preservation did not exhibit any problematic resistance to antibiotics nor possess potential cytotoxicity (Pilet & Leroi, 2011).

Using the direct application of bacteriocins to seafood, likewise, regulatory aspects of these substances must be met. In analogy, bacteriocins should not exert deleterious effects on sensory properties of the foods. The bacteriocin has to be stable during storage and, if the activity depends upon residual concentrations, it has to be consistent throughout the shelf-life of the food. Approved by the U.S. Food and Drug Administration (FDA) in 1988 for use in pasteurized processed cheese spreads, nisin is currently the only purified bacteriocin approved for food use in the U.S, which is used for fish and seafood product packaging in the form of coated or impregnated film (Jones, Salin, & Williams, 2005).

Although encouraging results had been achieved in controlling the growth of *L. monocytogenes* by using LAB protective cultures or/and their bacteriocins in seafood, so far potential bioprotective bacterial formulations for seafood products are not included in the list of micro-organisms judged suitable for QPS status (Leroi, 2010).

Table 5
Survey of literature dealing with biopreservation of fish and seafood products.

Product	Protective culture/bacteriocin employed	Reported effects	References
Fish fillet			
Catfish	*Lc. lactis* ssp. cremoris ATCC 19257	Improved odour and appearance	Kim & Hearnsberger, 1994
Catfish	*Bifidobacterium adolescentis, Bif. infantis*, or *Bif. longum*	Extended shelf-life	Kim, Hearnsberger, Vickery, White, & Marshall, 1995
Horse Mackerel	*Ped.* spp. (Bac+, Bac-)	Improved sensory quality	Cosansu, Mol, Ucok Alakavuk, & Tosun, 2011
Indian mackerel	*Ped. acidilactici, Ped. pentosaceous, Str. thermophilus, Lc. lactis, Lb. plantarum, Lb. acidophilus and Lb. helveticus.*	Controlled spoilage bacteria and amines	Sudalayandi & Manja, 2011
Rainbow trout	nisin-containing aqueous solution of *Lc. lactis* ssp. *lactis* NCFB 497	No effect	Kisla & Ünlütürk, 2004
Salmon	*Lb. sakei* LAD and *Lb. alimentarius* BJ33	Improve sensory attributes	Morzel, Fransen, & Arendt, 1997
Sardine	Nisin	Inhibited fish spoilage flora	Elotmani & Assobhei, 2004
Tilapia	*Lb. casei* DSM 120011 (A) and *Lb. acidophilus* 1M	Improved biochemical quality criteria and microbial aspects	Ibrahim & Salha, 2009
Tilapia	*Lb. casei* DSM 120011 and *Lb. acidophilus*	Extended shelf-life and safety	Daboor & Ibrahim, 2008
Turbot, VP and MAP	EntP-producing enterococci	Anti-listerial, anti-staphylococcal, and anti-bacilli	Campos et al., 2012
VP fresh plaice	*Bif. bifidum*	Inhibited *Pseudomonas* spp. and *Pseudomonas phosphoreum*	Altieri, Speranza, Del Nobile & Sinigaglia, 2005
VP rainbow trout	*Lb. sakei* CECT 4808 and *Lb. curvatus* CECT 904T	Extended shelf-life	Katikou, Ambrosiadis, Koidis & Georgakis, 2007
VP rainbow trout	Sakacin A-producing strain of *Lb. sakei* (Lb706)	Inhibited *L. monocytogenes*	Aras Husar, Kaban, Hisar, Yanik, & Kaya, 2005
Cold smoked fish			
CO_2 packed cold smoked salmon	Nisin	Reduced *L. monocytogenes*	Nilsson, 1997; Nilsson et al., 1999.
Cold smoked salmon	Sakacin P	Inhibited *L. monocytogenes*	Aasen et al., 2003
Cold smoked salmon	*C. maltaromaticum* CS526	Inhibited *L. monocytogenes*	Yamazaki, Suzuki, Kawai, Inoue, & Montville, 2003
Cold smoked salmon	*C. divergens* V41 *C. divergens* V1 *C. divergens* SF668	Inhibited *L. monocytogenes*	Brillet, Pilet, Prévost, Cardinal, & Leroi, 2005
Cold smoked salmon	*Lb. sakei*	Inhibited *L. innocua*	Weiss & Hammes, 2006
Cold smoked salmon	*Lb. casei*, *Lb. plantarum* and *C. maltaromaticum*	Inhibited innocua	Vescovo, Scolari, & Zacconi, 2006
Cold smoked salmon	*Lb. casei* T3 and *Lb. plantarum* PE2	Inhibited *L. innocua*	Vescovo et al., 2006
Cold smoked salmon	*Ent. faecium* ET05	Inhibited *L. innocua*	Tomé, Pereira, Lopes, Gibbs, & Teixeira, 2008
Cold smoked salmon	*C. divergens* M35 (bac+)	Inhibited *L. monocytogenes*	Tahiri, Desbiens, Kheadr, & Lacroix, 2009
VP cold smoked salmon	*C.* spp.	Improve sensory characteristics	Leroi, Arbey, Joffraud, & Chevalier, 1996
VP cold smoked salmon	*C. piscicola* V1, *C. divergens* V41 and Divercin V41,	Inhibited *L. monocytogenes*	Duffes, Corre, Leroi, Dousset, & Boyaval, 1999; Duffes, Leroi, Boyaval, & Dousset, 1999; Nilsson et al., 2004
VP cold-smoked rainbow trout	Nisin	Inhibited *L. monocytogenes*	Nykanen, Weckman, & Lapvetelainen, 2000
VP cold-smoked salmon	Sakacin P-producing *Lb. sakei* and Sakacin P	Inhibited *L. monocytogenes*	Katla et al., 2001
Shrimp			
Brine shrimp	Nisin Z, Carnocin UI49 and crude Bavaricin A	Improved quality and extended shelf life	Einarsson & Lauzon, 1995
Chilled shrimp	Nisin	Inhibited *Pseudomonas* spp. and H_2S producing bacteria	Shirazinejad, Noryati, Rosma, & Darah, 2010
Cooked shrimp	*Lc. piscium* CNCM I-4031	Inhibited *Brochothrix thermosphacta* and improved sensory indices	Fall, Leroi, Cardinal, Chevalier, & Pilet, 2010
Cooked shrimps	*C. maltaromaticum*	No effect	Laursen et al., 2005
VP cooked shrimp	*Lc. piscium* EU2241 and *Leuc. gelidum* EU2247	Inhibited *L. monocytogenes* and *Staph. aureus*	Matamoros et al., 2009

Comprehensive proof of antagonistic effects, precise taxonomic data and strong evidence in terms of safety are still needed for obtaining this status.

4. Conclusion and future perspectives

During the last 20 years, research activities were undertaken to study the application of protective cultures as biopreservatives in seafood matrices. Either deliberately added or produced *in-situ*, this strategy has been discovered to play a potential role in the control of undesirable microorganisms. In addition, the establishment of beneficial bacterial populations is promoted. However, LAB based antagonisms do not necessarily alleviate practical food safety issues in general, as they may be efficient only in a narrow range of food environment (pH, fat content, etc.) and this limits their application in many seafood products. Thus, a case-to-case consideration of applying such a bioprotectant to a certain single food matrix is essential.

It is expected that performing additional studies to select appropriate LAB strains and corresponding combinations to limit the growth of both the pathogenic and spoilage microflora and, furthermore, investigating of the individual nature of the strains and the mechanisms underlying the inhibitory potential will definitively result in the optimization of biopreservation.

Novel techniques such as genomics, proteomics, metabolomics, and system biology, will open up new avenues for the in-depth interpretation of biological data and may enable the development of predictive models estimating safety and shelf life issues of seafood products. By combining classical with molecular tools these new methods possess some big potential in exploring and designing valuable LAB functions allowing to develop not only safer traditional but also innovative seafood products.

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CHAPTER 3

Antimicrobial activity and partial characterization of bacteriocins produced by lactobacilli isolated from Sturgeon fish

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ABSTRACT

The antimicrobial spectrum and physico-chemical characteristics of bacteriocin like inhibitory substances produced by lactobacilli isolated from the intestinal flora of Sturgeon fish were determined in order to evaluate their inhibitory potential exerted against 42 food-borne and aquaculture-related bacterial pathogens as well as against food spoilage causing bacteria. In a first series a collection of 84 *Lactobacillus* strains previously isolated from Beluga (*Huso huso*) and Persian sturgeon (*Acipenser persicus*) were screened for their inhibitory activities and potential bacteriocin production against two indicator strains, *Listeria monocytogenes* ATCC 19115 and *Salmonella enterica* subsp. *enterica* serovar Typhimurium ATCC 14028. The isolates *Lactobacillus casei* AP8 and *Lactobacillus plantarum* H5 showed the highest activity and therefore were subjected to further examination to clarify the nature of the inhibitory effect. The physico-chemical properties of the harvested antimicrobial compounds were similar to those of bacteriocins of lactobacilli belonging to the group II with respect to molecular weight (5 and 3 kDa respectively), pronounced temperature stability (−20 °C to 120 °C), pH tolerance (3–12), chemical stability (SDS, EDTA, Tween 20, Tween 80) and sensitivity to proteolytic enzymes. Importantly, different food borne pathogens like *Escherichia coli*, *Listeria* spp., *Salmonella* spp., *Staphylococcus aureus*, *Aeromonas hydrophila*, *Vibrio anguillarum*, and *Bacillus cereus* were inhibited by cell-free supernatants of the strains selected. The broad inhibitory spectrum, the technological properties, especially the stability may lead to the assumption that the bacteriocins like inhibitory AP8 and H5 may be applied as biopreservative agents to control pathogens and spoiling bacteria in different food products. Furthermore their role as bioprotective agents in aquaculture systems is envisaged.

1. Introduction

It has been estimated that in industrialized countries annually as many as 30% of people suffer from a food-borne disease. In 2000 at least two million people even died from diarrhoeal disease worldwide (Gálvez, Abriouel, López, & Ben Omar, 2007). Evidently, indigenous bacteria present in marine environment or resulting from post-contamination during processing and packaging play some important role in some of these cases (Lewus, Sun, & Montville, 1992; Okafor, 1985; Pinto et al., 2009). Hence, despite ongoing progress in seafood production, seafood safety is still an important public health issue. Interestingly, traditional treatments like salting, smoking and canning and applied to seafood have disappeared in favour of the establishment of mild technologies involving lower salt concentrations, lower heating temperature and vacuum (VP) or modified atmosphere packing (MAP). Most of these treatments are usually not sufficient to fully inactivate microorganisms. In some cases psychrotolerant pathogens such as *Listeria monocytogenes* or spoilage-causing bacteria are able to develop during prolonged shelf-life of these products (Leroi, 2010; Todorov, Ho, Vaz-Velho, & Dicks, 2010). As several of these products are eaten raw, it is therefore essential that adequate precautious and preservation technologies are applied to guarantee their safety and quality. Among alternative preservation technologies, particular attention has been paid to biopreservation in order to extend the shelf-life and to enhance the hygienic status of products like seafood, thereby minimizing the impact on nutritional and sensorial properties (Lewus et al., 1992). In this context, lactic acid bacteria (LAB) possess a major potential in biopreservation. Besides the long history of consumption, which proves the safety of consuming lactic acid bacteria, they often naturally dominate the micro-flora of

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many foods and may act as powerful competitors/inhibitors to contaminating spoilage microorganisms. Their antagonistic and inhibitory properties are based on different properties. Among these, the competition for nutrients and the production of one or more antimicrobial metabolites such as organic acids (lactic and acetic acid), hydrogen peroxide, and antimicrobial peptides like bacteriocins are relevant (Cleveland, Montville, Nes, & Chikindas, 2001; Holzapfel, Geisen, & Schillinger, 1995; Kandler & Weiss, 1986; Maragkoudakis et al., 2006). Bacteriocins are ribosomally synthesized peptides that exert their antimicrobial activity against either strains of the same species as the bacteriocin producer (narrow range), or even to more distantly related species (broad range) (Belguesmia, Naghmouchi, & Chihib, 2011; Galvez, Lopez, Abriouel, Valdivia, & Omar, 2008). It has been noted that a pronounced number of lactic acid bacterial strains of different species and archaea may produce bacteriocins (Belguesmia et al., 2011; Bhattacharya & Das, 2010; Campos, Rodríguez, Calo-Mata, Prado, & Barros-Velázquez, 2006; Chahad, El Bour, Calo-Mata, Boudabous, & Barros-Velàzquez, 2012). Practically, many LAB based bacteriocins exert their activity at nanomolar concentrations and against a number of bacterial pathogens (Chahad et al., 2012; Indira, Jayalakshmi, Gopalakrishnan, & Srinivasan, 2011; Leroi, 2010; Todorov et al., 2010). Even multidrug-resistant nosocomial pathogens such as methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococci (VRE) may be inhibited (Karska-Wysocki, Bazo, & Smoragiewicz, 2010; Mojgani & Amirinia, 2007). Therefore, this property offers some interesting application potential in medical and veterinary applications.

Although fermented food and feed have been known as a well-known source for bacteriocin-producing LAB, isolates from the intestinal tract of animals and humans have become an increasingly important source for protective cultures. In fish LAB are naturally present, and the bio-protective potential of some strains and/or their bacteriocin has been highlighted in the last years (Bucio, Hartemink, Schrama, Verreth, & Rombouts, 2006; Campos et al., 2006; Ghanbari, Rezaei, Jami, & Nazari, 2009; Ghanbari, Rezaei, Soltani, & Shahosseini, 2009; Itoi et al., 2008). However, in comparison with other food products of dairy or meat origin, only few bacteriocinogenic LAB strains have been recovered from seafood. In a previous study we have isolated and described a number of *Lactobacillus* strains originating from the intestinal microbiota of two sturgeon species from the Caspian Sea (Ghanbari, Rezaei, Jami, et al., 2009). The present study focuses on the characterization of antimicrobial properties produced by two lactobacilli isolates and their individual ability to inhibit the growth of food-borne pathogens, food spoilage bacteria and contaminants in aquaculture.

2. Materials and methods

2.1. Bacterial isolates and media

A total of 84 *Lactobacillus* strains isolated from two species of sturgeon fish inhabiting the Caspian Sea (Iran) were used in this study (Ghanbari, Rezaei, Jami, et al., 2009). The strains were stored at −80 °C in MRS broth (Merck, Germany) containing 15% (v/v) glycerol. Prior to further investigation, cultures strains were thawed at room temperature and cultivated successively in MRS broth for 24 h at 30 °C under anaerobic conditions. The indicator strains were obtained from different sources (Table 1) and kept frozen in 20% (v/v) glycerol at −20 °C and then cultivated under appropriate conditions (Table 1).

2.2. Screening of Lactobacillus strains for their inhibitory potential

In a first test series, the ability of each of the 84 *Lactobacillus* isolates to exert an antibacterial effect against *L. monocytogenes* ATCC 19115 and *Salmonella enterica* subsp. *enterica* serovar Typhimurium ATCC 14028 was examined by using three methods: the spot-on-lawn method, a standardized agar disc diffusion method and the well diffusion method as described by Benkerroum, Ghouati, Sandine, and Tantaoui-Elaraki (1993); Schillinger and Lücke (1989), and Tagg and McGiven (1971), respectively. Throughout, cell-free supernatants (CFS) of strains were obtained by centrifugation at 10,000× *g* for 20 min and then adjusted to pH 6.5 by applying NaOH to exclude the inhibitory effect of organic acids. Finally, the solution was filter-sterilized applying a pore-size of 0.2 μm (16534K, Sigma, UK).

Table 1
Antimicrobial activity of presumptive bacteriocins produced by *Lb. casei* AP8 and *Lb. plantarum* H5 as examined with selected bacterial indicator strains.

	Medium[b]	Temp. [°C]	Inhibitory activity[c]	
			AP8	H5
Gram-negative bacteria (source[a])				
Aeromonas hydrophilus MJ 1120	BHI	37	++	0
Aeromonas hydrophilus MJ 1240	BHI	37	+++	+
Aeromonas salmonicida CC 1546	BHI	37	+	+
Aeromonas salmonicida RT 7895	BHI	37	++	+
Brochothrix thermosphacta RF 35	BHI	37	++	+
Escherichia coli ATCC 25922	BHI	37	++	0
Escherichia coli PTCC 1325	BHI	37	++	++
Photobacterium damselae ssp. *piscicida* PC 35	BHI	37	0	0
Pseudomonas aeruginosa PTCC 1310	BHI	37	++	+
Pseudomonas fluorescens HFC 1236	BHI	37	++	0
Salmonella enteritidis ATCC 13076	BHI	37	++	++
Salmonella enterica ssp. *enterica* Typhimurium ATCC 14028	BHI	37	+++	+++
Salmonella typhi PTCC 1609	BHI	37	+++	++
Vibrio anguillarum MI 12	BHI	37	++	+
Vibrio parahaemolyticus MI 23	BHI	37	+++	0
Vibrio parahaemolyticus MI 56	BHI	37	+++	+
Gram-positive bacteria (source[a])				
Bacillus cereus ATCC 11778	BHI	37	+++	+++
Bacillus coagulans CH 7	BHI	37	+++	++
Bacillus licheniformis PTCC 1331	BHI	37	++	0
Bacillus atrophaeus ATCC 9372	BHI	37	+++	+
Clostridium perfringens ATCC 3624	RCM	37	++	+
Clostridium sporogenes PTCC 1265	RCM	37	++	+
Lactobacillus acidophilus ATCC 4356	MRS	30	++	+
Lactobacillus alimentarius AP 10	MRS	30	+	++
Lactobacillus brevis H 56	MRS	30	++	++
Lactobacillus brevis AP 83	MRS	30	++	++
Lactobacillus casei PTCC 1608	MRS	30	0	++
Lactobacillus casei RN 78	MRS	30	0	0
Lactobacillus casei LB 10	MRS	30	0	+
Lactobacillus casei LB 46	MRS	30	0	+
Lactobacillus plantarum PTCC 1050	MRS	30	0	0
Lactobacillus plantarum AP 76	MRS	30	+	0
Lactobacillus plantarum H12	MRS	30	+	0
Lactobacillus sakei AP 43	MRS	30	0	+
Lactobacillus sakei ssp. *sakei* PTCC 1712	MRS	30	0	0
Lactococcus lactis ssp. *lactis* PTCC 1403	MRS	30	+	0
Lactobacillus curvatus RF 5	MRS	30	0	+
Listeria innocua AN 15	BHI	37	++	++
Listeria monocytogenes ATCC 7644	BHI	37	+++	+++
Listeria monocytogenes PTCC 1163	BHI	37	++	++
Listeria monocytogenes PTCC1297	BHI	37	++	++
Listeria monocytogenes ATCC 19115	BHI	37	+++	+++
Staphylococcus aureus ssp. *aureus* ATCC 25923	BHI	37	+++	+
Staphylococcus aureus PTCC 1112	BHI	37	+++	+

[a] PTCC: Persian type culture collection; ATCC: American type culture collection.
[b] BHI: Brain heart infusion; MRS: De Man–Rogosa–Sharpe agar; RCM: Reinforced clostridial medium.
[c] 0: no inhibition zone; +: 1–5 mm inhibition zone; ++: 5–8 mm inhibition zone; +++: >8 mm inhibition zone.

Based on the screening tests, the inhibitory spectrum of potential bacteriocin-producing isolates was assessed against 42 indicator strains using a standardized agar disc diffusion test. Accordingly, an aliquot of 20 μl CFS was applied on sterile filter paper discs (6 mm diameter) and set on agar plates previously inoculated (0.1 ml) with each individual indicator strain suspension corresponding a cell density of approx. 10^5 CFU/ml. Plates then were incubated 24 h at optimum temperatures of the test organisms (Table 1). Antimicrobial activity was detected as a translucent halo in the bacterial lawn surrounding the discs.

2.3. Further characterization of the inhibitory effect

In order to determine the biological nature of the antimicrobial activity of bacteria, CFS (pH 6.0) of selected *Lactobacillus* isolates, incubated in MRS broth at 30 °C for 24 h, were tested for their sensitivity to the proteolytic enzymes. One millilitre of CFS was treated for 2 h at 30 °C with 1 mg/ml final concentration of papain, trypsin, proteinase K, pronase E and α-amylase (P 4762, T6567, P6556, P 6911, A6211, Sigma, London). To clarify whether the antimicrobial activity detected derives from the production of hydrogen peroxide, 2600 IU/ml catalase (C-100, Sigma, London) were added to 1 ml portions of extracellular extracts (CFS) of LAB exhibiting antimicrobial activity, and incubated for 24 h at 30 °C. Further, different organic solvents (Butanol, Ethanol, Methanol, Ethyl ether), complexing agents and detergents (sodium dodecyl sulphate (SDS), Tween 20, Tween 80, ethylenediaminetetraacetic acid (EDTA), sodium deoxycholate, sulfobetaine 14) were added to the CFS, and the samples were incubated for 5 h before being tested for antimicrobial activity (for details see Table 2). To find out the temperature sensitivity of potential bacteriocin activities, samples of CFS were incubated under defined conditions (for details see Table 3). The effect of pH on the antimicrobial activity was ascertained by adjusting the pH of the CFS with appropriate volumes of diluted 1 M HCl and 1 M NaOH (Table 3). After incubating for 2 h, the pH of the CFS samples was readjusted to 6.5 followed by filter-sterilization (0.2 μm). The remaining antimicrobial activity was assessed exemplarily by using *L. monocytogenes* ATCC 19115 as indicator bacterium and by applying the agar disc diffusion plate bioassay. Untreated cell-free supernatants were used as controls and experiments were performed in duplicate.

Table 2
Effect of enzymes and chemical treatment on the antimicrobial activity of cell free supernatant derived from *Lb. casei* AP8 and *Lb. plantarum* H5.

Treatment	Concentration	Residual antimicrobial activity [%][a]	
		AP8	H5
Enzymes			
Trypsin	1 mg/ml	0	0
Papain	1 mg/ml	0	0
Proteinase K	1 mg/ml	0	0
Pronase E	1 mg/ml	0	0
α-amylase	1 mg/ml	100 ± 0	100 ± 0
Catalase	1 mg/ml	100 ± 0	100 ± 0
Organic solvents			
Butanol	10% (v/v)	100 ± 0	100 ± 0
Ethanol	10% (v/v)	100 ± 0	100 ± 0
Methanol	10% (v/v)	92.3 ± 0.05	100 ± 0
Ethyl ether	10% (v/v)	100 ± 0	100 ± 0
Surfactants			
EDTA	5 mmol/l	100 ± 0	83 ± 1
Sodium deoxycholate	1 mg/ml	100 ± 0	100 ± 0
Sulfobetaine 14	1 mg/ml	92.8 ± 0.06	100 ± 0
SDS	1% (w/v)	100 ± 0	100 ± 0
Tween 20	1% (v/v)	96.1 ± 0.8	98.6 ± 0
Tween 80	1% (v/v)	94.0 ± 0.02	97.2 ± 1

[a] Results are presented as the mean value of triplicate trials ± standard deviation (SD).

Table 3
Effect of cold storage, temperature and pH on the inhibitory activity against *L. monocytogenes* ATCC 19115.

Treatment	Residual antimicrobial activity [%][a]	
	AP8	H5
Storage		
4 °C, −20 °C/30 d	98.0 ± 0.57	99.0 ± 0.77
Temperature		
40–100 °C/30 min	85.6 ± 0.06	96.3 ± 2
121 °C/10 min	65 ± 0.06	94.6 ± 0
121 °C/15 min	0	90.3 ± 0.03
pH		
pH = 2	0	100 ± 0
pH = range 3–10	87.5 ± 3	95.5 ± 0.2
pH = 11	0	92.1 ± 0.04
pH = 12	0	92.0 ± 0.03

[a] Results are presented as the mean value of triplicate trials ± standard deviation (SD).

2.4. Growth dynamics and production of antimicrobial compounds

The kinetics of the production of inhibitory substances were investigated by inoculating 10 ml of an overnight culture of selected *Lactobacillus* isolates into 100 ml of MRS broth followed by incubation at 30 °C. Samples were taken at appropriate time intervals and measured for changes in pH, bacterial growth (600 nm; Hitachi U 1100 spectrophotometer, Tokyo, Japan) and antibacterial activity (CFS) against *L. monocytogenes* ATCC 19115. For this purpose the agar well diffusion bioassay was used by measuring the diameter of the inhibition zone around the wells, and antimicrobial activity was expressed as arbitrary units (AU) per ml. One AU is defined as the reciprocal value of the highest dilution showing a clear zone of growth inhibition (Ghanbari, Rezaei, Soltani, et al., 2009).

In a separate experiment, the inhibitory effect of CFSs against *L. monocytogenes* ATCC 19115 as indicator strain was also examined in a liquid medium. For this purpose, 20 ml of each filter-sterilized cell-free supernatant were added to a 100 ml culture of the indicator organism at early exponential phase (aged 4 h). These experiments were also repeated with stationary phase cells. The optical density at 600 nm and the viable cell count were monitored hourly during an observation period of 20 h. Indicator cells incubated without CFSs served as negative-control.

2.5. Adsorption of antimicrobial compounds to producer cells

Inhibitory substance producing cells were cultured for 18 h at 30 °C. The pH of the cultures was adjusted to 6.0 with 1 M NaOH to allow maximal adsorption of the potential bacteriocin to the producer cells, according to the method described by Yang, Johnson, and Ray (1992). The cells were then harvested (10,000× *g* 20 min, 4 °C) and washed with sterile 0.1 M phosphate buffer (pH 6.5). The pellet was re-suspended in 10 ml of 100 mM NaCl (pH 2.0) and stirred slowly for 1 h at 4 °C. The suspension was then centrifuged (10,000× *g* 20 min, 4 °C), and the corresponding CFS neutralized to pH 7.0 with sterile 1 M NaOH followed by testing the bacteriocin activity as described above.

2.6. Partial purification and characterization of the inhibitory substances

Potential bacteriocin producing strains were cultured in MRS broth and incubated without agitation for 18 h at 30 °C. The cells

were harvested (10,000× *g*, 20 min, 4 °C) and the bacteriocin was precipitated from the CFS with a saturated 60% (v/v) ammonium sulphate solution (Yang et al., 1992). The precipitated pellet and the floating on the surface were collected and re-suspended in one-tenth volume 25 mM ammonium acetate buffer (pH 6.5). The sample was stored at −20 °C up to one week and activity tests were performed as described above. Tricine-SDS-PAGE technique was employed to determine the molecular mass of the inhibitory compounds according to Schägger and von Jagow (1987). Low molecular weight markers, ranging from 2.5 to 45 kDa (Pharmacia, Sweden) were used. One half of the gel containing the molecular marker was fixed for 20 min in 5% (v/v) formaldehyde, then rinsed with water and stained with Coomassie Brilliant Blue R250 (161-0400, Bio-Rad, USA) overnight. The other half of the gel (not stained and extensively pre-washed with sterile distilled water) was overlaid with a culture of 10^6 CFU/ml *L. monocytogenes* ATCC 19115 (2 ml) embedded in BHI agar. The position of the bacteriocin was visualized by an inhibition zone around the active protein band (Todorov, Nyati, Meincken, & Dicks, 2007).

3. Results

3.1. Screening of Lactobacillus strains for antimicrobial activity and potential bacteriocin production

Eighty-four previously isolated lactobacilli strains originating from two species of Sturgeon fish (Ghanbari, Rezaei, Jami, et al., 2009) were assayed for their antimicrobial activity and potential bacteriocin production against *L. monocytogenes* ATCC 19115 and *S. enterica* subsp. *enterica* serovar Typhimurium ATCC 14028 by using spot-on-lawn method, standardized agar disc diffusion method and well diffusion method. Out of these strains, 15 isolates (18%) exhibited inhibitory activities against both indicator organisms. Subsequently, all candidate isolates displaying an inhibition zone greater than 8 mm were subjected to further tests such as growth at different temperatures, pH values, salt condition, antibiotic resistance, etc. (Ghanbari et al., unpublished results). Based on the results of the previous screening tests, *Lactobacillus casei* AP8 and *Lactobacillus plantarum* H5 were found to be most active and therefore were used in further experiments.

3.2. Inhibitory spectrum

According to the screening tests, the inhibitory potential could be optimally visualized by agar disc diffusion tests of CFSs. Hence this method was chosen as suitable technique for examining the antibacterial activity of *Lb. casei* AP8 and *Lb. plantarum* H5 CFSs against a series of 42 Gram-positive and Gram-negative bacterial strains. The CFS preparations from both strains showed some inhibitory effect against a wide range of LAB of different species, some food-borne pathogens and spoilage-causing bacteria including *Listeria innocua*, *L. monocytogenes*, *S. aureus*, *Aeromonas hydrophila*, *Aeromonas salmonicida*, *Bacillus cereus*, *Bacillus subtilis*, *Brochothrix thermosphacta*, *Escherichia coli*, *Salmonella* spp. and *Pseudomonas* spp., *Clostridium perfringens* and *Vibrio parahaemolyticus* (Table 1). The results indicate that the tested Gram-positive bacteria are more sensitive to the antimicrobial compound than Gram-negative bacteria. The largest antimicrobial spectrum was exerted by CFS of *Lb. casei* AP8, which inhibited 33 out of 42 indicator strains.

3.3. Characterization of the inhibitory effect

Tables 2 and 3 depict the stability characteristics of the presumptively inhibitory compound under different physico-chemical conditions. Foremost, proteolytic and neither lipolytic nor glycolytic enzymes, negated the antimicrobial effect exerted by both *Lb. casei* AP8 and *Lb. plantarum* H5 CFSs cell-free supernatants, confirming the proteinaceous nature of the inhibitory compound (Table 2). Hence it was concluded that the inhibitory effect was due to a bacteriocin. To detect the hydrophobic nature of the bacteriocin the CFSs were treated with a group of detergents. The CFSs remained active even after 5 h of treatment with substances such as catalase, SDS, Tween 20, Tween 80 and EDTA (Table 2). It is worth mentioning, that in the case of *Lb. casei* AP8 the addition of anionic detergent (SDS), EDTA resulted in increased antibacterial activity against *L. monocytogenes* ATCC 19115, whereas the addition of nonionic detergents (Tween 20 and 80) decreased the activity (Table 2). Regarding their stability, the antimicrobial compounds of *Lb. casei* AP8 and *Lb. plantarum* H5 were able to resist most of these physical and chemical treatments even when exposed during prolonged incubation time (Table 3).

Fig. 1 shows the relationship between bacterial growth of *Lb. casei* AP8 and *Lb. plantarum* H5 at 30 °C and the corresponding antimicrobial against the Listeria test strain due to the production of antimicrobial substance. Cellular multiplication of *Lb. casei* AP8 reached the stationary phase after 12 h of cultivation. The resulting antimicrobial effect started after 4 h, during onset of the exponential phase, while maximum activity was observed at the early stationary phase (Fig. 1A). The antimicrobial activity of *Lb. plantarum* H5 detectable in the culture supernatant was evident after 5 h and increased throughout the logarithmic growth phase. In analogy to the other *Lactobacillus* strain, maximum anti-listerial activity of *Lb. plantarum* H5 was observed in the stationary phase. Then the inhibitory activity gradually declined followed by a constant level. During the stationary phase, extracellular pH also remained constant, while bactericidal activity decreased, suggesting that the antimicrobial effect detected obviously was not due to the production of lactic acid.

Examining the culture reduction of *L. monocytogenes* in the presence of inhibitory substances produced by the two candidate strains showed that while in the control samples indicator strain's viable cell count reached 10^{11} CFU/ml after 24 h incubation at 37 °C, addition of the supernatant containing the bacteriocin of *Lb. casei* AP8 to the target culture in early logarithmic-phase resulted in some growth inhibition after 1 h, followed by a phase of slow decline. The supernatant of the AP8 culture even was bactericidal against *L. monocytogenes* ATCC 19115 (Fig. 2).

In the case of *Lb. plantarum* H5, the inhibition had some bacteriostatic characteristic. Adding the supernatant harvested from *Lb. plantarum* H5 to *L. monocytogenes* ATCC 19115 resulted in a growth inhibition after 1 h, but subsequently slow growth of *Listeria* occurred. Experiments with cells from the stationary phase did not show any inhibitory effect. When treating the producer cells with 100 mmol/l NaCl at low pH aiming at detecting any adherence of the bacteriocins to the surface of the producer cells, no change in the inhibitory activity of both supernatants from AP8 and H5 was detected.

Finally, an attempt was made to partially purify the inhibitory compounds using 60% (v/v) ammonium sulphate precipitation. Test series carried out with the corresponding supernatants showed an increase (10–15%) in the inhibitory activity against *L. monocytogenes* for both purified bacteriocins. The SDS-PAGE analysis of the partially purified samples yielded typical peptide bands for both corresponding with a molecular weight of approximately 5 and 3 kDa, respectively (Fig. 3).

4. Discussion

It could be shown that two strains, *Lb. casei* AP8 and *Lb. plantarum* H5 isolated from the intestinal bacterial flora of beluga (*Huso huso*) and Persian sturgeon (*Acipenser persicus*) produced antibacterial

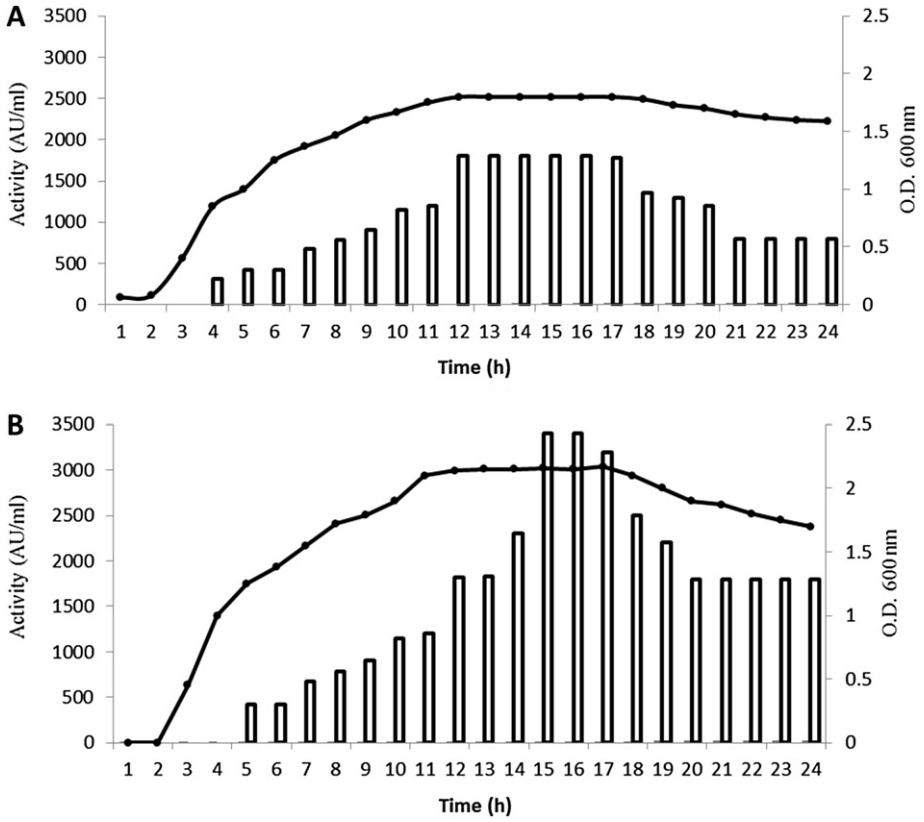

Fig. 1. Antimicrobial activity (bars) of *Lb. casei* AP8 (A) and *Lb. plantarum* H5 (B) against *L. monocytogenes* ATCC 19115 observed during growth in BHI medium (●) and expressed in AU/ml. Results represent the arithmetical mean of three independent experiments.

substances. According to the findings it was likely that the antibacterial effect was due to the formation of bacteriocin-like inhibitory substances (BLIS), as results from proteolytic enzyme inactivation indicated that the individual antimicrobial activity of isolates AP 8 and H5 was lost or unstable after such a treatment. Furthermore, treatment with lipolytic or glycolytic enzymes did not affect the activity of antimicrobial compounds, suggesting that BLISs produced do not belong to the controversial group IV of the bacteriocins, which carry carbohydrates or lipids in their active molecular structure (Cotter, Hill, & Ross, 2005; Lewus et al., 1992). It is important to note that the detected activities were not due to hydrogen peroxide or acidity, as antimicrobial activity was neither lost after treatment with catalase nor after neutralizing the pH value. Both of these presumptive inhibitory compounds were considered to be heat stable. Although heat stability of antibacterial substances produced by *Lactobacillus* spp. has been well demonstrated (Bhattacharya & Das, 2010; Chung & Yousef, 2010; Drider, Fimland, Héchard, McMullen, & Prévost, 2006; Parente & Ricciardi, 1999; Tagg & McGiven, 1971) pronounced heat stability of *Lb. casei* AP8 derived substances at 121 °C for 10 min is a novel phenomenon. The results of pH stability were not in agreement with previous findings indicating the tolerance of bacteriocins to acidic pH rather

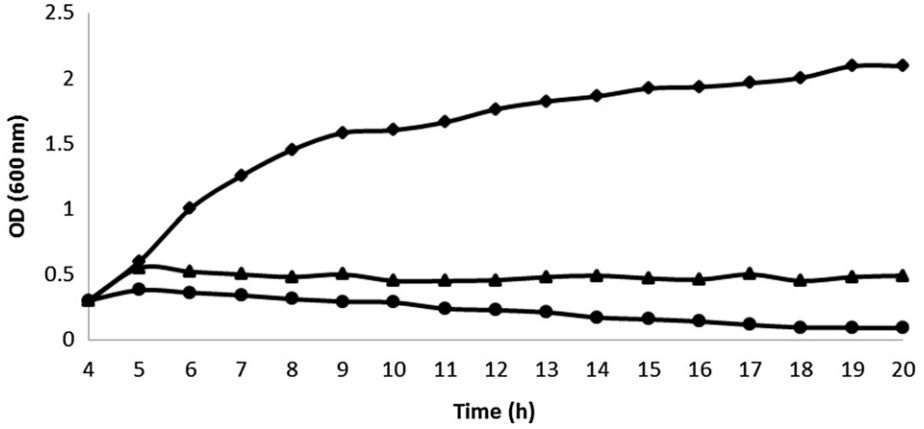

Fig. 2. Antimicrobial effect of the CFS of *Lb. casei* AP8 (●) and *Lb. plantarum* H5 (▲) on the growth of *L. monocytogenes* ATCC 19115 at 30 °C. Growth of *L. monocytogenes* ATCC 19115 without added CFS (control, ◆).

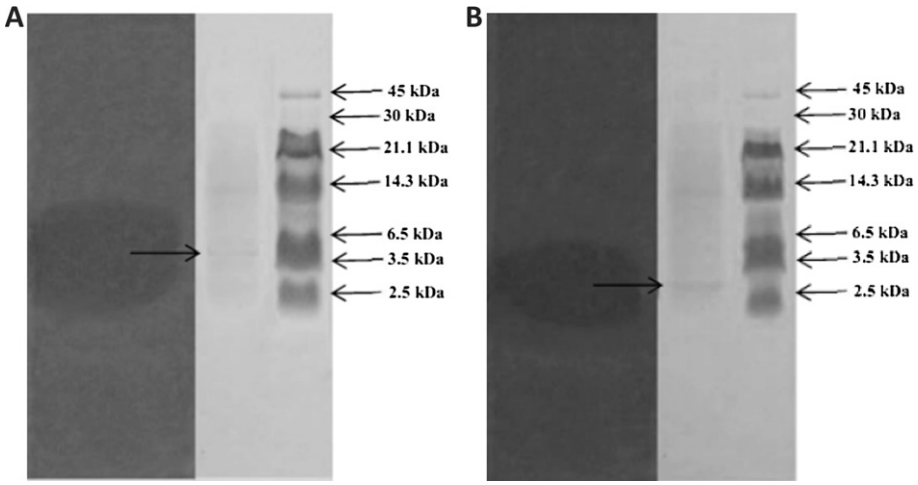

Fig. 3. Tricine-SDS-PAGE of partially purified bacteriocins (precipitated by 60% saturated ammonium sulphate) *Lb. casei* AP8 (A) and *Lb. plantarum* H5 (B) along with the standard molecular weight markers.

than to alkaline (Bhunia, Johnson, Ray, & Kalchayanand, 1991; Yang et al., 1992). The loss of antimicrobial activity of AP8 BLIS at pH > 10 might be ascribed to some proteolytic degradation, protein aggregation or the instability of proteins at this extreme pH (Bhattacharya & Das, 2010; Todorov et al., 2007). *Lb. casei* AP8 BLIS showed an increase in the inhibitory activity after treatments with agents like SDS and EDTA, with the latter presumably by chelation of divalent cations from the protective outer cell membrane of bacteria, making them sensitive to hydrophobic peptides such as bacteriocins (Castellano, Belfiore, & Vignolo, 2011). Also, the anionic detergent SDS is known to unfold proteins by complexing their interior hydrophobic core and thus affecting their three dimensional conformation (Mojgani & Amirinia, 2007).

In similarity to Lactocin RN78 and Plantaricin LC74, both bacteriocin-like substances produced by *Lb. casei* AP8 and *Lb. plantarum* H5 were stable after treatment with organic solvents like butanol, ethanol and methanol, supporting their proteinaceous and soluble nature (Gálvez et al., 2007; Mojgani & Amirinia, 2007; Verschuere, Rombaut, Sorgeloos, & Verstraete, 2000). Although bacteriocins from LAB usually are ineffective against Gram-negative bacteria and rather relate to a narrow antimicrobial spectrum (Cleveland et al., 2001; Drider et al., 2006; Parente & Ricciardi, 1999), both presumptive bacteriocin-like substances AP8 and H5 showed some broad antimicrobial activity against several genera of Gram-positive and Gram-negative bacteria based on agar disc diffusion bioassay. Even representatives of *Pseudomonas* spp., *Salmonella* spp., *E. coli*, *A. hydrophila*, *A. salmonicidia* and *Vibrio anguillarum* could be inhibited. Moreover, a high inhibitory potential against *L. monocytogenes* was observed. In recent years, a large number of new bacteriocins produced by *Lb. plantarum* have been identified and characterized. Their molecular masses have been reported to be in the range of 3–10 kDa (Todorov et al., 2007, 2010). However, to our knowledge, there is no bacteriocin produced by any *Lb. casei* strain with a molecular mass of 5 kDa with similar characteristics to the one investigated in this study (e.g. from fish intestine sources).

The physiochemical properties of bacteriocins from *Lb. casei* AP8 and *Lb. plantarum* H5 found in this study may allow to allocate them to those of group IIa lactic acid bacteria, as they display similar properties in terms of molecular weight, heat and pH stability and sensitivity to proteolytic enzymes (Cleveland et al., 2001; Cotter et al., 2005; Drider et al., 2006). Characteristics unifying all members of class IIa bacteriocins are (i) they are below 10 kDa (ii) their pronounced activity against *Listeria* spp., (iii) their resistance to elevated temperatures and pH value, and (iv) their cystibiotic feature attributed to the presence of at least one disulphide bridge, which is crucial for antibacterial activity (Belguesmia et al., 2011; Bhunia et al., 1991; Cotter et al., 2005; Drider et al., 2006). Class IIa bacteriocins were formerly considered as "narrow"-spectrum antibiotics, with antimicrobial activity directed against related strains. However, recently, some class IIa bacteriocins, such as bacteriocin OR-7, enterocin E50-52, and enterocin E760, have been shown also to be active against both Gram-negative and Gram-positive bacteria, including *Campylobacter jejuni*, *Yersinia* spp., *Salmonella* spp., *E. coli* O157:H7, *Shigella dysenteriae*, *S. aureus*, and *Listeria* spp. (Belguesmia et al., 2011; Bhunia et al., 1991; Cotter et al., 2005; Drider et al., 2006; Pinto et al., 2009).

Besides the broad inhibitory spectrum, their technological properties and especially cold, heat and storage stability indicate that bacteriocins AP8 and H5 possess some potential for being applied not only as biopreservative agents to control pathogens and spoiling bacteria in food products but also as bioprotective compounds for aquaculture, since the use of bacteria releasing antimicrobial substances like the isolates described is now gaining importance as a natural alternative to the administration of antibiotics in fish farming (Indira et al., 2011; Venkat, Sahu, & Jain, 2004; Verschuere et al., 2000). Hitherto only few bacteriocins from *Lb. plantarum* isolated from fish have been identified and, to our knowledge, this is the first report describing a *Lb. casei* strain isolated from fish and displaying pronounced inhibitory effects due to bacteriocin formation.

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CHAPTER 4

A new view of the fish gut microbiome: advances from next-generation sequencing

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A new view of the fish gut microbiome: Advances from next-generation sequencing

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ABSTRACT

The fish gut microbiota contributes to digestion and can affect the nutrition, growth, reproduction, overall population dynamics and vulnerability of the host fish to disease; therefore, this microbial community is highly relevant for aquaculture practice. Recent advances in DNA sequencing technologies and bioinformatic analysis have allowed us to develop a broader understanding of the complex microbial communities associated with various habitats, including the fish gut microbiota. These recent advances have substantially improved our knowledge of bacterial community profiles in the fish intestinal microbiota in response to a variety of factors affecting the host, including variations in temperature, salinity, developmental stage, digestive physiology and feeding strategy. The goal of this review is to highlight the potential of next-generation sequencing platforms for analysing fish gut microbiota. Recent and promising results in this field are presented along with a focus on new perspectives and future research directions of fish gut microbial ecology.

Contents

1. Introduction

In-depth knowledge of community membership as well as the structure and relationships between resident microbes (microbiota) and their hosts can provide insight into both the function and dysfunction of the host organism. Comprising a large diversity of approximately 28,000 species, fish make up nearly half of all vertebrate species and exhibit a wide variety of physiologies, ecologies and natural histories (Wong and Rawls, 2012). Thus, fish represent an important group for understanding the variety and nature of symbioses in vertebrate gut microbial communities (Nayak, 2010). The digestive tract of fish receives water and food that are populated with microorganisms from the surrounding environment; these microbes undoubtedly affect the

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resident microbiota. Correlations between changes in the composition and activity of the fish gut microbiota with fish physiology and disease have been proposed, increasing the scientific community's interest in this field of research. From this perspective, a comprehensive and detailed view of the fish gut microbiota, including phylogenetic composition as well as the genetic and metabolic potential, is essential to understand the dynamics and possible mechanisms of the cause/effect relationships between gut microbiota and physiology (Austin, 2006, 2011). As numerous studies have indicated that culture-dependent techniques possess dubious sensitivity and often detect only a limited fraction of microbial communities (Austin, 2006; Kim et al., 2007; Namba et al., 2007; Wu et al., 2010, 2012a; Lan and Love, 2012; Larsen et al., 2013), several methods for culture-independent microbial analysis have been developed within the past few decades (Head et al., 1998; Muyzer and Smalla, 1998) and applied in studies of the fish gut. Molecular methods provide faster results and novel, high-resolution insights into the structure and diversity of microbial communities within the digestive tracts of freshwater and marine fish (Austin, 2006; Kim et al., 2007; Namba et al., 2007; Wu et al., 2010, 2012a; Lan and Love, 2012; Larsen et al., 2013).

Despite these recent findings, the cost, technical difficulties and low coverage associated with Sanger sequencing have limited the ability to analyse a large number of samples. Recently, however, rapid and low-cost approaches for next-generation sequencing (NGS) technologies have been introduced to study the composition and genetic potential of densely populated microbial communities such as gut microbiota (Foster et al., 2012). Within the past few years, these techniques have been applied to analyse the composition and functional properties of fish microbial communities. Fish microbiota studies have most frequently utilised the 454/Roche pyrosequencing (e.g., the Roche 454 FLX Titanium and FLX+) and Illumina technologies (e.g., the Illumina MiSeq and HiSeq 2000). Unlike the Sanger sequencing approach, these NGS platforms provide a larger number of reads in a single run, enabling the rapid and cost-effective acquisition of in-depth and accurate sequence data and allowing for the detection of both dominant and low-abundance (rare) microbial community members (Roeselers et al., 2011; Wu et al., 2012b; Star et al., 2013; Wong et al., 2013). With the emergence and availability of NGS techniques for studying complex microbial ecosystems and the growing appreciation of the importance of the indigenous microbiota of fish, this review highlights the potential of NGS platforms for the analysis of fish gut microbial ecology.

2. NGS platforms and technologies

Although capillary sequencers such as the ABI 3730 (Applied Biosystems, USA) continue to be used for small-scale sequencing (Metzker, 2010; Zhou et al., 2010) in fish microbiota studies, there is an increasing trend towards the use of NGS platforms. While there are several different NGS technologies, one benefit common to all of them is the ability to rapidly generate large volumes of sequencing data in parallel. Table 1 compares the main characteristics of NGS sequencers to emphasise the similarities and differences among the platforms. An overview of the NGS technologies that have been used for microbiome studies is given in the following sections. For a more in-depth discussion about the technical aspects of next-generation sequencing platforms and principles, we refer to comprehensive reviews in the literature (Mardis, 2008; Kircher and Kelso, 2010; Metzker, 2010; Foster et al., 2012; Highlander, 2012; Kuczynski et al., 2012; Liu et al., 2012; Di Bella et al., 2013; Hui, 2014; Hodkinson and Grice, 2015).

2.1. The 454 platform family

Developed by 454 Life Sciences, the 454 Roche platforms based on sequencing-by-synthesis with pyrophosphate chemistry (Mardis, 2008; Metzker, 2010) are among the most commonly used NGS techniques for studying fish-associated microbiota. These platforms use "pyrosequencing" chemistry, which involves the incorporation of deoxynucleotide triphosphate (dNTPs) bases into a synthesised DNA chain followed by the release of a pyrophosphate. The pyrophosphate subsequently serves as a substrate for the enzymatic production of ATP. In the presence of luciferase, the ATP produces a quantifiable amount of light, which is then detected by a camera. This reaction is performed on beads containing millions of copies of a single DNA molecule. For DNA amplification, the single DNA molecules are clonally amplified in an oil–water emulsion containing PCR reagents in micelles occupied by only one bead, a process known as emPCR (Dressman et al., 2003). After harvesting, beads carrying single-stranded DNA are placed into the wells of a picotiterplate, which contains millions of wells, in such a way that each well is occupied by a single bead and the enzymatic reagents. Sequencing occurs through the repeated cyclic flow of thin films of dNTPs across the wells. Base incorporation leads to the production of photons that are detected by a camera every 7 s. Each cycle contains a different dNTP, and an image is captured after each cycle to measure the light produced in each well. Thus, sequential collections of images are analysed for light intensity patterns. The amount of light discloses whether a specific dNTP in each flow was incorporated as well as how many dNTPs were incorporated in that flow when homopolymer runs are present. This information is then translated into a DNA sequence for each bead (Ahmadian et al., 2006; Siqueira et al., 2012). The massively parallel 454 platform family has been further improved in recent years to optimise sequence read length and the amount of sequence data obtained (modal read length 750 bp and average read length 700 bp using FLX+ chemistry; see Table 1), resulting in an increase in the performance at different phylogenetic depths. However, the higher costs per base as well as the total output per run of 454 platforms in terms of reads (1 million) and bases (700 Mb) are clearly limiting factors in projects in which coverage is more important than read length, though it is valid for some metagenomic or amplicon sequencing projects.

2.2. Illumina sequencing platforms

The Illumina sequencing platforms were introduced in 2006 and quickly embraced by many researchers because a larger amount of data could be generated in a more cost-effective manner. Major progress in the Illumina platforms has been made in recent years with regard to sequence read length and output (number of reads per run) by technically improving the instruments, the chemistry and the base-calling algorithms. The working principle of this platform is based on sequencing-by-synthesis chemistry and, like the Sanger method, relies on the incorporation of dye terminator nucleotides into the sequence by a DNA polymerase (Siqueira et al., 2012). In this approach, DNA fragments are immobilised on a flow cell surface, which is coated with adapters and complementary adapters. Each of these single DNA fragments then creates a bridge with the complementary adapters. DNA sequencing is initiated by the addition of the reaction mixture containing DNA polymerase, sequencing primers and four reversible terminator nucleotides, each labelled with a different fluorescent dye. After incorporation, the terminator nucleotide and its position on the support surface are detected and recorded by a four-channel fluorescence scanner (Metzker, 2010).

Among the Illumina platforms, HiSeq sequencers produce the largest amount of data per run (up to 1500 Gb) at the lowest cost per base and with the shortest reads (Table 1). The Illumina MiSeq platform (introduced in 2011) generates 44–50 million paired-end reads 2 × 300 bp in length using the new V3 chemistry. The costs per sequenced base are higher compared with the HiSeq instrument; however, the longer read length in combination with the lower read number can be of particular interest for amplicon sequencing projects. The Illumina NextSeq 500 platform is another recently released product that performs at an

Table 1
Technological and data output specifications of currently commercially available NGS platforms (Adapted from Knief, 2014).

Company	Platforms	Template prep.	Sequencing chemistry	Type of sequencing error	Sequencing run conditions and read length	Sequencing run time	Maximum data output per run	Maximum output read
Roche	454 FLX Titanium 454 FLX + 454 GS Junior	emPCR on microbeads	Pyrosequencing	Indels in homopolymeric regions	Modal 450 bp, max. 600 bp Modal 700 bp, max. 1000 bp ~450 bp	10 h 23 h 10 h	450 Mb 700 Mb 35 Mb	1 Million per plate 1 Million per plate 0.1 Million per plate
Illumina	Illumina GAIIx Illumina HiSeq1000 Illumina HiSeq1500 Illumina HiSeq2000 Illumina HiSeq2500 Illumina MiSeq Illumina NextSeq 500 Illumina HiSeq X Ten	Bridge-PCR on flow cell surface	Reversible terminator Sequencing-by-synthesis	Substitutions, in particular at the end of the read	HiSeq 2000/2500 (high output mode): max. 2 × 125 bp HiSeq 2500 (rapid run mode): max. 2 × 250 bp MiSeq: 2 × 300 bp NextSeq 500 (high output mode): max. 2 × 150 bp NextSeq 500 (mid output mode): max. 2 × 150 bp	6 days 40 h 55 h 29 h 26 h	900–1000 Gb 250–300 Gb 13.2–15 Gb 100–120 Gb 32–39 Gb	250 Million per lane 125–150 Million per lane 22–25 Million per flow cell 400 Million per flow cell 130 Million per flow cell
Life Technologies	SOLiD 4 SOLiD 5500 SOLiD 5500xl SOLiD 5500 W SOLiD 5500xl W	emPCR on microbeads; PCR on FlowChip surface for the 5500 W models	Sequencing-by-ligation	Substitutions, in particular at the end of the read	SOLiD 5500xl: max. 60 bp + 60 bp SOLiD 5500xl W: max. 2 × 50 bp	8 days 8 days	260 Gb 320 Gb	160 Million per lane 256 Million per lane
Life Technologies	Ion PGM Ion Proton	emPCR on microbeads	Semiconductor-based sequencing-by-synthesis	Indels	Ion PGM (318): max. 400 bp Ion Proton (318): 200 bp	7.3 h 2–4 h	1.2–2 Gb Up to 10 Gb	4–5.5 Million per chip 60–80 Million per chip
Pacific Biosciences	PacBio RS	Not applied	Single-molecule, real-time DNA sequencing-by-synthesis	Indels	C2/P4 chemistry, mean read length ~8000 bp	2–3 h per cell	400 Mb per cell	0.05 per SMRT cell

intermediate scale in terms of output, read length and costs per base compared with HiSeq, MiSeq and the HiSeqX series (Table 1).

2.3. Other NGS platforms

Applied Biosystems SOLiD (Sequencing by Oligo Ligation Detection), Ion Torrent PGM and Ion Proton are among the other NGS platforms with potential for microbiome applications (Table 1) (Feehery et al., 2013; Milani et al., 2013; Mitra et al., 2013). Beginning with an emulsion PCR step, the SOLiD platform uses the sequencing-by-ligation approach for the sequencing step (Metzker, 2010). Although the SOLiD platform can produce large numbers of sequences in a run (up to 3 billion reads), the persistently short read length (maximum single read 75 bp) has greatly limited its application for microbiomics projects (Hodkinson and Grice, 2015).

The Ion Torrent PGM (Personal Genome Machine) sequencer has been available on the market since the end of 2010. Sequencing on this platform is performed using semiconductor chips of different scales that allow sequencing between 0.4 and 5.5 million reads. The Ion Torrent system begins in a manner similar to 454, with a plate of microwells containing beads to which DNA fragments are attached. It differs from all of the other systems, however, in the manner in which base incorporation is detected. When a base is added to a growing DNA strand, a proton is released, which slightly alters the surrounding pH. Microdetectors sensitive to pH are associated with the wells on the plate, which is itself a semiconductor chip, and they record when these changes occur. As the different bases (A, C, G, T) are sequentially washed through, additions are recorded, allowing the sequence of each well to be inferred. Read length on this platform has successively increased from approximately 100 bp to 400 bp.

Table 2
Studies evaluating the effects of environmental and host-related factors on fish gut microbiota composition using NGS platforms.

Sequencing platform	Fish species	Number of fish	Type of sample	Major finding(s)	References
454 GS FLX	Atlantic cod	11 Samples	Intestinal contents	✓ Substantially high inter-individual variation of bacterial community, with predominance of the Vibrionales order.	Star et al. (2013)
‘’	Zebrafish	3 Samples	Intestinal mucosa and contents	✓ The presence of a core set of bacterial taxa (within Gammaproteobacteria, Betaproteobacteria, Fusobacteria, Bacilli, Flavobacteria, Actinobacteria classes and *Aeromonas*, *Shewanella* as the most frequent genera) in domesticated wild zebrafish despite salient differences in their life histories and local environments.	Roeselers et al. (2011)
454 GS FLX Titanium	Grass carp Crucian carp Bighead carp	3/fish species	Intestinal contents	✓ Bacterial richness higher in filter-feeding fish than in grazers. ✓ Same rearing environment did not result in similar intestinal microbiota compositions. ✓ The compositions of the intestinal microbiota of grass carp and crucian carp shared higher similarity compared with that of bighead carp.	Li et al. (2015)
‘’	Trinidadian guppy	3–5 /sampling site	Intestinal mucosa	✓ Local environmental or host genetic factors contribute to the observed microbiome differentiation.	Sullam et al. (2015)
‘’	12 Finfish 3 Shark species	2–4 /fish or shark species	Intestinal mucosa and contents	✓ The composition of the gut microflora community responds to external factors such as habitat and diet. ✓ Significant correlation between increased intestinal microbiota diversity and a more varied diet. ✓ Proteobacteria were one of the most abundant phyla across the majority of samples.	Givens et al. (2014)
‘’	8 Freshwater fish	3/fish species	Intestinal contents	✓Proteobacteria and Firmicutes were the dominant phyla in all fish species. ✓ The bacterial richness of the intestinal microbiota of carnivorous fish was significantly lower than that of herbivorous, omnivorous and filter-feeding fish. ✓ Feeding habits and genotype influence the gastrointestinal microbiota of fish.	Li et al. (2014a)
‘’	Channel catfish Largemouth bass Bluegill	5/fish species	Intestinal contents	✓ Fusobacteria was the most abundant phylum in all fish intestinal microbiota, followed by Proteobacteria. ✓ An increase, though not statistically significant, in the number of predicted OTUs from carnivory to herbivory. ✓ Significantly higher species evenness in bluegill and Channel catfish than in largemouth bass.	Larsen et al. (2014)
‘’	Silver carp Gizzard shad	28 Silver carp 24 Gizzard shad	Intestinal contents	✓ Significant differences in the intestinal microbiota (foregut and hindgut) of different species are related to food source types and intestinal morphology.	Ye et al. (2014)
‘’	Gibel carp	2 Samples	Intestinal contents	✓ Sediment is the most important factor determining the intestinal microbiota community of gibel carp. ✓Proteobacteria was predominant in the gibel carp intestinal microbiota, followed by Firmicutes.	Wu et al. (2013)
‘’	Common carp	3–5/treatment/time	Intestinal mucosa and contents	✓ Significant differences in the intestinal microbiota communities of transgenic and wild-type fish. ✓ Significant relationships of Proteobacteria, Bacteroidetes, and Firmicutes with host metabolic characteristics.	Li et al. (2013)
‘’	Grass carp	3 Samples	Intestinal mucosa, Intestinal contents	✓ Local environmental conditions (mainly water and sediment, followed by feed) may significantly drive the intestinal microbiota composition of the grass carp.	Wu et al. (2012b)
‘’	Common carp	10 Samples	Intestinal mucosa and contents	✓ Microbial communities in the GI tract of fish were shown to be highly diverse and structured. ✓ The major abundant bacterial phylum was Fusobacteria, with almost all belonging to the genus *Cetobacterium*.	van Kessel et al. (2011)
Illumina MiSeq	Paddlefish Bighead carp	6/ fish species	Intestinal contents	✓ Different intestinal microbiota compositions in fish species reared under the same environment, possibly influenced by fish genetic factors, intestinal structure or physiology.	Li et al. (2014b)
‘’	4 Midas cichlid fish species	3/ fish species	Intestinal mucosa	✓ Significant differences in the microbiota of limnetic and benthic adult cichlid fish.	Franchini et al. (2014)

Sequencing on Ion instruments is very fast, taking only a couple of hours. The Ion Proton is a larger-scale instrument that produces 10-fold more bases per run using the Ion PI chip. An even larger-scale chip (Ion PII) has been announced for this platform. In terms of sequencing costs per base, the Ion PGM falls between the 454 and Illumina technologies.

3. Caveats and limitations of NGS approaches

As with any other method, NGS techniques also have their limitations. Most of these drawbacks, such as the effect of DNA extraction and primer selection on the interpretation of bacterial diversity data, are common to other culture-independent molecular methods and have been reviewed elsewhere (Kuczynski et al., 2012). Some limitations inherent to the NGS approaches include the short reads, which pose difficulties when assembling and mapping to reference sequences, particularly at repetitive regions. In addition, not all sequences are equally processed and sequenced, and DNA regions with enriched GC content are particularly prone to low coverage (Hui, 2014). With respect to amplicon library preparation, PCR amplification bias may be introduced and affect the estimated diversity (Kuczynski et al., 2012; Siqueira et al., 2012). Last but not the least, sequencing errors are present in essentially all NGS platforms. Longer reads (for example, those produced by 454 pyrosequencing) are prone to error readings, particularly towards the ends of the sequences, inflating richness estimates (Balzer et al., 2010; Siqueira et al., 2012). Repetitive sequences and homopolymers are also of concern for some third-generation sequencers (Balzer et al., 2010). In recent years, rapid improvements in technical aspects (increased coverage and deep sequencing) and bioinformatic tools have been made to overcome and correct these problems (Balzer et al., 2010; Quince et al., 2011; Bragg et al., 2012).

Analyses of bacterial communities associated with fish gut microbiota have almost exclusively used NGS-based 16S rRNA (SSU rRNA) sequencing. However, there is an important caveat that should be taken into account when interpreting the 16S sequencing results. Due to its universal phylogenetic distribution, 16S rRNA has been one of the most important targets of NGS (and other culture-independent) applications for investigating the phylogenetic relationships among bacterial communities and bacterial diversity in fish gut analyses as well as for quantifying the relative abundances of taxa of various ranks (Hugenholtz, 2003; Vetrovsky and Baldrian, 2013). Despite the wide use of this marker, there are several aspects that limit the interpretation of 16S rRNA-derived results. The most important is the fact that its copy numbers per genome vary from 1 to 15 or more copies (Klappenbach et al., 2001; Hugenholtz, 2003). In general, abundance estimates based on 16S rRNA sequence counts tend to underestimate the abundance of taxa with low 16S rRNA copy numbers, such as Acidobacteria, and overestimate taxa with high 16S rRNA copy numbers, such as Gammaproteobacteria and Firmicutes (Vetrovsky and Baldrian, 2013), which have been frequently reported in the gut microbiota of fish (Table 2). Furthermore, while it is assumed that copies of rRNA genes within an organism are subject to homogenisation through gene conversion, it has been shown that 16S sequences from the same species or even the same genome are often different (Vetrovsky and Baldrian, 2013). Both of these characteristics of the 16S rRNA marker represent problems for the measurement of bacterial diversity in current investigations of bacterial communities and, if not carefully taken into account, could make the relative abundance estimates in complex bacterial populations unreliable (Farrelly et al., 1996; Vetrovsky and Baldrian, 2013; Bolnick et al., 2014b).

4. Application of NGS technologies in recent studies of fish gut-associated microbiota

One approach to studying animal behaviour involves the examination of gut microbial communities. Many studies on humans, mice, chickens and termites have successfully correlated gut microbial communities with host physiology, nutrition and growth (Ye et al., 2014).

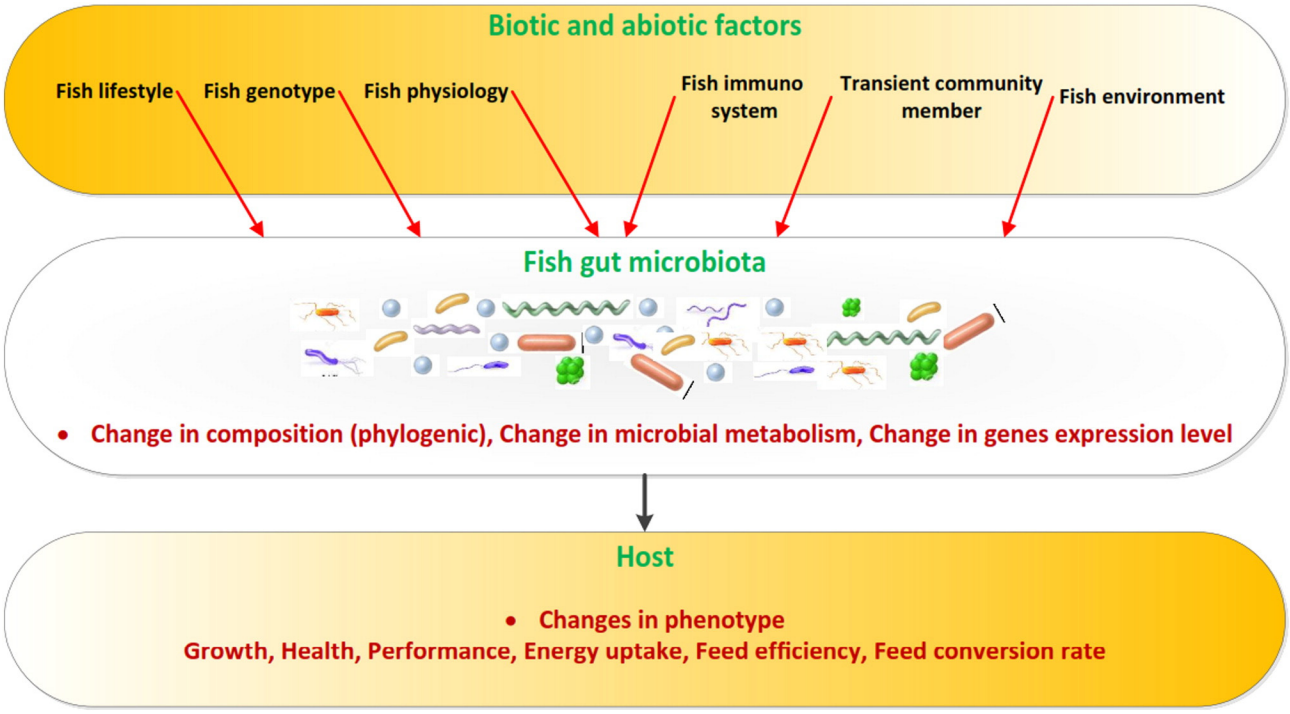

Fig. 1. A combination of biotic and abiotic factors (red arrows) such as genotype, fish physiological status (including properties of the innate and adaptive immune systems), fish pathobiology (disease status), fish lifestyle (including diet), fish environment and the presence of transient populations of microorganisms affect the composition, function and metabolic activity of the fish gut microbiota. These changes affect processes involved in growth, performance, energy storage and health in fish. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

Table 3
Studies investigating the effects of dietary and genetic factors on fish gut microbiota composition using NGS platforms.

Sequencing platform	Fish species	Number of fish sampled	Type of sample	Major finding(s)	References
454 GS FLX	Zebrafish	30 /treatment	Intestinal mucosa and contents	✓ Microbiota regulate intestinal absorption and fatty acid metabolism in the zebrafish.	Semova et al. (2012)
454 GS FLX Titanium	Atlantic salmon	4 /diet/time	Faecal	✓ Successional dynamics over a period of 13 months in salmon hindgut communities with respect to season and fish growth phases but not significantly related to differences in commercial diet.	Zarkasi et al. (2014)
"	Sea bream	3 /treatment	Intestinal mucosa	✓ Different feeding modes reflect the gut prokaryotic community structure in conventionally reared, organically reared, and wild *S. aurata* individuals.	Kormas et al. (2014)
"	Siberian sturgeon	9 /treatment	Intestinal contents	✓ A combination of dietary probiotics and AXOS resulted in a significant reduction in bacterial richness compared with the control diet.	Geraylou et al. (2013a)
"	Siberian sturgeon	12–16 /treatment	Intestinal contents	✓ The administration of 2% AXOS-32–0.30, but not 4%, showed beneficial shifts in gut microbiota, primarily in the phylum Firmicutes, and higher concentrations of SCFAs.	Geraylou et al. (2013b)
"	Sea bass	4 /diet/time	Intestinal mucosa, Intestinal contents	✓ Administration of functional diets containing essential oils caused significant changes in the gut microbiota of sea bass.	Carda-Diéguez et al. (2013)
"	Rainbow trout	6–9 /treatment/time	Intestinal mucosa and contents	✓ Variations in diet and rearing density caused minor changes in intestinal microbiota composition but significant effects on fish growth, performance, fillet quality, and welfare.	Wong et al. (2013)
Illumina HiSeq 2000	Threespine stickleback	150 Samples	Intestinal mucosa and contents	✓ MHC IIb polymorphisms contribute to inter-individual variation in gut microbiota within a single wild population of threespine stickleback.	Bolnick et al. (2014a)
"	Grass carp	4 Sample/site	Intestinal mucosa	✓ External variables, especially the abundance of food in the fish gut and feed composition, significantly affected the composition and function of the intestinal microbiota.	Ni et al. (2014)
"	Asian sea bass	6/treatment	Intestinal mucosa and contents	✓ The intestinal microbiota composition changed in response to starvation towards a significant enrichment of Bacteroidetes and depletion of Betaproteobacteria. ✓ Antibiotic activity-related genes were significantly enriched in response to starvation. ✓ Host immune system-related genes were generally up-regulated.	Xia et al. (2014)
"	Rainbow trout	48/treatment time	Intestinal mucosa	✓ The colonisation of the intestinal microbiota in rainbow trout fry is influenced by first feeding and diet with the marine-favoured presence of Proteobacteria and plant-based diet-favoured presence of Firmicutes.	Ingerslev et al. (2014b)
"	Rainbow trout	20 /treatment/time	Intestinal mucosa	✓ Dietary and pathogenic challenge-mediated differences in the composition of the intestinal microbiota were observed. ✓ Protective effect of a plant-based diet against *Y. ruckeri*, likely due to the higher number of bacteria from the family Lactobacillaceae .	Ingerslev et al. (2014a)
Illumina HiSeq 2000 Illumina MiSeq	Perch Threespine stickleback	192 Perch 192 Wild stickleback 50 Lab-reared stickleback	Intestinal mucosa and contents	✓ Genotype (sex)-by-environment (diet) interactions regulate intestinal microbiota composition in fish.	Bolnick et al. (2014c)

It has been well documented that in aquatic animals, the gut microbiota is responsible for the digestion of algal cells, the production of amino acids and the secretion of inhibitory compounds that protect against colonisation of the gut by bacterial pathogens (Austin, 2006; Nayak, 2010). Furthermore, the composition and interactions of the gut microbiota may affect the amount of energy extracted from the diet and play an important role in the metabolism of dietary substrates and immune system modulation (Fig. 1) (Xia et al., 2014). Thus, deciphering the make-up of fish gut microbiota is of great importance for understanding the dynamic functions of this community and the manner in which these organisms affect their host's physiology. This information has the potential to help manage fish populations and their feeding and growth in captivity.

The antiquity, diversity and dietary variations among fish species present an exceptional opportunity to identify the parameters that influence membership within the intestinal microbiota. NGS technologies are increasingly being used for amplicon sequencing of bacterial marker genes to characterise fish gut-associated bacterial communities. Tables 2 and 3 provide a compilation of reports from these studies. The full details of the studies listed in Tables 2 and 3 are reported in Supplementary file Tables S1 and S2. The majority of these studies applied Roche 454 sequencing platforms; however, in recent years, the trend has shifted towards Illumina sequencing technologies because they generate far more data and are more cost-effective. Most sequencing projects to date have addressed the same issues, e.g., the diversity of bacterial taxa present in fish gut microbiota or the influence of factors such as fish species, culturing condition, feeding behaviour, genetic background or sex. Some of the studies listed in Tables 2 and 3 are further discussed in the following sections.

Most amplicon sequencing studies on the fish gut have aimed to describe the diversity of bacterial taxa associated with the fish gut ecosystem (Table 2). A detailed look at the literature (Tables S1 and S2) shows that the read numbers generated in 16S rRNA-based sequencing projects are mainly in the range of a few hundred to ten thousand reads per sample. Indeed, there is no "gold standard" for the necessary number of reads per sample for metagenomic surveys, as the number depends on several factors, such as sample type, the aim of the sequencing project, the richness and diversity of the samples and primer selection. Measurement of alpha diversity metrics like rarefaction curves is regarded as the best way to determine whether the sequencing effort is sufficient to capture the true species richness of the sample. To build rarefaction curves, each community is randomly subsampled without replacement at different intervals, and the average number of Operational Taxonomic Units (OTUs) at each interval is plotted against the size of the subsample (Gotelli and Colwell, 2001). The point at which the number of OTUs does not increase with further sampling is the point at which enough samples have been taken to accurately characterise the community. In a study of grass carp, the 16S rRNA V1–V3 region was targeted, and 259 to 1400 OTUs were identified from 6990 to 18,993 sequence reads (Wu et al., 2012b). This sequence coverage resulted in rarefaction curves that nearly reached the saturation plateau, and Good's coverage estimations revealed that 94 to 98%

of the phylotypes were obtained. In a study of other herbivorous cyprinids, between 280 and 2325 OTUs were identified from 5480 to 12,659 sequence reads (Li et al., 2014a). This sequence coverage resulted in rarefaction curves near the plateau level, and Good's coverage estimations revealed that 82 to 98% of the phylotypes were obtained. However, studies of omnivorous cyprinids revealed that rarefaction curves generated from 16S sequence data did not reach a plateau even after 11,000 sequence reads per individual. In these studies, between 449 and 2340 OTUs were detected per individual, and the average coverage reported was 89%. However, the literature indicates that bacterial diversity is much higher in planktivorous fish compared with omnivorous and herbivorous fish species. Ye et al. (2014) examined the intestinal microbiota of planktivorous silver carp and gizzard shad and revealed that rarefaction curves failed to reach a saturation phase even after 100,000 sequence reads. In total, more than 3000 and 7000 OTUs were identified in the intestinal microbiota of silver carp and gizzard shad, respectively. Li et al. (2015) also reported that the intestinal microbiota of the filter-feeding bighead carp was more diverse (410–1260 OTUs per individual, coverage 89–94%) compared with grass carp (280–307 OTUs per individual, coverage 97–98%) and crucian carp (125–214 OTUs per individual, coverage 98–99%). However, in a study by Desai et al. (2012) targeting the cpn60 gene in the carnivorous rainbow trout, estimates of Good's coverage ranged from 94.5 to 97.8% when using an average of only 1660 reads per sample. A study by Kormas et al. (2014) on the sea bream (*Sparus aurata*) also showed that a Good's coverage estimate of 99% was achieved with an average of 6860 reads per individual sample. Although a limited number of studies are available, this comparison between fish with different feeding strategies is suggestive of potentially large differences in levels of microbial diversity. These studies reveal a trend in the diversity of gut microbiota, with the highest level in planktivorous fish, followed by omnivorous and then herbivorous fish species, with the lowest diversity in carnivorous fishes (Givens et al., 2014; Li et al., 2014a, 2015). Therefore, it seems that more than 10,000 sequences per sample are necessary to obtain at least 80% coverage of the fish gut microbial communities in fish with planktivorous, omnivorous and herbivorous feeding habits, although, as already mentioned, several other variables may influence the number of reads required to accurately characterise the bacterial community being studied.

4.1. The core gut microbiota concept in fish

Although the gut is likely among the most densely populated microbial ecosystems within a fish's body, sequence-based analyses have demonstrated that it comprises a peculiarly low phylogenetic diversity. NGS-based studies have shown that among all bacterial phyla formally described to date, the following bacterial groups are primarily represented in the fish gut microbiota: Proteobacteria, Fusobacteria, Firmicutes, Bacteroidetes, Actinobacteria, Clostridia, Bacilli and Verrucomicrobia. Among these, Proteobacteria, Firmicutes and Bacteroidetes represent up to 90% of the fish intestinal microbiota in different species (Ringø et al., 2006; Desai et al., 2012; Carda-Diéguez et al., 2013; Li et al., 2013; Givens et al., 2014; Ingerslev et al., 2014b). The presence of several similar bacterial taxa/OTUs in the gut-associated microbiota of the same or multiple fish species from different populations and geographic locations indicates that these ribotypes may be important contributors to host gut functions, such as digestion, nutrient absorption and the immune response (Roeselers et al., 2011), a concept often referred to as the “core gut microbiota” (Rawls et al., 2004; van Kessel et al., 2011; Desai et al., 2012; Wong et al., 2013; Givens et al., 2014; Ingerslev et al., 2014b). Although the theory of a core gut microbiota has primarily been explored in mammalian hosts, some authors believe that this concept may also apply to bony fish, particularly with respect to the physiology, evolutionarily conserved points of digestive tract morphology, immunity and the preferred salinity levels in the surrounding water (Roeselers et al., 2011). The hypothesis that the shared microbiota is shaped by evolutionarily conserved aspects of digestive tract anatomy, physiology and immunity is currently receiving increased attention, though additional studies are required to confirm this concept. Additional information on the core gut microbiota will likely facilitate the development of safe and effective methods for manipulating the composition of the gut microbiota to promote the health and growth of fish (Turnbaugh et al., 2009; Roeselers et al., 2011; van Kessel et al., 2011; Wu et al., 2012b).

NGS approaches offer the ability to obtain a large number of sequence reads in a single run, yielding more accurate and reliable sequence datasets that are orders of magnitude larger than those produced by previous culture-independent techniques. These technologies thus allow researchers to apply a massively parallel sequencing approach to address the core gut microbiota concept and to examine the relationship between gut microbiota composition and lifestyle in a large number of fish species with different or similar features, such as gastrointestinal (GI) physiology and feeding behaviour.

4.2. The influence of environmental and host-related factors

Understanding the factors that regulate community membership and the structure of the microbiota of the fish intestine is crucial for improving fish performance (Wu et al., 2012b). Colonisation from the surrounding environment is thought to be one of the primary mechanisms of microbiota acquisition for fish (Nayak, 2010) and has been demonstrated in some carp species (Table 2). Wu et al. (2012b) applied 454 pyrosequencing to track the origins of bacteria present in the intestinal lumen of grass carp and determined that the microbiota is notably enriched from the surrounding water and sediment. This finding is similar to that of Wu et al. (2013), who confirmed that the culture environment, i.e., sediments and surrounding water, was the most important source of gut bacteria in gibel carp. Sullam et al. (2012) performed a meta-analysis based on 25 bacterial 16S rRNA gene sequence libraries obtained from the intestinal microbiota of different fish species (e.g., salmon, trout and zebrafish) to address the relationship between the gut microbiota of fish to biotic and abiotic variables such as trophic levels and habitats. The authors observed increased representation of OTUs from the bacterial order Aeromonadales in freshwater fishes and from Vibrionales in saltwater fishes. A recent study of the intestinal microbiota of zebrafish and their housing water under fed and starved conditions revealed that some bacterial taxa observed in the fish intestine were found at similar frequencies in the water, while other taxa were enriched specifically in the intestine (Semova et al., 2012). This finding suggests that the fish gut more closely resembles the surrounding environment than does the mammalian intestinal microbiota, even more so under starvation conditions due to a limited selective environment (Semova et al., 2012; Sullam et al., 2015). By using metagenomics alongside 16S rRNA analysis, Xing et al. (2013) characterised the taxonomic and metabolic profiles of the farmed adult turbot (*Scophthalmus maximus*) and investigated how the microbiome is related to the surrounding aquatic environment. The results showed that both the GI tract and mucus samples from the fish were largely made up of Proteobacteria and Firmicutes. The authors also found that the GI tract might possess bacteria that are initially associated with seawater.

However, other studies have shown that the composition of the fish intestinal microbiota is not a simple reflection of the microorganisms in their local habitat; instead, other host-related factors can also shape fish gut microbial communities (Roeselers et al., 2011; Larsen et al., 2014; Ye et al., 2014; Li et al., 2015). To quantify the effect of habitat selection and the surrounding environment on the fish gut microbial community, Roeselers et al. (2011) characterised and compared the bacterial membership and structure among laboratory-reared zebrafish in different geographic locations with zebrafish collected from their natural habitat using 16S rRNA

clone library sequencing and 454 pyrosequencing approaches. Notably, they found that regardless of salient differences in the life histories and local environments of the two groups of zebrafish, a core set of bacterial genera, including the classes Gammaproteobacteria and Fusobacteria (genera including: *Aeromonas*, *Shewanella*, *Pseudomonas*, *Stenotrophomonas*, *Vibrio*, *Burkholderia*, *Diaphorobacter*, *Cetobacterium*, *Streptococcus*, *Bacillus*, *Cloacibacterium* and *Propionibacterium*), were present in both domesticated and wild-caught zebrafish (Roeselers et al., 2011). According to the study of Li et al. (2012), distinct dominant intestinal microbiota compositions were observed in four fish species (silver carp (*Hypophthalmichthys molitrix*), grass carp (*Ctenopharyngodon idella*), bighead carp and blunt snout bream (*Megalobrama amblycephala*)) reared in the same environment. Another study on the juvenile paddlefish (*Polyodon spathala*) and bighead carp detected species-specific intestinal microbiota even when the fish were reared in the same pond with similar plankton available, with significantly lower diversity among the paddlefish microbiota (Li et al., 2014b). Franchini et al. (2014) also found significant differences in the microbiota of limnetic and benthic adult cichlid fish reared under common environments. Recent analyses of pyrosequencing data from three species of carp (Li et al., 2015) clearly revealed that the intestinal bacterial communities of the three species harboured specific features despite the fish cohabiting in the same environment. Other studies on rainbow trout (*Oncorhynchus mykiss*), transgenic common carp (*Cyprinus carpio* L.), silver carp (*H. molitrix*), and gizzard shad (*Dorosoma cepedianum*) have also shown that even under a common living/rearing/feeding environment, significant differences are present in the intestinal bacterial communities of different species (Navarrete et al., 2012; Li et al., 2013; Franchini et al., 2014).

The host may contribute to the bacterial community assemblage by selecting for microbial populations that include specialised bacteria to aid in the digestion and absorption of nutrients from a variety of food sources (i.e., protein versus chitin or structural polysaccharides) (Roeselers et al., 2011). Recent studies based on 16S rRNA gene sequencing in cyprinids have demonstrated a significant role for intestinal microbiota in the digestion of plant material within the fish intestinal tract (e.g., van Kessel et al., 2011; Wu et al., 2013; Li et al., 2015). van Kessel et al. (2011) analysed the taxonomic profiles of the bacterial communities associated with the GI contents of the common carp using the 454 pyrosequencing technique. Notably, bacterial taxa known to be involved in vitamin production and food digestion comprised the majority of the retrieved sequences, and, interestingly, some of these bacteria had not been previously reported in common carp (van Kessel et al., 2011). Fusobacteria and Bacteroidetes were two of the well-represented groups within the retrieved sequences, the latter being known for fermentative metabolism and degradation of oligosaccharides derived from plant material, followed by Planctomycetes, Gammaproteobacteria, Clostridia, Verrucomicrobiae and Bacilli. Comparisons of the intestinal microbiota of three different carp species by pyrosequencing also revealed higher abundances of Fusobacteria, Firmicutes, Proteobacteria and Bacteroidetes among the other phyla, though with significant differences between species (Li et al., 2015). Although *Cetobacterium* was the most dominant genus in crucian carp (97.29 ± 0.46%), it composed only 40 and 60% of the sequences read in grass carp and bighead carp (*Hypophthalmichthys nobilis*), respectively. The presence of *Cetobacterium* genus members that can perform fermentative metabolism of peptides and carbohydrates and produce vitamin B12 (cobalamin) (Larsen et al., 2014) has been reported in several species of the Cyprinidae family that are not dependent on dietary vitamin supplements, including goldfish, zebrafish and the common carp (*C. carpio*) (Rawls et al., 2006; Tsuchiya et al., 2008; Silva et al., 2011; van Kessel et al., 2011). Thus, the combination of a fermentative metabolism with de novo vitamin production may explain the relevance of *Cetobacterium* sp. in the gut microbiota of carp species.

The above-mentioned findings suggest that phylogenetic factors such as host physiology and gut anatomy may interact with environmental and ecological factors (e.g., biogeography of host fishes), such that many factors must be considered when assessing relationships between microbiota composition and host biology.

4.3. Contribution of feeding habit, diet and genetic factors

Feeding habit is an important factor affecting gastrointestinal microbiota. Bacterial diversity has been observed to increase from carnivores to omnivores to herbivores in humans, mice and certain fish species (Ley et al., 2008; Ward et al., 2009; Givens, 2014; Larsen et al., 2014; Li et al., 2014a).

Givens et al. (2014) applied 454 pyrosequencing to investigate correlations between gut microbiota composition, lifestyle and feeding behaviour in 12 finfish and three shark species. Proteobacteria ribotypes were present in all sampled marine fish and often dominated the gut microbiota. Firmicutes comprised the second-most prevalent ribotypes. The results indicated that the gut microbiota of omnivorous fish were more diverse than the gut bacterial communities of carnivorous fish, with the diversity indices for the herbivorous adult pinfish being greater in richness and diversity than those of the piscivores (Givens et al., 2014). These findings were generally in accordance with the conclusions of Larsen et al. (2014), who performed a study of gut-associated bacterial communities in channel catfish (*Ictalurus punctatus*, omnivorous), largemouth bass (*Micropterus salmoides*, piscivorous) and bluegill (*Lepomis macrochirus*, herbivorous) and found that in terms of the bacterial species evenness index, the gut microflora of the omnivore was more diverse than the carnivore gut communities. Fusobacteria were found at high levels (82.6% in *L. macrochirus*, 90.6% in *M. salmoides* and 94.9% in *I. punctatus*) in the intestinal lumen-associated bacterial communities, followed by *Proteobacteria* (Larsen et al., 2014). Givens (2014) also reported that the diversity of intestinal microbiota was lowest in *Lagodon rhomboides* fed an invertebrate diet (krill), greater in *L. rhomboides* fed an omnivorous diet (seaweed and krill), and greatest among those fed a vegetarian (seaweed) diet. These findings agree with the conclusions of Ley et al. (2008), who found that diversity was lowest in the faecal microflora of carnivores in a study of humans and terrestrial mammals. Fish with diverse diets may support richer gut-associated bacterial communities as a result of the wider range of potential substrates available to the microbes (Givens et al., 2014).

The interaction between environment and genotype and their possible effects on fish gut microbiota composition have also been investigated (Bolnick et al., 2014c; Sullam et al., 2015) (Table 3). Sex is regarded as a possible genetic polymorphism that could regulate environmental effects (Bolnick et al., 2014c). By applying Illumina HiSeq 2000 sequencing, Bolnick et al. (2014c) reported that diet changes gut microbial communities in a sex-specific manner in wild-caught threespine stickleback (*Gasterosteus aculeatus*) and Eurasian perch (*Perca fluviatilis*). This observation was also made in laboratory-reared fish, with a significant sex∗diet interaction effect on the abundance of OTUs in male and female fish (Bolnick et al., 2014c). Bolnick et al. (2014a) addressed to what degree a single immune system-related gene of the host, i.e., the major histocompatibility complex class II (MHCII) receptors of antigen-presenting cells, influences the establishment and maintenance of gut microbial communities. Interestingly, they found an inverse and sex-dependent relationship between MHCII allele diversity and gut bacterial community diversity in the threespine stickleback. That is, individuals with greater MHCII allele diversity had less diverse bacterial communities, suggesting that adaptive immunity can constrain commensal bacterial communities (Bolnick et al., 2014a; Stagaman et al., 2014).

It is now widely accepted that diet modulates the species composition of symbiotic gut microbiota in vertebrates (Bolnick et al., 2014c). Studies have suggested that dietary composition is one of the most important factors shaping the fish GI microbial community and altering the metabolism and population sizes of key symbiont species, resulting in biological changes to the host (Fig. 1) (Ringø et al., 2006, 2014; Sullam

et al., 2012; Askarian et al., 2013). Emerging NGS instruments have made it possible to better understand how diet influences the fish GI bacterial community. As the contribution of aquaculture to global fish consumption continues to increase, much research has focused on plant-protein diets as an alternative to the traditional protein source in aquaculture feed.

From studies on rainbow trout, it is evident that the gut microbiota of this fish is dominated by four phyla (Bacteroidetes, Proteobacteria, Firmicutes and Actinobacteria). These four phyla constitute the 'core' microbiota in the gut of rainbow trout, regardless of whether the fish has initiated first feeding and whether the feed has a marine or a plant-based origin (Desai et al., 2012; Wong et al., 2013; Ingerslev et al., 2014a, 2014b). Wong et al. (2013) also found a core set of intestinal bacteria in rainbow trout that were resistant to dietary changes. Indeed, variations in diet and rearing density caused consistent alterations in only a limited number of bacterial community members, including the phylum Firmicutes, the family Lactobacillaceae and its genera *Lactobacillus* and *Streptococcus*. Several studies have demonstrated the impact of diet composition and the origin of the ingredients (marine versus plant-derived) on gut microbiota in rainbow trout. Pyrosequencing data revealed that in rainbow trout, a diet containing protein from vegetables such as pea and soy generally led to a higher abundance of bacteria belonging to the phylum Firmicutes relative to the phylum Proteobacteria compared with a fish meal-based diet (Desai et al., 2012). Recently, Ingerslev et al. (2014b) were able to detect direct relationships between ontogenetic development, the onset of first feeding, diet type and the intestinal microbiota in rainbow trout using the Illumina HiSeq sequencing platform. During the period of the first feeding, the microbiota was dominated by the four phyla, Bacteroidetes, Proteobacteria, Firmicutes and Actinobacteria, but administering a plant-based diet containing rape seed oil and pea meal favoured a significantly higher abundance of the genera *Weissella*, *Streptococcus* and *Leuconostoc* from the *Firmicutes* phylum. The marine-based diet instead favoured the presence of Proteobacteria. The same trend was seen in adult rainbow trout using plant-based diets containing meal or protein concentrate from pea or soy (Desai et al., 2012). Using 16S rRNA pyrosequencing, Givens (2014) investigated the effect of diet-associated bacteria on the composition of the gut microflora community in cultured mummichogs (*Fundulus heteroclitus*) and in wild juvenile and adult pinfish (*Lagodon rhomboids*). Notably, the authors found that in both sterilised and unsterilised diet treatments, the taxonomic profile of the simple gut of *F. heteroclitus* was more directly influenced by diet-associated bacteria than the community of the more differentiated gut of *L. rhomboides*, possibly due to more complete digestion of the microbial biomass by *L. rhomboides* than by *F. heteroclitus*. The results also revealed that diet directly impacts the microbial community present in the fish gut via contributing diet-associated ribotypes to the community and also through the proliferation of certain bacterial ribotypes.

Collectively, the positive relationships between fish genetic attributes and the intestinal microbiota diversity observed in different studies (Table 2) suggest an influence of host genetic factors and trophic ecology on intraspecific variation in the gut microbiome. However, diet may also rapidly shift the gut microbiota in ways that can be influenced by sex or host genotype (Bolnick et al., 2014a, 2014c; Sullam et al., 2015). More studies are still needed to better understand whether and how diet is involved in shaping the fish gut-associated microbiota (Semova et al., 2012; Geraylou et al., 2013a; Bolnick et al., 2014b, 2014c; Ingerslev et al., 2014a; Zarkasi et al., 2014; Sullam et al., 2015).

4.4. Essential oil, pro- and prebiotic applications

The impact of specific treatments applied during rearing, such as the administration of probiotic and prebiotic supplements, has also been addressed in some studies. Indeed, one of the most important goals in studying fish microbiota in aquaculture is to develop effective strategies for manipulating gut microbial communities to promote and sustain the health of the farmed fish (Merrifield et al., 2010; Ringø et al., 2010). Approaches to manipulate the microbiome include probiotics, prebiotics, immunostimulants, vitamins and nucleotides (Ringø et al., 2010; Carda-Diéguez et al., 2013). Promoting beneficial intestinal bacterial species with the use of pro- and prebiotics is a promising method for improving the overall condition of the animals (see review by Merrifield et al., 2010; Ringø et al., 2010). Altering the intestinal microbiota in favour of beneficial bacteria may increase the production of beneficial metabolic products and result in the elevation of pathogen resistance, growth enhancement, improved lipid metabolism, stimulation of immune responses and a better physiological status for the gut. Although a growing number of studies have asserted the positive influence of administering supplementary compounds on fish growth performance, digestive function, and immunity, many of these reports did not investigate the effect of these compounds on the gastrointestinal microbiota. Indeed, few studies have applied NGS techniques to monitor the effects of these supplements on the fish gut microbiota. Carda-Diéguez et al. (2013) investigated the effect of administering β-1,3/1,6-glucans (immunostimulants) and plant essential oils on the gut-associated bacterial communities in cultured European sea bass (*Dicentrarchus labrax*) through 454 pyrosequencing technology. After 8 weeks of treatment with the supplemented diets, the authors found that the addition of essential oils, but not β-glucans, resulted in a significant shift in the gut microbiota.

In Siberian sturgeon (*Acipenser baerii*), 454 pyrosequencing analysis of 16S rRNA genes was used to investigate the effect of the prebiotic carbohydrate AXOS (AXOS-3-0.25, low degree of polymerisation; and AXOS-32-0.30, high degree of polymerisation) on juvenile Siberian sturgeon growth performance, immune responses and allochthonous hindgut microbiota. Both supplementations enhanced the phagocytic activity of macrophages, while the alternative haemolytic complement activity and total serum peroxidase content improved only in fish fed AXOS-32-0.30. Neither AXOS preparation affected growth performance or the activities of serum lysozyme or superoxide dismutase. However, AXOS administration in juveniles led to significant and beneficial shifts in gut-associated bacterial communities towards butyrate-producing/enhancing bacteria, including lactic acid bacteria and *Clostridium*, and therefore increased the concentration of short chain fatty acids (higher in fish fed AXOS-32-0.30 than AXOS-3-0.25) (Geraylou et al., 2013b). The study concluded that AXOS improved sturgeon health through prebiotic actions, but the induced effect was dependent on the degree of polymerisation. By applying 16S rDNA pyrosequencing, Geraylou et al. (2013a) observed that the inclusion of *Lactococcus lactis* spp. *lactis* in combination with AXOS resulted in a significant reduction in bacterial diversity while improving growth performance and positively affecting the immunological parameters of Siberian sturgeon. It is clear from these studies that a greater emphasis on understanding the effects of probiotics and prebiotics on the complex microecology of the intestinal microbiota of fish is essential. Future studies must incorporate more sensitive molecular-based methods, including next-generation sequencing, into probiotic studies to determine microbial abundances as well as elucidate microbial functionality and activity. Such studies will help to identify suitable prebiotics and optimum levels of supplementation for each species, expanding our knowledge of beneficial endogenous intestinal microbial communities.

5. Future lines of research: Moving from phylogenetic characterisation to functional metagenomics

Although studies applying NGS techniques to provide greater in-depth knowledge of the fish gut microbiota are continually increasing in number, there are still some issues that warrant further examination. For example, one overriding problem is whether it is possible to differentiate members of the indigenous (fish) microbiota from transients that could be present in the water film around the fish or in the water/food within the digestive tract. How can NGS methods be

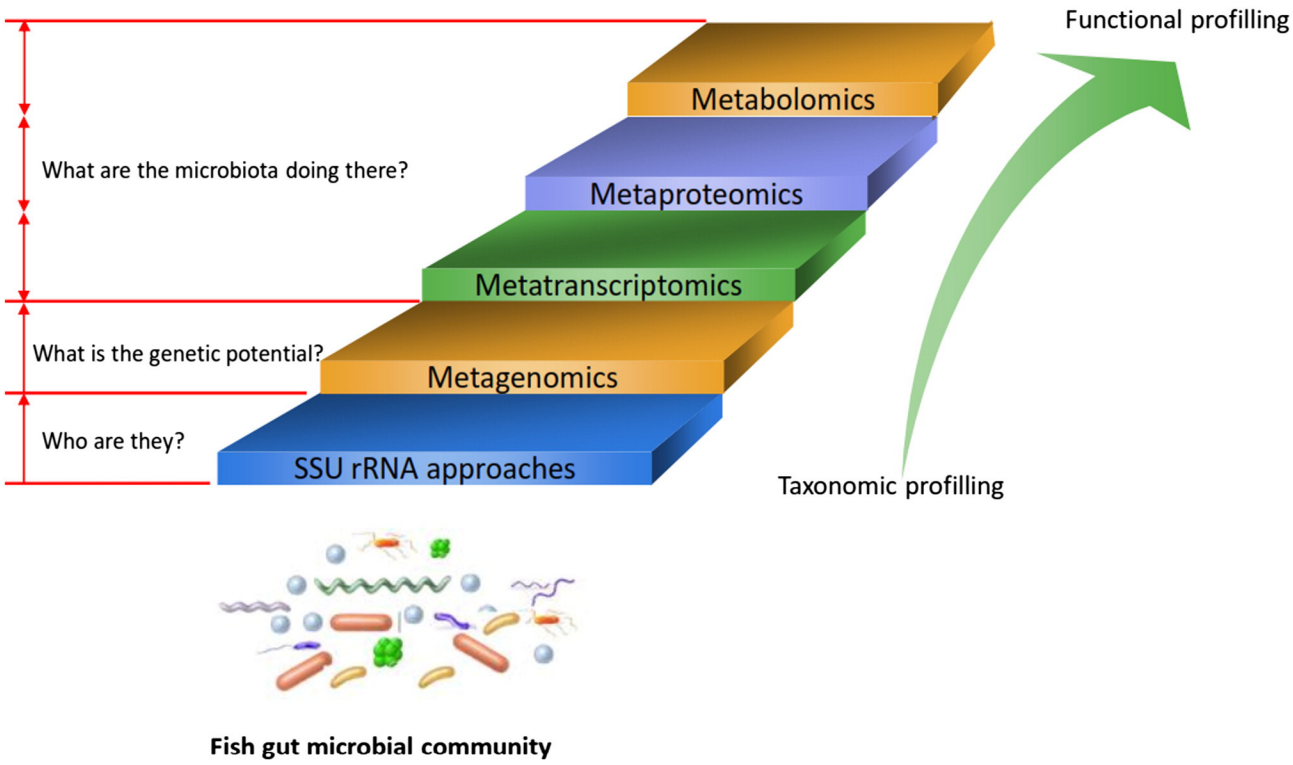

Fig. 2. Enhancing the basic amplicon-based metagenomic approach through the complementary use of other meta-omic approaches. Metagenomic analyses of microbial communities by random shotgun sequencing should be complemented by parallel detection and analysis of transcripts ('metatranscriptomics'), expressed proteins ('metaproteomics') and produced metabolites (metabolomics) to correctly address the relationship between fish gut bacteria and fish behaviour.

used to distinguish these two groups? Unfortunately, most publications have not addressed this issue. Therefore, optimisation of sample preparation should be taken into account in future studies to truly address the spatial distribution of the gut-associated bacterial community.

Studies in humans and other vertebrates have shown that the gut microbiota plays a direct role in the etiopathogenesis of a number of systemic disorders (Llewellyn et al., 2014). While most of the studies in fish have mentioned that changing husbandry treatments (such as administration of plant-based diets) favours a shift of the fish gut bacterial community towards specific taxa (primarily lactic acid bacteria), the functional impact of these bacteria on the fish intestine is unknown and needs to be systematically analysed. With access to NGS technology, future research on fish gut microbiota should aim to integrate cutting-edge genomic platforms to truly address these concerns and unravel the potential roles of different microbes in the fish gut microbiota. However, only limited metagenomic information on the fish GI microbiome is currently available (Xing et al., 2013; Ni et al., 2014; Xia et al., 2014). To address this issue, researchers in the field of fish microbiology need to consider the application of metagenomics from a broader perspective and as complementary to other meta-omic approaches (Fig. 2). While small-subunit ribosomal RNA (16S rRNA) has been widely used in fish gut microbiota studies to provide a general overview of the taxonomic profile of prokaryotes, metagenomic analyses actually make the entire genetic complement of a microbial community accessible. Metagenomics is defined as the large-scale application of random shotgun sequencing to DNA extracted directly from environmental samples and resulting in at least 50 megabase pairs (Mbp) of sequence data (Warnecke and Hugenholtz, 2007). Transcriptomic and proteomic analyses have been used on microbial isolates for several years to observe their expressed metabolic potential. These approaches have recently been applied in a high-throughput fashion to microbial communities, giving rise to the terms 'metatranscriptomics' and 'metaproteomics' (Fig. 2). Metatranscriptomics includes the retrieval and sequencing of mRNA from a complex microbial ecosystem to determine the active bacterial taxa in the gut and to assess what genes are expressed in that community, revealing responses to environmental fluctuations (Sorek and Cossart, 2010; Franzosa et al., 2015). Metaproteomics aims to identify the microbial proteins translated in a complex mixed population by matching their sequences with the available metagenome database and assigning putative functions to reflect the catalytic potential of the microbiota (Sorek and Cossart, 2010; Franzosa et al., 2015). Metabolomics seeks to quantify all of the metabolites in a cellular system under defined conditions and at different time points so that the dynamics of any biotic, abiotic or genetic perturbation can be accurately assessed. In the near future, the challenge for the scientific community will be to integrate these complementary meta-omics data into an ecosystem-level approach to study the fish microbiome (Warnecke and Hugenholtz, 2007; Franzosa et al., 2015).

Numerous strategies for the manipulation of the fish gut microbiome have recently emerged (Llewellyn et al., 2014). Probiotics are microorganisms that are expected to benefit host health. The aquaculture industry has been overwhelmed by probiotic supplements that base their activity on selected bacterial strains and promise to benefit fish immunology or digestive processes, although these effects have generally not been well studied, and the mechanisms are unknown. It is possible that the effect of a probiotic is strain-specific and cannot be extrapolated to other probiotics. For these reasons, new systematic studies using NGS platforms and more detailed characterisations of the mechanisms of action are required.

In relation to the significance of microbial genetic content for the health and disease of the host, a new concept has been presented called the 'minimal gut genome,' defined as those genes required for gut bacteria to compete in the gut environment (e.g., housekeeping and gut-specific functions, such as adhesion to the host epithelium) (Qin et al., 2010). This minimal gut genome is expected to be present in most or all gut bacterial inhabitants, and its characterisation should improve our understanding of how evolution has shaped successful gut-colonising

bacteria (Maccaferri et al., 2011). Additionally, the 'minimal gut metagenome' has been identified in humans as a set of functions involved in the homeostasis of the whole ecosystem, half of which (approximately 45%) are present in approximately 10% of the sequenced bacterial genomes and are hence considered 'rare'. In other vertebrates such as fish, the majority of these genes are poorly characterised, highlighting the still-limited knowledge of gut ecosystem functionality and the need for more sequencing efforts and functional analyses to explore the genetic and metabolic potential of the gut microbiome in animals such as fish. Hopefully, these future studies will lead to the refinement and augmentation of our understanding of microbiota-driven changes in fish physiology and growth.

6. Conclusions

Characterisation of the diverse microbial communities that are ubiquitous in any environment represents a significant approach towards a better understanding of the factors that determine the composition of the microbiota and their respective roles in the ecosystem. The role of microorganisms in the ecosystem is also of great relevance for developing effective strategies to manage and manipulate microbial communities. This review provides information regarding how new nucleic acid-based sequencing platforms and their applications are helping to improve our knowledge of bacterial community profiles in the fish gut-associated microbiota, including how these communities respond to a variety of factors affecting the host. NGS approaches have already transformed microbiology research, allowing for the expansion of large community studies of the microbiota of diverse animal species. Despite the above-mentioned shortcomings of NGS technologies, rapid improvements in technical and bioinformatic tools (e.g., the increasing availability of bioinformatic tools to denoise sequences) are likely to further support these platforms as major methodologies in the field of fish microbiology research. The results produced by rapid, low-cost, and reliable NGS techniques will continue to improve our knowledge of the bacterial community profiles of fish GI microbiota. These high-throughput sequencing technologies have enabled us to monitor and quantify the impact of the host fish's environment on its gut microbiota, both within a single group of fish over time and globally across different locations. The knowledge gathered from such studies will be useful for improving nutrient management for fish and helping the aquaculture industry increase fish harvesting efficiency and optimise feeding management.

Conflict of interest statement

The authors have no conflict of interest to declare.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.aquaculture.2015.06.033.

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CHAPTER 5

Community membership and structure of mucosa and luminal-associated intestinal microbiota in snow trout (*Schizothorax zarudnyi*) as revealed by pyrosequencing

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Abstract

To provide some in-depth knowledge of the community membership and the relationship between the constituent members of the resident microbes (microbiota) in male and female Snow trout (*Schizothorax zarudnyi,)* this study aimed at applying a high-throughput parallel sequencing approach. For this purpose the mucosa and luminal-adherent intestinal microbiota were investigated. Moreover, the developed bioinformatic pipeline PICRUSt was implemented to address the functionality of the bacterial community in snow trout intestinal microbiota. The results showed that Firmicutes phylum members represented the dominant phyla of mucosal-associated microbiota regardless of the fish gender, constituting 87.1% and 68.5% of total sequences in males and females, respectively. However, Fusobacteria phylum group made up the most dominant group in fish intestinal lumen of male and female with 75.9% and 66.6%, respectively. *In silico* functional analysis using PICRUSt revealed that the gut microbiome of snow trout has pronounced activity in terms of metabolism of carbohydrates, amino acids, vitamins etc. with comparatively similar gene ontology among the fish intestinal lumen and mucosa-associated microbiota. It is concluded that the community membership and structure of bacterial populations at the mucosal epithelium differs from the luminal-associated microbiota of snow trout, indicating that some microbial species obviously poorly colonise gut mucosal layer and that host-related factors are of relevance to the gut ecosystem.

Introduction

Indigenous microbiota play a crucial role in their vertebrate hosts. It is increasingly clear that the intestine is a multifunctional organ system involved in the digestion, resorption and absorption of food components, in electrolyte balance, endocrine metabolic regulation of and immunity against pathogens (Leser & Molbak, 2009). As in mammals, studies on the intestinal microbiota of fish have confirmed these assumptions and revealed that the bacterial community may exert several effects (Ringø *et al.*, 1995, Ringø & Birkbeck, 1999, Austin, 2002, Ghanbari *et al.*, 2009, Nayak, 2010). Studies also have shown that the composition of the gut microbiota varies among fish species, and that dominant bacteria are typically either aerobes or facultative anaerobes (e.g., see reviews by (Cahill, 1990, Ringø *et al.*, 1995, Austin, 2002, Nayak, 2010). Many fish species possess a gut microbiota that is dominated by γ-Proteobacteria, Firmicutes and Bacteroidetes (Austin, 2002, Kim *et al.*, 2007, Desai *et al.*, 2012, Carda-Diéguez *et al.*, 2013, Larsen *et al.*, 2014). Relevant genera in the gut microbiota of freshwater fish species are usually comprised of *Aeromonas* sp., *Pseudomonas* sp., *Flavobacterium*/*Cytophaga* species, *Enterobacter* sp., and/or *Acinetobacter* sp. (Cahill, 1990, Ringø *et al.*, 1995, Ringø & Gatesoupe, 1998, Ringø & Birkbeck, 1999, Austin, 2002, Ringø *et al.*, 2007, Wu *et al.*, 2013, Larsen *et al.*, 2014). Lactic acid bacteria (mainly *Lactobacillus* sp.) have also been found to be minor hosted by both freshwater and marine fish (Ringø & Gatesoupe, 1998, Ghanbari *et al.*, 2013a, Ghanbari *et al.*, 2013b). Some microbiota in the intestine are considered to be transient, whereas others seem to be members of the established microbiota associated with the intestinal mucosa. As the intestine mucosal microbiota may possess the function to prevent pathogens from colonization and beneficially contributes to the induction of the host innate immune response and nutrient exchange (Kim *et al.*, 2007), it is likely that the bacteria inhabiting this environment are of significance to the host. It is apparent that most of the previous publications do not thoroughly address this issue and rather rely on the identification of bacteria from intestinal lumen when reporting about the diversity of the microbiota in fish intestine. Thus our present knowledge of the bacterial diversity associated with the fish gut still lacks the spatial characterization of fish intestine (Rawls *et al.*, 2004, Roeselers *et al.*, 2011). Fish represent an important group for understanding the variety and nature of symbioses in vertebrate gut microbial communities (Nayak, 2010). Comprising a large diversity of approximately 28,000 species, fish include

nearly half of all vertebrate species and exhibit a wide variety of physiologies, ecologies and natural histories (Wong, Rawls, 2012). The digestive tract of fish receives water and food from the surrounding environment that is populated with microorganisms, and these microbes undoubtedly affect the resident microbiota (Nayak, 2010).

Snow trout (*Schizothorax zarudnyi*), a cold water riverine and short migratory fish is one of the most commercially relevant and important native fish in Hamoun Lake and Hirmand River system (Khajeh & Ghanbari, 2011). It belongs to the family *Cyprinidae* and sub-family *Schizothoracinae,* which are widely distributed in the Himalayan and sub-Himalayan region and the rest of Asia (Khajeh & Ghanbari, 2011). Almost nothing is known about the nature and the ecology of intestinal bacteria and their impact on health and physiology of this important fish. Hence this study aims at applying the pyrosequencing technique 1) to create a survey of the intestinal microbiota from both the male and female snow trout, 2) to clarify whether the composition of the bacterial community in the intestinal mucosa differ from that in the intestinal lumen and 3) to investigate if possible differences in composition could be related to different functional profiles.

Materials and methods

Sample collection and DNA extraction

In this study, 16 snow trouts were caught from Hirmand River (Sistan, Iran) and Hamoun Conservation Area wetlands in Iran during September and October 2013. The sex ratio of both fish species was half male and half female. Prior to dissection, all fish‘s morphometric and meristometric data were recorded and then the fish were euthanized with an overdose of tricaine methanesulfonate. All instruments, surfaces, and the exterior of each fish were treated with 70% EtOH and instruments were flame-sterilized prior to dissection. After opening the body cavity, the intestines were separated. Digesta from the fish intestinal lumen were gently squeezed out and the intestines were washed three times by 3 ml of sterile 0.9% saline solution containing 0.1% (v/v) Tween 80. Then these samples (digesta and intestine) were transferred to a sterile screw cap tube containing Phosphate Buffer Solution (PBS) and stored at -20 °C. After transportation to the Laboratory of Food Microbiology at the University of Natural Resources and Life Sciences in Vienna, Austria. Digesta from each sample were washed once by suspension in 2 ml of saline containing 0.1% (v/v) Tween 80 and centrifuged at 4 °C for 5 min. After removal of the pellet, bacterial

cells were recovered by centrifugation at 20,000 *g* for 20 min at 4 °C. Bacterial samples prepared in this manner were immediately subjected to DNA extraction process. The intestinal mucosa-associated bacterial were prepared as described by Gong *et al.* (2002) with some modifications. Collected parts of the intestines were opened longitudinally and briefly washed three times in sterile 0.9% saline solution by gentle agitation to remove unattached or loosely attached bacteria from the wall. Bacterial cells were released from the intestine wall in three 1-min washes using a 10-ml conical tube containing saline and 0.1% (v/v) Tween 80 by vigorously shaking the tube. The washes were pooled and then centrifuged (20,000 *g*, 20 min) at 4 °C to pellet the cells. Genomic DNA of each sample was extracted and purified using repeated bead beating plus column method (Zhongtang & Morrison, 2004).

454 pyrosequencing and bioinformatic analysis

In total, 32 genomic DNA samples were obtained from the intestinal mucosa and lumen of 16 fish. The samples were coded as follow: SMG (the intestinal mucosa of male fish), SFG (the intestinal mucosa of female fish), SMC (the intestinal lumen of male fish) and SFC (the intestinal lumen of female fish). From the resulting DNA, the V3-V6 region of bacterial 16S rRNA genes was amplified using degenerate F338 (ACTYCTACGGRAGGCWGC) and R1061 (CRRCACGAGCTGACGAC) primers tagged with the Lib.A and Lib.B Roche 454 Titanium sequencing adapters using hot start and proofreading KAPA HiFi PCR kit (KAPA Biosystems, South Africa). The Forward primers were modified to contain a 10-base barcode unique to each sample. The amplified PCR products were visualised by 2% low-melting gel electrophoresis and purified with PureLink PCR purification Kit (Invitrogen, CA, USA). A Qubit fluorometer (Invitrogen, Carlsbad, CA, USA) was used to quantify PCR products. Equimolar amounts of the replicate PCR products (three) were pooled together and sequenced unidirectional on a 454 GS FLX Titanium platform applying the so-called GS FLX++ update system (Roche Applied Science, Indianapolis, IN, USA) at the Eurofins-Genomics centre (Ebersberg, Germany). Negative controls (no sample added) were included in both the DNA extraction and 16S PCR amplification stages to test for contamination; these PCRs yielded negligible DNA concentrations during Qubit quantitation, indicating contamination was not a problem. Sequence data were prefiltered and analyzed with the Quantitative Insights Into Microbial Ecology (QIIME) v 1.8.0 pipeline (Caporaso *et al.*, 2010b) using default parameters with the following exceptions: we removed

sequences with ≥50 consecutive bases possessing an average quality score of <30 or with lengths of <200 or >1,000 bases. Sequences were then grouped according to their barcodes and then error-corrected and truncated by applying Acacia algorithm (Bragg *et al.*, 2012) based on Quince *et al.* (2009) model. The filtered sequences were binned by the UCLUST into operational taxonomic units (OTUs) using a threshold of 0.97 similarity (Edgar, 2010). Reads that did not match a reference sequence at >97% identity were discarded. Representative sequences from each OTU were then aligned to the Greengenes core set (version gg_otus_13_8) using PyNast (Caporaso *et al.*, 2010a). The representative sequences from each OTU were also taxonomically classified using UCLUST consensus taxonomy assigner (Edgar, 2010). Consensus lineages were assigned at each taxonomic level if ≥97% of the sequences in the OTU agreed with the classification. We then used ChimeraSlayer algorithm to identify and exclude from subsequent analysis any OTUs with chimeric representative sequences (Haas *et al.*, 2011). We rarefied the data to 3,000 sequences per sample to calculate the alpha diversity indices and the conditional uncovered probability for each sample using QIIME v 1.8.0 pipeline (Caporaso *et al.*, 2010b). Because traditional rarefaction or extrapolation to equal-sized samples can misrepresent the relationships between the richness of the communities being compared (Chao & Jost, 2012, Chao *et al.*, 2013), we used a coverage-based estimated richness rarefaction approach to estimate the rate of increase in species richness with increasing sample size (Chao *et al.*, 2013, Hsieh *et al.*, 2014). Bootstrap methods were used to construct confidence intervals for species richness of any rarefied or extrapolated sample. All estimates were obtained by the software iNEXT (interpolation/extrapolation) (Chao *et al.*, 2013). Beta diversity was computed using the weighted and unweighted UniFrac metrics (Lozupone & Knight, 2005, Lozupone *et al.*, 2011) at exactly 3,000 sequences per sample.

The following statistical analyses were conducted in R (R Development Core Team, 2013). For each microbial taxonomic rank (phyla, class, order, family, genus and species), we determined the relative abundance of each taxon identified by Greengenes, out of all identified sequence reads for each individual (not rarefied). Visualization of microbial communities in samples was conducted using Circos (Krzywinski *et al.*, 2009). Permutational Multivariate Analysis of Variance (PerMANOVA) analysis was used to test whether overall microbial community covaries with sex (Bolnick *et al.*, 2014).

Analysis of metabolic potential

In addition to the evaluation of microbial diversity, we implemented the newly developed bioinformatic pipeline PICRUSt (phylogenetic investigation of communities by reconstruction of unobserved states) (Langille *et al.*, 2013b) to address the functionality of the bacterial community in snow trout gut microbiome. For this purpose, another round of the OTU picking protocol (closed-reference) applying QIIME v 1.8.0 was performed for use in PICRUSt pipeline in the following environment: NumPy (1.7.1), biom-format (1.3.1), PyCogent (1.5.3), PICRUSt (1.0.0dev), and PICRUSt script (1.0.0-dev). Sequences were searched against the Greengenes database (version gg_otus_13_8), and taxonomically assigned using UCLUST with default parameters (Edgar, 2010), with normalization to control for differences in 16S rDNA copy number among OTUs. The PICRUSt pipeline scans two main functional databases, the catalogue with the Kyoto Encyclopedia of Genes and Genomes (KEGG) and the Clusters of Orthologous Groups (COG) databases and uses the OTU table of assigned taxa and their relative distribution in different samples to generate the relative abundance of functional categories. To assess the likely accuracy of the PICRUSt analysis, the Nearest Sequenced Taxon Index was calculated. This index indicates the average divergence between each 16S rRNA read within an OTU and the 16S rRNA gene of the closest reference organism with a sequenced genome. The average Nearest Sequenced Taxon Index values for our gut libraries (0.14 ± 0.04) suggests comparable predictive accuracy for our communities as those achieved for mammalian guts (Langille *et al.*, 2013a). In a second quality check, we found that after closed-reference 97% OTU assignment, our communities still showed strong taxonomic resemblance to those of our complete data set. Combined, these analyses suggested fairly high accuracy of subsequent functional predictions. Functional predictions were assigned up to KO tier 3 for all genes. To simplify the analysis, however, only tier 1 functions of “metabolism,” “genetic information processing,” “environmental information processing,” and “cellular processes” were analysed further, as the categories of “organismal systems” and “human disease” were considered as being poorly relevant to environmental samples.

Results

Microbial complexity of fish gut flora

A total of 108,693 quality-filtered sequences were obtained in this study. After removing chimera sequences, the microbial complexity in the gut and gut content of snow trout samples were estimated on the basis of alpha-diversity indices. The estimators of the microbial complexity in the intestinal mucosa and lumen of snow trout on the basis of alpha-diversity indices are shown in Table 1. While we found no significant difference in of alpha-diversity indices between male and female fish, there were statistically significant differences of ACE, Chao 1, PD, observed_species, between mucosal and luminal intestine samples (t-test, $P<0.05$), demonstrating the significantly higher diversity found in the mucosal samples compared to the intestinal lumen samples. Figures 1a and 1b show the sample-size-based rarefaction and extrapolation sampling curve for different samples. In accordance to the statistical analysis of the alpha diversity indexes there is higher richness in the intestinal mucosa then the luminal samples, while there is no difference between species richness among male and female fish,. Figures 1c and 1d show the sample completeness curve (as measured by sample coverage) with respect to sample size. The curve provides a bridge between sample-size and coverage-based rarefaction and extrapolation. It can be seen that the sequencing approach was carried out sufficiently to reach a plateau, and thus provided an accurate assessment on the species diversity of the fish intestinal microbiota. Figures 1e and 1f show coverage-based rarefaction and extrapolation sampling curves for different samples. These curves plot the species richness estimates for rarefied sample and extrapolated sample with respect to sample coverage. The curve reveals that, on average, 50 ± 10 bacterial species cover (dominant) approximately 98-99% of bacterial community in the intestinal microbiota of male and female fish, suggesting the presence of the highly heterogeneous and uneven bacterial community in snow trout intestinal microbiota.

Good's coverage estimations revealed that more than 99.9% of the species were obtained in all of the samples. Conditional uncovered probability based on the model reported by Lladser *et al.* (2011), which estimates the amount of unseen species in the samples, was also lower than 0.01.

Microbial community composition

Figures 2 and 3 illustrate the abundance of major phyla, class, family as well as genus commonly observed in all samples. As it can be seen, Firmicutes plus the Fusobacteria comprised ca. 95 - 99% of total bacterial community, though with different ratios in the intestinal mucosa and lumen samples. Firmicutes were the prominent phyla among the intestinal mucosa-associated bacteria, regardless of the fish gender, and representing 87.1% and 68.5% of total sequences in SMG and SFG, respectively. However, in the intestinal lumen, Fusobacteria phylum made up the most dominant group in the SMC and SFC samples with 75.9% and 66.6%, respectively. Of class phylogenetic level, Clostridia were the most prevalent group in the mucosa and accounted for 79.5% and 64.2% in male and female fish, respectively. In the intestinal lumen, these percentages decreased to 21.7% for male and 30.6% for the female. Fusobacteria class was found to be the dominant group of bacterial community among the luminal samples. Clostridiaceae accounted for 65.4% and 42.0% of total sequences in family level in SMG and SFG , respectively, but only was 18.8% in SMC and 21.0% in SFC. The abundance of Fusobacteriaceae ranged from 75.9 to 66.6% in SMC and SFC, but decreased to 0.3% and 27.6% in SMG and SFG, respectively. As can be seen in Figure 3, *Romboutsia* sp. is the dominant group in SMG and SFG samples and made up 60.2% and 33.9% of respective sequences. In the lumen samples, the majority of community belongs to members of *Cetobacterium* genus, with a percentage of 75.9% and 66.6% in male and female samples.

Based on the Spearman rank correlation's results (Fig. 4 and 5), the correlations between the relative abundance of gut microbiota in male and female snow trout were

ρ= 0.79, 0.64, 0.74 and 0.64 for classes, families, genera and total OTUs, respectively, with all $P<0.0001$ in all cases. PerMANOVAs of Jaccard distance and UniFrac matrices calculated from relative abundance data found no significant between-sex differences in microbiota composition at the levels of Phylum, Class, Order, Family and Genus.

Functional characterization of bacterial communities

To evaluate the functional significance of varying OTU relative abundance, we used 16S sequences to indirectly infer microbiome gene composition using PICRUSt. The greatest number of genes (>45%) in all four type samples where a function could be

assigned encoded proteins involved in “metabolism” among tier 1 KO categories in PICRUSt datasets (Fig. 5). Furthermore, PICRUSt functional inferences revealed >17% and ~16% of sequences among the samples encoded proteins involved in “genetic information processing” and “environmental information processing” respectively (Fig. 5). Notably, among different samples, PICRUSt prediction showed little variation in the abundances and distributions of second and fourth tier KO functional gene annotations with 0.5% and 0.8% standard deviation respectively. Among all samples, the highest standard deviation observed within a tier 1 functional category was 2.19% of sequence reads. A similar PerMANOVA-based approach calculated from genes relative abundance data showed no significant between-sex differences in gene compositions.

Nucleotide sequence accession number

All sequences have been deposited to the NCBI SRA under the accession numbers SRP057633, SRS921008, SRX1008165 and SRR1994878.

Discussion

Fish gut-associated microbial communities exert some pronounced effects on host nutrition, physiology, and growth (Ye *et al.*, 2014). Although studies on gut microbiota in some carp species have been reported (van Kessel *et al.*, 2011, Wu *et al.*, 2012, Wu *et al.*, 2013), no knowledge exists about gut microbial community in Snow trout.

Surveys on the vertebrate gut samples, especially terrestrial mammalian gut microbiota, suggest that Firmicutes and Bacteroidetes are numerically the most dominant phyla (Leser & Molbak, 2009, The Human Microbiome Consortium, 2012, Wu *et al.*, 2012). The present study indicates that Firmicutes and Fusobacteria are the most abundant bacterial phyla in the intestinal mucosa and lumen of the snow trout. Other phyla including Tenericutes, Cyanobacteria, Proteobacteria and Actinobacteria are relatively less pronounced. The results are generally consistent with those of Han *et al.* (2010), van Kessel *et al.* (2011), Wu *et al.* (2012) and Roeselers *et al.* (2011) who demonstrated for different members of the Cyprinidae family that Firmicutes were among the most ubiquitous and common ones, and other phyla such as Bacteroidetes were rather under-represented in the intestinal lumen.

The number of OTUs observed within the gut of snow trout in this study (Table 1) is higher than that reported about the gut of grass carp (Wu *et al.*, 2012), zebrafish

(Roeselers *et al.*, 2011) channel catfish, largemouth bass and bluegill (Larsen *et al.*, 2014), but lower than found in the intestinal contents in grass carp (Wu *et al.*, 2012), silver carp (*Hypophthalmichthys molitrix*) and gizzard shad (*Dorosoma cepedianum*) (Ye *et al.*, 2014). Interestingly, both coverage estimation indices used in our study indicate a higher coverage of OTUs present in the samples tested (99,9%) compared to those calculated in previous studies on fish gut microbiota (96-98%) (Wu *et al.*, 2012, Larsen *et al.*, 2014).

With the highest abundance of Clostridia class members, Firmicutes were identified as prevalent group of the intestinal bacterial communities in the male and female fish examined. In contrast to previous studies attempting to characterize fish gut microbes (van Kessel *et al.*, 2011, Wu *et al.*, 2012, Larsen *et al.*, 2014, Ye *et al.*, 2014) the relatively high proportion of Firmicutes OTUs found in the luminal-associated bacteria of snow trout intestine is different. Notably it looks similar to that found in herbivorous mammals (Ley *et al.*, 2008, Zhu *et al.*, 2011). Clostridia are well-known members of the endogeneus microflora in the intestines of various organisms including fish play some role in the fermentation of various carbon sources (Ray *et al.*, 2009, van Kessel *et al.*, 2011, Larsen *et al.*, 2014, Ye *et al.*, 2014). In this context Ringø *et al.* (1995) reported that Clostridia provide essential fatty acids and vitamins and thus enhance nutrition. Being anaerobic, Gram-positive, mesophilic, motile, spore-forming group they can utilize both simple and complex carbohydrates such as cellulose, cellobiose, xylose, glucose (Munir *et al.*, 2014), and this implies a pronounced role in the utilization of plant materials in the gut of snow trout. Anaerobic metabolism, in general, is an important step in the digestion of plant material, which usually is the major food source for the fish in aquatic ecosystems. *Romboutsia,* the most prevalent genus of Clostridia members in the snow trout gut microbial community, also seems to be of relevance in the intestine of rodents (Gerritsen *et al.*, 2014). This bacterial group is regarded as a flexible anaerobe, adapted to a nutrient-rich environment in which carbohydrates and exogenous sources of amino acids and vitamins are abundantly available (Gerritsen *et al.*, 2014). Applying the whole genome transcriptome analysis, these authors revealed the presence of key pathways involved in the degradation of different carbohydrates in this genus.

According to our findings, genera like *Lactobacillus* and *Pediococcus* were identified as two other important bacterial groups in gut of snow trout. Being lactic acid

bacteria (LAB), most representatives present in the GIT of animals are clearly autochthonous, since they form stable populations throughout the life of the animal host. They also can be propagated to large numbers, and they are present in almost all gut microbiota of animals (Ringø & Gatesoupe, 1998, Walter, 2008, Ghanbari *et al.*, 2009). Previous studies have indicated that lactic acid bacteria like *Lactobacillus*, S*treptococcus*, *Pediococcus* and *Lactococcus* are important biological control agents in aquaculture (Ringø & Gatesoupe, 1998, Balcázar *et al.*, 2006, Ghanbari *et al.*, 2009, Ferguson *et al.*, 2010).

We were further capable of recovering sequences of Tenericutes with prevalence of Mycoplasmataceae from the snow trout gut samples. Although some Mycoplasmataceae have been classified as pathogens, other members have been observed to be simply commensal and act as natural components of bacterial communities without exerting harmful effects on the host (Roediger, 2004, Bano *et al.*, 2007). *Mycoplasma* sp. are associated with the gut microflora of a variety of freshwater and marine fish (Holben *et al.*, 2002, Bano *et al.*, 2007, Ward *et al.*, 2009, Nayak, 2010, Givens *et al.*, 2014).

While Firmicutes were the prevalent group of the autochthonous bacterial communities, surprisingly Fusobacteria made up the biggest proportion of allochthonous bacterial community in the intestinal lumen samples investigated. Fusobacteria are anaerobic, Gram-negative bacilli occasionally providing several benefits to the host, including of the provision of energy to gastrointestinal cells via the production of short fatty acids (Larsen *et al.*, 2014). Representative genera of this phylum, *Cetobacterium* and *Fusobacterium* spp., were identified in the microbiota of fish (Roeselers *et al.*, 2011, Wu *et al.*, 2012, Di Maiuta *et al.*, 2013, Larsen *et al.*, 2014). Almost 70-75% of fusobacterial 16S rRNA sequences from the snow trout intestinal lumen could be allocated to the *Cetobacterium somerae* species. *Cetobacterium somerae*, initially named Bacteroides type A, is a poorly known, microaerotolerant, nonspore-forming, Gram-negative rod with fermentative metabolism of peptides and carbohydrates and capable of producing vitamin B12 (cobalamin) (Larsen *et al.*, 2014). It was originally found in children with late-set autism (Finegold *et al.*, 2003). Since the original description (Sakata *et al.*, 1981), this Fusobacterium (Larsen *et al.*, 2014) has been identified in several species of Cyprinidae family like goldfish, the zebrafish and the common carp (*Cyprinus carpio*) that being not dependent on dietary vitamin supplements (Silva *et al.*, 2011)

(Tsuchiya *et al.*, 2008, van Kessel *et al.*, 2011), (Rawls *et al.*, 2006). Thus the combination of a fermentative metabolism with original vitamin production may explain the relevance of *Cetobacterium* sp. in the luminal-associated bacterial communities of snow trout intestine.

Like in humans (Zoetendal *et al.*, 2002), pig (Looft *et al.*, 2014), mice (Nava *et al.*, 2011) and alligators (Keenan *et al.*, 2013) some different microbial communities were detected between the intestinal mucosa-associated and lumen-associated microbiota in the snow trout, indicating that some microbial species of transient nature usually poorly colonise the mucus layer. *Per*MANOVA analysis revealed that there were no differences in microbial community composition between male and female fish. We therefore conclude that there seems to be no sex-specific effect, a fact that is consistent with just weak or even no such effects in other vertebrates (Fierer *et al.*, 2008, Kovacs *et al.*, 2011, Wu *et al.*, 2011, Bolnick *et al.*, 2014). Our results, however, raise a fundamental question: why are Firmicutes and specially Clostridia family members more prevalent in the gut bacterial community of snow trout? We presume that Clostridia may play a functional role in the gut of the fish related to the maintenance of gut health and nutrient acquisition (e.g. degradation of carbohydrates). Interestingly, the Clostridiales are primary butyrate producers being of relevance in mucin production (Looft *et al.*, 2014, Veiga *et al.*, 2014). Mucin is a glycoprotein that is believed to maintain the integrity of the gut epithelium (Corfield *et al.*, 2000). Recent studies have highlighted the importance of several mucosa-associated bacteria such as butyrate-producing bacteria in vertebrates (Louis & Flint, 2009, Wilson & Castro, 2010, Li *et al.*, 2012, Looft *et al.*, 2014). As butyrate possesses anti-inflammatory properties (Pryde *et al.*, 2002, Hamer *et al.*, 2008), it improves the intestinal barrier by increasing tight junction assembly (Lewis *et al.*, 2010). Our results indicate the presence of genes predicted to encode for enzymes of the butyrate synthesis pathway (thiolase EC 2.3.1.9 (THL); beta-hydroxybutyryl-CoA dehydrogenase (BHCD; EC 1.1.1.35); crotonase (CRO; EC 4.2.1.17); butyryl-CoA dehydrogenase (BCD; EC 1.3.99.2); ElectronTransfer Flavoprotein alpha and beta sub-units (ETFα and ETFβ; E.C. 1.5.5.1); butyrate kinase (BK; EC 2.7.2.7); butyryl-coA acetyl-coA transferase (ButCoA; EC 2.8.3.8). Thus, the higher prevalence of Firmicutes may be seen as an indication for the maintenance of gut integrity.

The functional capacities of the predicted bacterial metagenome in both the intestinal mucosa and the luminal-associated microbiota were also compared the catalogue with the KEGG database and the COG databases (Fig. 5), suggesting that the gut microbiome of snow trout possess some pronounced metabolic activities related to carbohydrates, amino acids, xenobiotics, nucleotides, glycans, vitamins, and lipids (Figures 6a and 6b). In total, 47% of KEGG pathway and 37% of COG categories were related to metabolism category. This finding is obviously due to the fact that carbohydrate utilising bacteria like Fusobacteria and Clostridia are present at relevant numbers. Although the composition of the gut wall and content-related microbiota was different, Gene Ontology (GO) compositional details displayed a rather similar among individuals, at least based on the resolution available with PICRUSt. This may suggest that there is some substantial functional redundancy among many taxonomically disparate microbiota within each population.

Conclusions

Next-generation sequencing studies may significantly contribute to refining and augmenting the knowledge about the nature, the community membership and structure of intestinal microbiota in animals. The microbiota develops and changes in response to a variety of factors affecting the host, including changing environmental conditions such as temperature and salinity, developmental stage, digestive physiology and feeding strategy. The mucosal-associated bacterial communities in the intestine of snow trout are significantly differ from the intestinal lumen. It is quite obvious that host-related factors determine the gut ecosystem in many ways. Further investigations of the gut-specific microbial ecology, in combination with the assessment of physiological parameters, may offer a more in-depth insight into the complexity of this natural environment.

Conflict of Interest

The authors declare no conflict of interest.

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Table 1. Summary of alpha-diversity indices and coverage estimation of difference samples.

#Fish code	Gender	Sample type	Sequences passed quality check	Chao1	ACE	Shannon	PD_whole_tree	Good's coverage estimations
SMG	M	Mocusa	37246	209	215	4.33	15.92	0.99
SMC	M	Lumen	51993	145	159	1.63	9.42	0.99
SFG	F	Mocusa	34121	223	228	3.40	13.85	0.99
SFC	F	Lumen	35035	167	185	2.13	10.70	0.99

Abbreviations: SMG, snow trout/male/mucosa; SMC, snow trout/male/lumen; SFG, snow trout/female/mucosa; SFC, snow trout/female/luemn.

FIGURE LEGENDS

Figure 1. Size-based rarefaction (solid) and extrapolation (dashed) curves (a, b), sample completeness curves (c, d) and the proposed coverage-based rarefaction/extrapolation curve (e, f) with 95% confidence intervals (shaded areas) based on a bootstrap method with 1000 replications) for the comparison of bacterial species richness in samples originating from male versus female and from the fish intestinal mucosa versus the intestinal lumen.

Figure 2. Circular representation of microbial communities in fish gut samples at phylum and class level. Phyla and classes with relative abundance (all samples) lower than 1% were not shown.

Figure 3. Circular representation of microbial communities in fish gut samples at Family and Genus level. Families and genera with relative abundance (all samples) lower than 1% were not shown.

Figure 4. Statistical comparison of microbiota originating from samples drawn from the intestinal mucosa versus the intestinal lumen as well as female versus male. Spearman rank correlations between the $\log_{10}$ relative abundance of microbial taxa are provided for Classes, Families, Genera, and OTUs, all $P<0.00001$. To plot taxa absent in one host, a small value was added to all frequencies before log transformation.

Figure 5. Functional analyses of identified genes in the predicted sequenced genomes inferred from 16S sequences and published microbial genome databases, using PICRUSt. The relative representation of each KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway or COGs (Clusters of Orthologous Groups) category in the core genome and in variable genes in each genome is graphed as a stacked bar chart. A, KEGG pathways. B, COG categories (level 2).

Figure 6. Projection on KEGG metabolic pathways of functions encoded by the snow trout intestinal mucosa-associated microbiota (A) and lumen-associated microbiota (B) reconstructed genome using iPath tool.

Figure 1

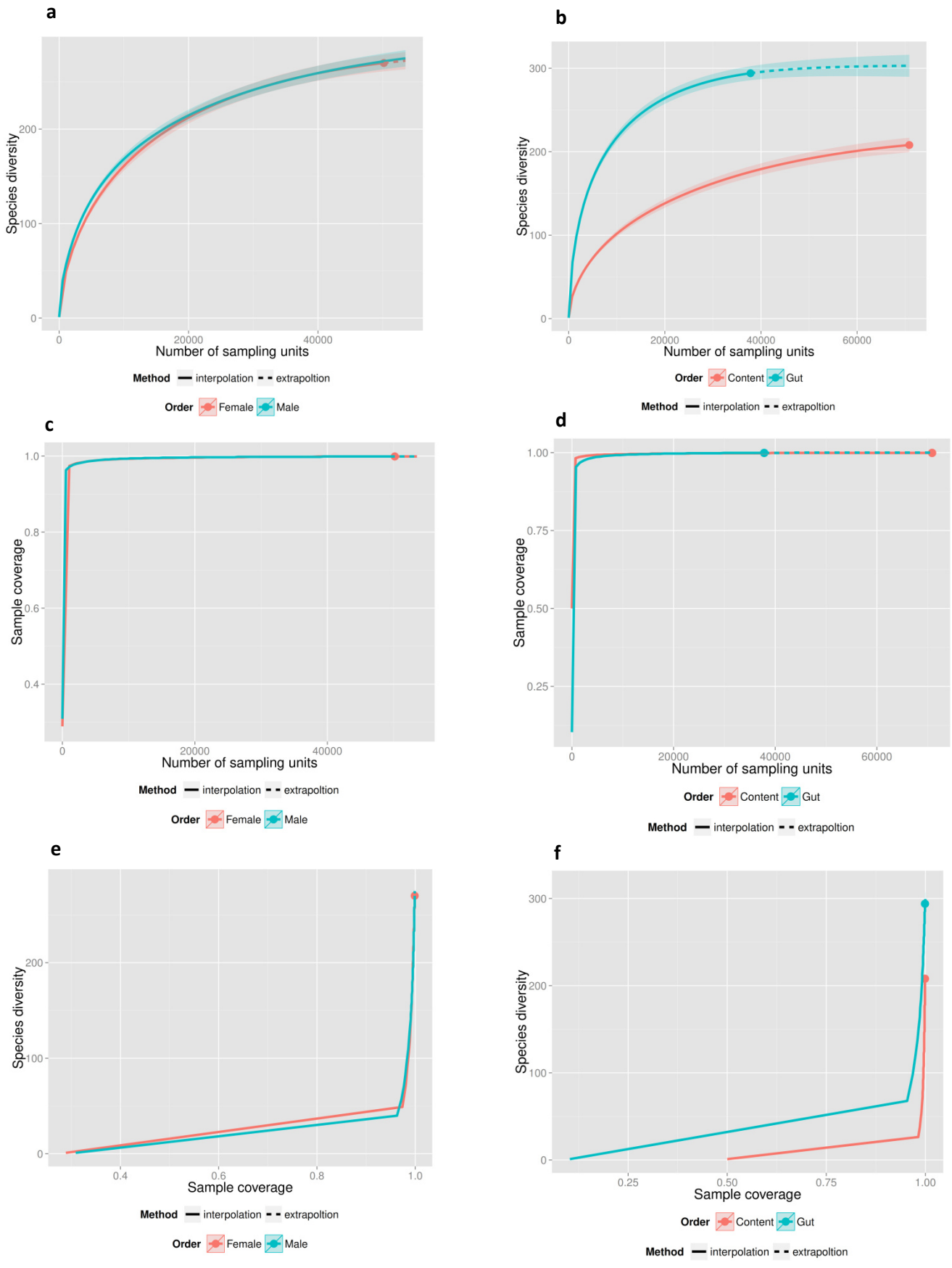
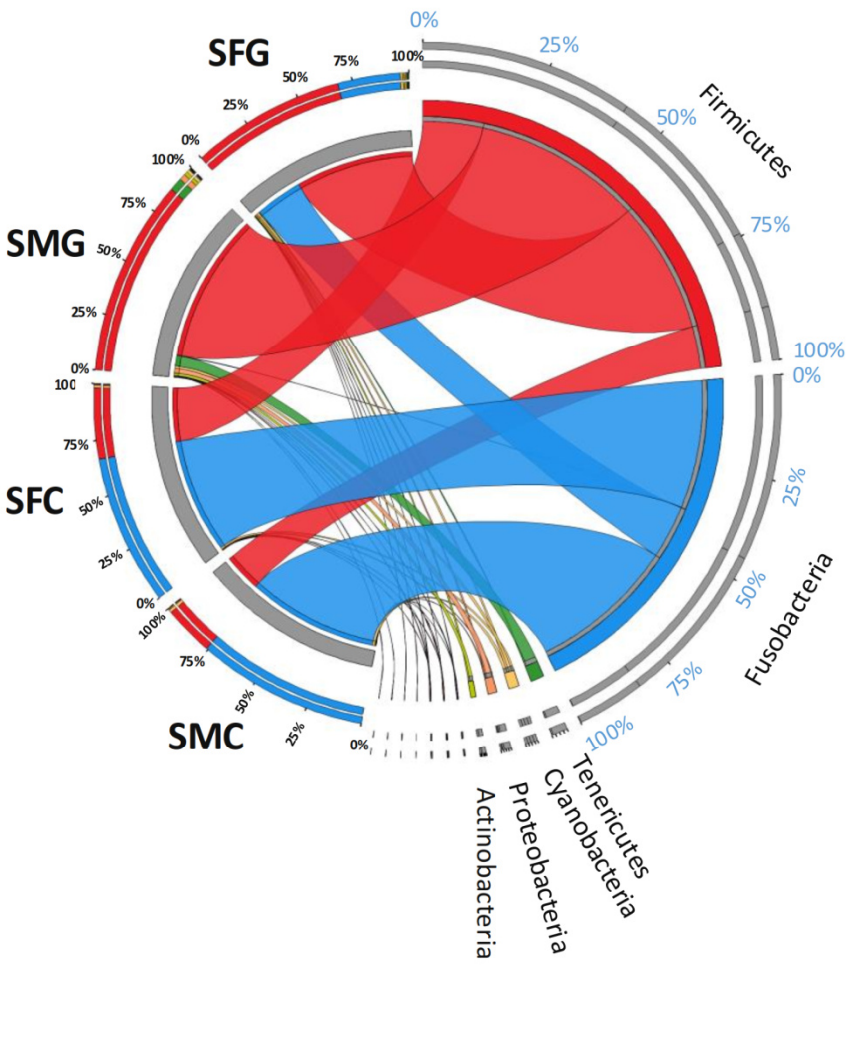
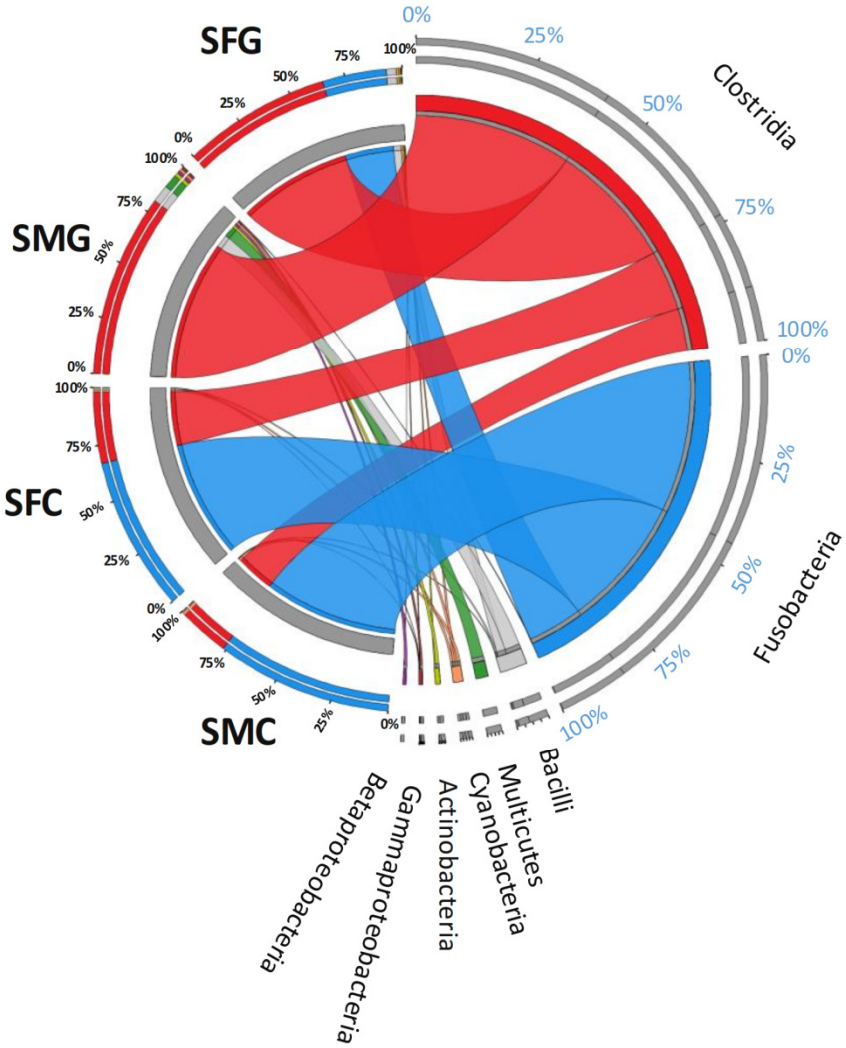

a
Species diversity
Number of sampling units
Method interpolation extrapoltion
Order Female Male
b
Species diversity
Number of sampling units
Order Content Gut
Method interpolation extrapoltion
c
Sample coverage
Number of sampling units
Method interpolation extrapoltion
Order Female Male
d
Sample coverage
Number of sampling units
Order Content Gut
Method interpolation extrapoltion
e
Species diversity
Sample coverage
Method interpolation extrapoltion
Order Female Male
f
Species diversity
Sample coverage
Order Content Gut
Method interpolation extrapoltion

Figure 2

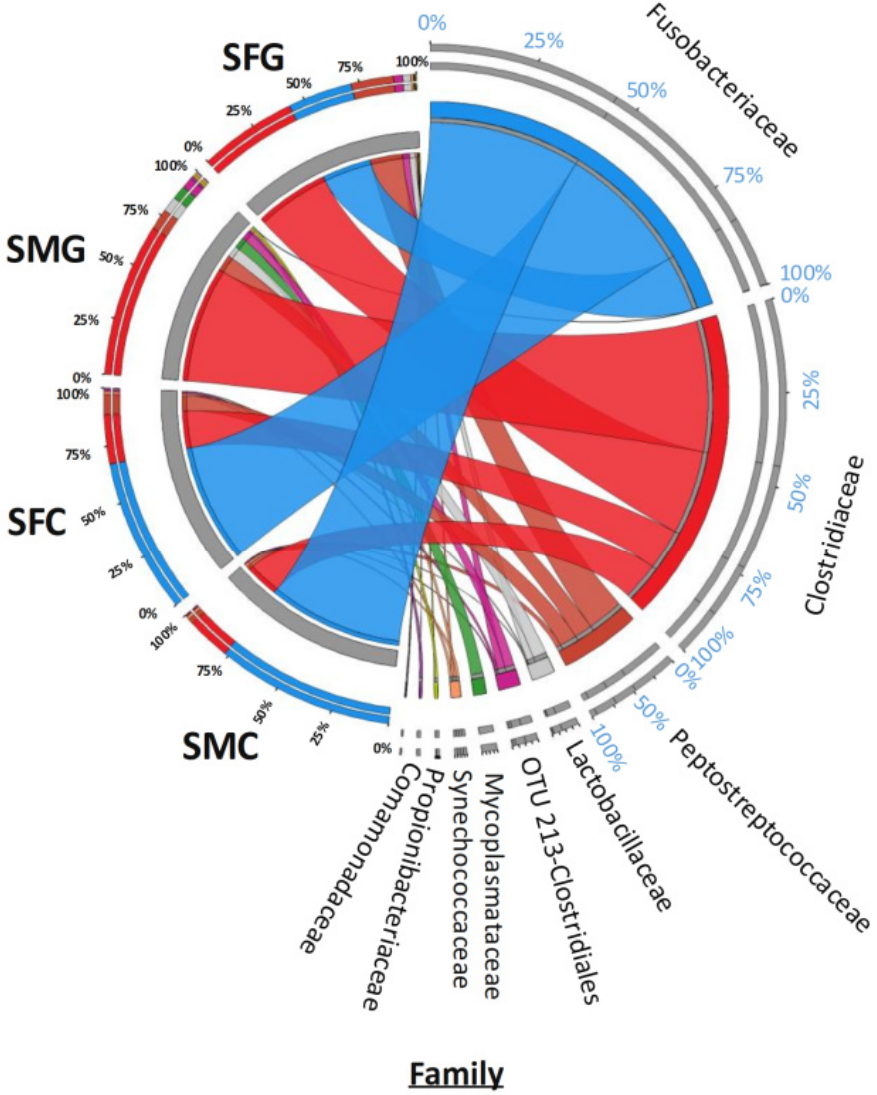


Phylum

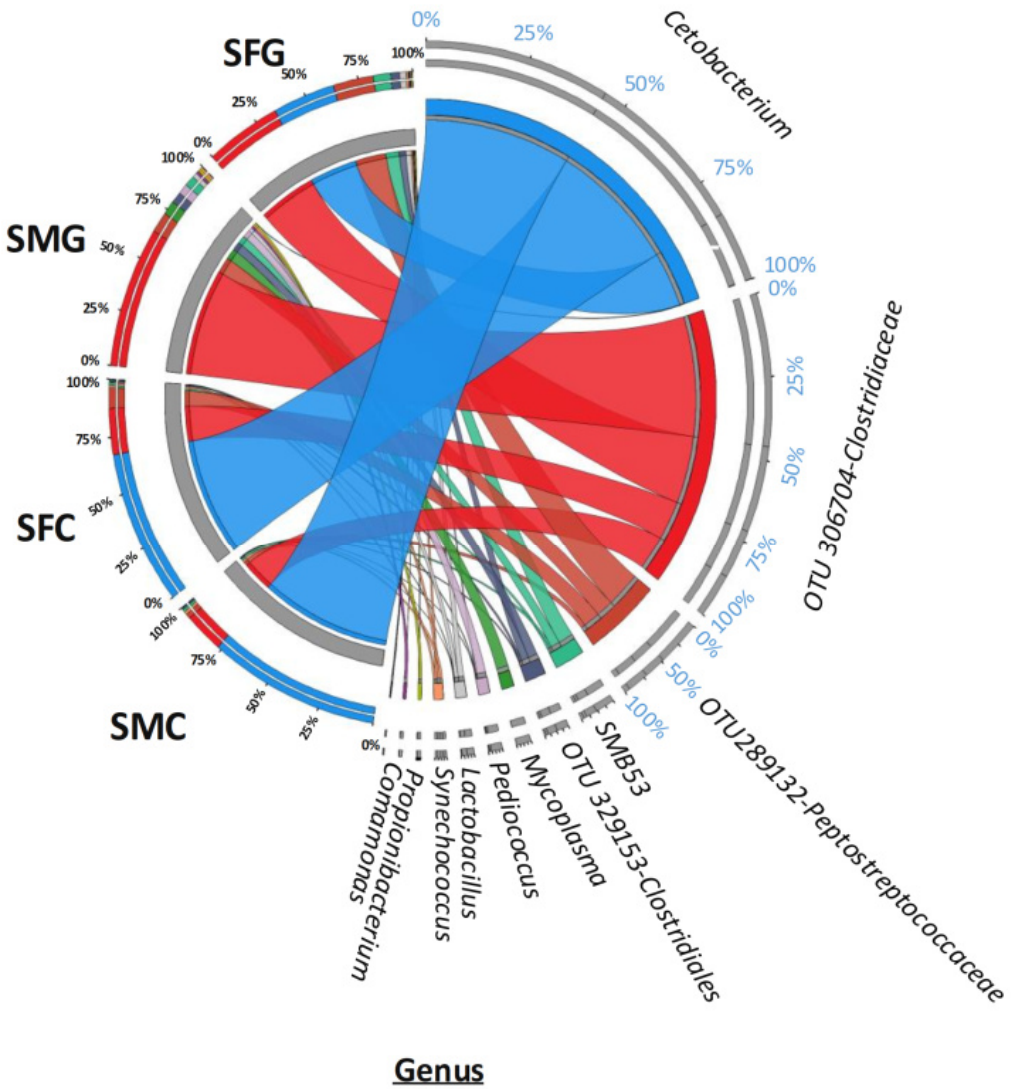


Class

Figure 3



Family



Genus

Figure 4

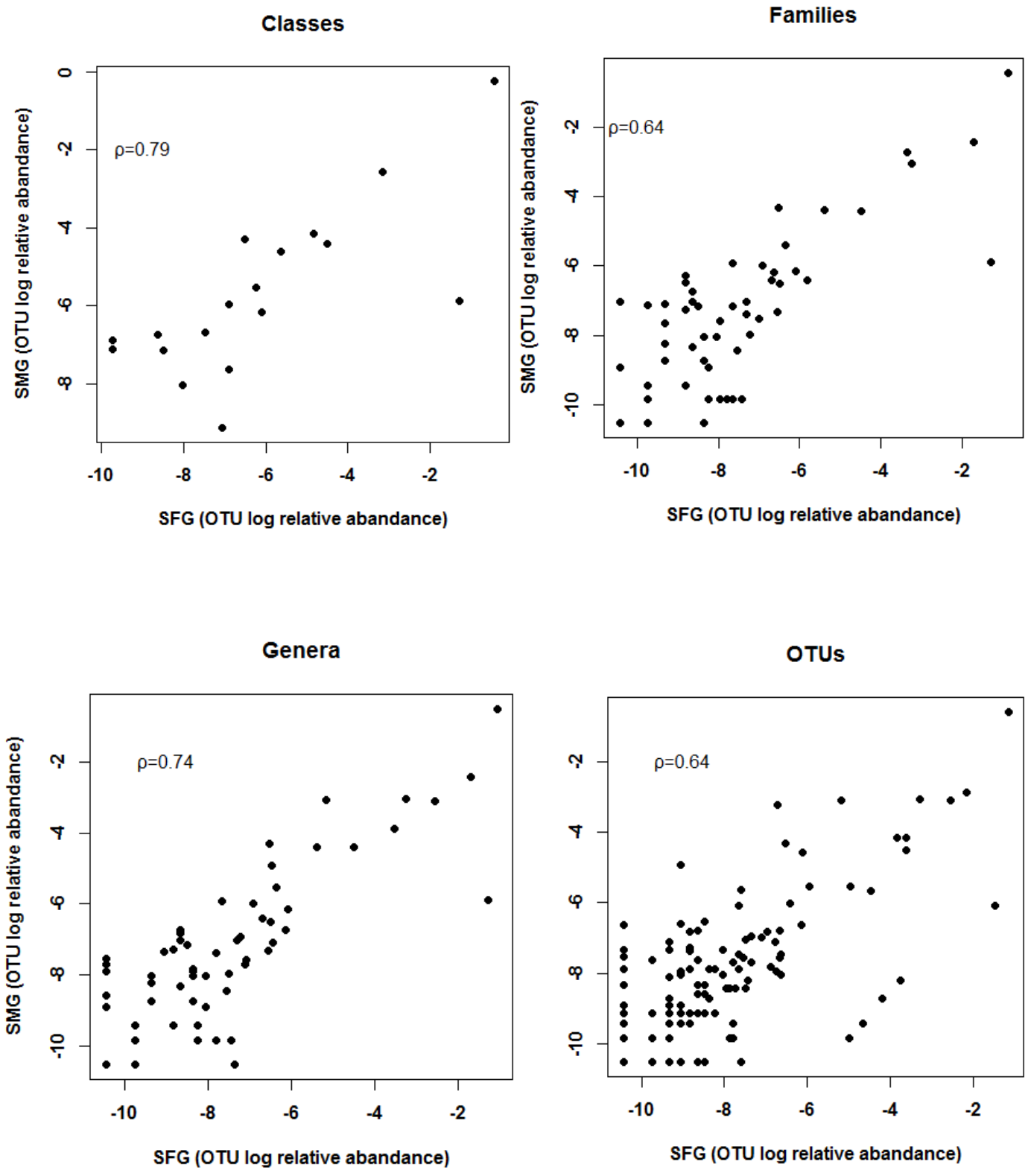

Classes
ρ=0.79
SMG (OTU log relative abandance)
SFG (OTU log relative abandance)
Families
ρ=0.64
SMG (OTU log relative abandance)
SFG (OTU log relative abandance)
Genera
ρ=0.74
SMG (OTU log relative abandance)
SFG (OTU log relative abandance)
OTUs
ρ=0.64
SFG (OTU log relative abandance)

Figure 5

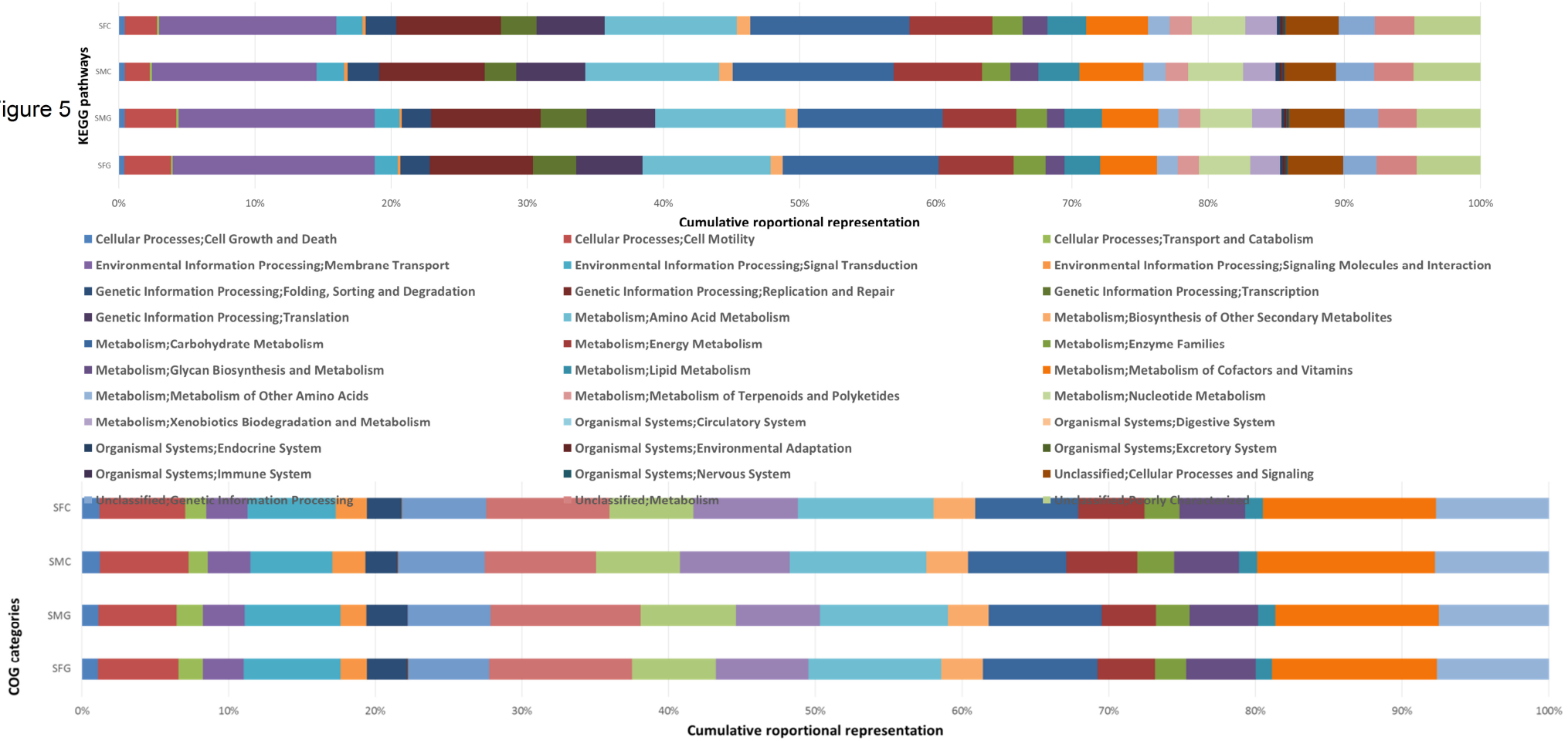
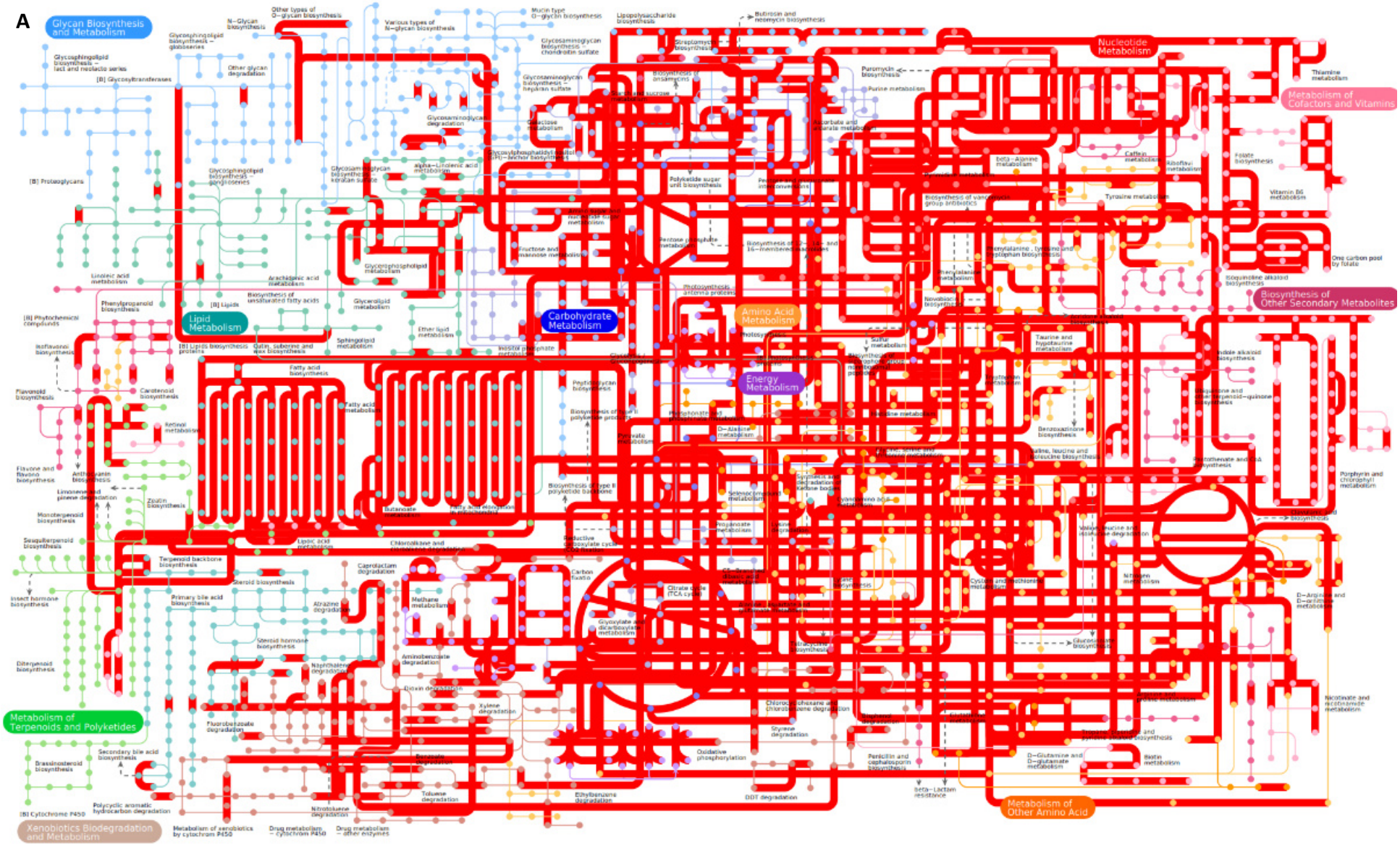
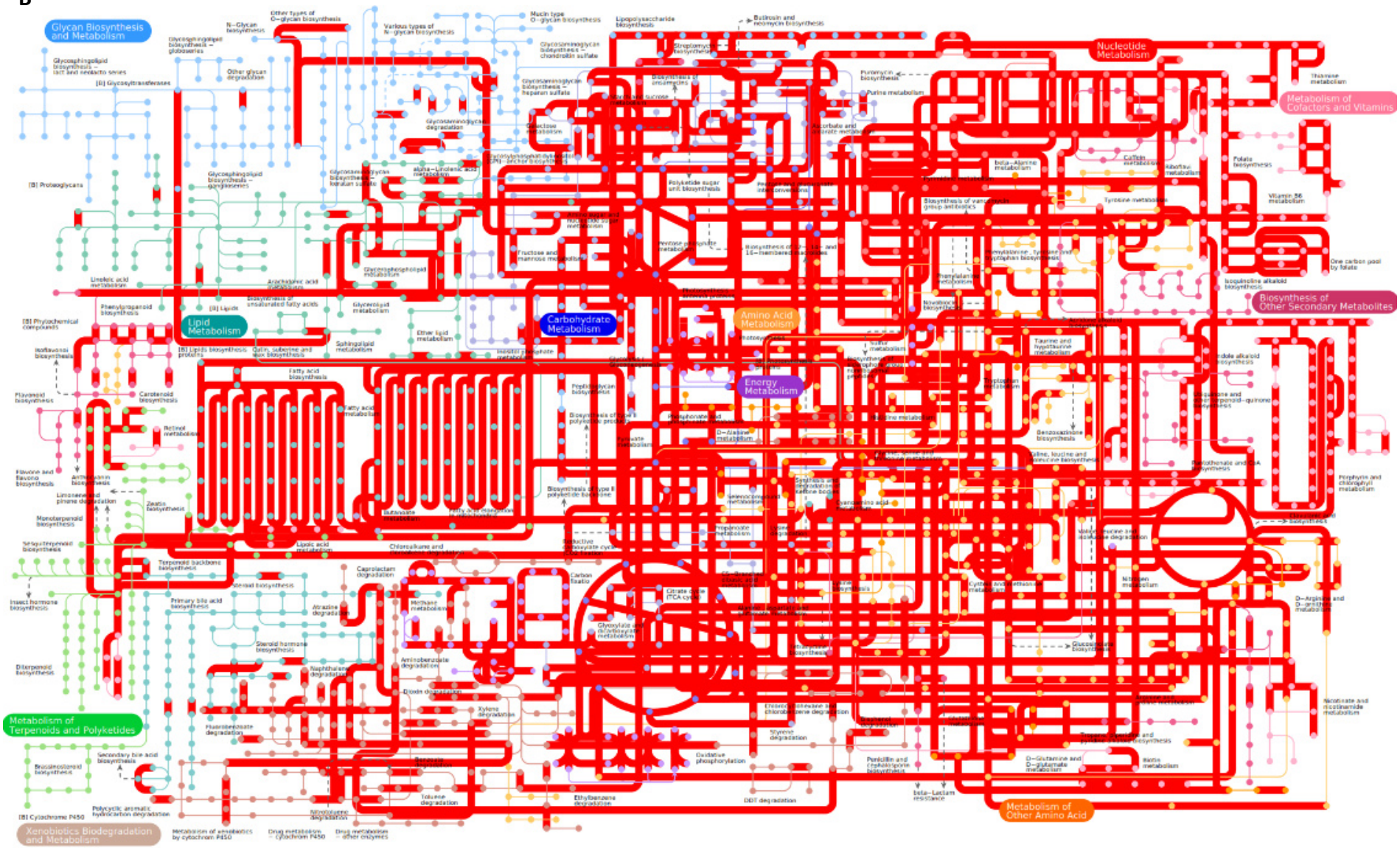

KEGG pathways
SFC
SMC
SMG
SFG
0%
10%
20%
30%
40%
50%
60%
70%
80%
90%
100%
Cumulative roportional representation
Cellular Processes;Cell Growth and Death
Cellular Processes;Cell Motility
Cellular Processes;Transport and Catabolism
Environmental Information Processing;Membrane Transport
Environmental Information Processing;Signal Transduction
Environmental Information Processing;Signaling Molecules and Interaction
Genetic Information Processing;Folding, Sorting and Degradation
Genetic Information Processing;Replication and Repair
Genetic Information Processing;Transcription
Genetic Information Processing;Translation
Metabolism;Amino Acid Metabolism
Metabolism;Biosynthesis of Other Secondary Metabolites
Metabolism;Carbohydrate Metabolism
Metabolism;Energy Metabolism
Metabolism;Enzyme Families
Metabolism;Glycan Biosynthesis and Metabolism
Metabolism;Lipid Metabolism
Metabolism;Metabolism of Cofactors and Vitamins
Metabolism;Metabolism of Other Amino Acids
Metabolism;Metabolism of Terpenoids and Polyketides
Metabolism;Nucleotide Metabolism
Metabolism;Xenobiotics Biodegradation and Metabolism
Organismal Systems;Circulatory System
Organismal Systems;Digestive System
Organismal Systems;Endocrine System
Organismal Systems;Environmental Adaptation
Organismal Systems;Excretory System
Organismal Systems;Immune System
Organismal Systems;Nervous System
Unclassified;Cellular Processes and Signaling
Unclassified;Genetic Information Processing
Unclassified;Metabolism
COG categories
SFC
SMC
SMG
SFG
0%
10%
20%
30%
40%
50%
60%
70%
80%
90%
100%
Cumulative roportional representation
[D] Cell cycle control, cell division, chromosome partitioning
[M] Cell wall/membrane/envelope biogenesis
[N] Cell motility
[O] Post-translational modification, protein turnover, and chaperones
[T] Signal transduction mechanisms
[U] Intracellular trafficking, secretion, and vesicular transport
[V] Defense mechanisms
[W] Extracellular structures
[Y] Nuclear structure
[Z] Cytoskeleton
[A] RNA processing and modification
[B] Chromatin structure and dynamics
[J] Translation, ribosomal structure and biogenesis
[K] Transcription
[L] Replication, recombination and repair
[C] Energy production and conversion
[E] Amino acid transport and metabolism
[F] Nucleotide transport and metabolism
[G] Carbohydrate transport and metabolism
[H] Coenzyme transport and metabolism
[I] Lipid transport and metabolism
[P] Inorganic ion transport and metabolism
[Q] Secondary metabolites biosynthesis, transport, and catabolism
[R] General function prediction only
[S] Function unknown

Figure 6


A
Glycan Biosynthesis and Metabolism
Lipid Metabolism
Carbohydrate Metabolism
Energy Metabolism
Amino Acid Metabolism
Nucleotide Metabolism
Metabolism of Cofactors and Vitamins
Biosynthesis of Other Secondary Metabolites
Metabolism of Terpenoids and Polyketides
Xenobiotics Biodegradation and Metabolism
Metabolism of Other Amino Acid

B


Glycan Biosynthesis and Metabolism
Lipid Metabolism
Carbohydrate Metabolism
Amino Acid Metabolism
Energy Metabolism
Nucleotide Metabolism
Metabolism of Cofactors and Vitamins
Biosynthesis of Other Secondary Metabolites
Metabolism of Terpenoids and Polyketides
Xenobiotics Biodegradation and Metabolism
Metabolism of Other Amino Acid

CHAPTER 6

FUTURE ASPECTS

6.1 Biopreservation: lactic acid Bacteria and bacteriocins

During the last decades, many research activities were undertaken to study the application of protective cultures as biopreservatives in food matrices. Either deliberately added or produced in-situ, this strategy has been discovered to play a potential role in the control of undesirable microorganisms. In addition, the establishment of beneficial bacterial populations is promoted. However, LAB based antagonisms do not necessarily alleviate practical food safety issues in general, as they may be efficient only in a narrow range of food environment (pH, fat content, etc.), and this limits their application in many seafood products. Thus, a case-to-case consideration of applying such a bioprotectant to a certain food matrix is essential. Additional studies are encouraged to select appropriate LAB strains and corresponding combinations to limit the growth of both the pathogenic and spoilage microflora and, furthermore, to investigate the individual nature of the strains. There is some need to clarify the mechanisms underlying the inhibitory potential in order to be able to optimize this way of biopreservation. Novel techniques such as genomics, proteomics, metabolomics, and system biology, have opened up new avenues for the in-depth interpretation of biological data. By combining classical with molecular tools, these new methods possess some big potential in exploring and designing valuable LAB functions, allowing to develop not only safer traditional but also innovative seafood products.

Besides the broad inhibitory spectrum, their technological properties indicate that bacteriocins AP8 and H5 found in our research seem to possess some potential value for being applied as biopreservative agents to control pathogens and spoiling bacteria in food products. Moreover, they possess some bioprotective properties that are useful for aquaculture, since the use of bacteria releasing naturally occurring antimicrobial substances like the isolates described is now gaining importance as a natural alternative to the administration of antibiotics in fish farming.

6.2 Next-generation sequencing of fish gut microbiota

Although the number of studies applying NGS techniques to provide greater in-depth knowledge of the fish gut microbiota is continually increasing, there are still some gaps that warrant further examination. For example, one overriding problem is whether it is possible to differentiate members of the indigenous (fish) microbiota from transients that could be transmitted by the water film around the fish as a relevant vector or via the water/food system. To address the question ´how can NGS methods be used to distinguish these two groups?´ most publications available so far are inconsistent or have not addressed this issue. Therefore, tailored sample preparation should be taken into account in future studies to sufficiently elucidate the spatial distribution of the gut-associated bacterial community.

Another important factor to consider is the study design. Cross-sectional study designs are useful for identifying systematic patterns across a population, for testing the hypothesis that some component(s) of microbial variation within a population is correlated with a study parameter (e.g., diet, genetic factors). Applying a cross-sectional study design to very large cohorts, for instance thousands of subjects, may provide the statistical power to elucidate subtle phenomena when faced with many confounding factors, as is common in microbiome studies where lifestyle, diet, genetics, and disease play important roles in shaping community structure of animals. Although cross-sectional studies are useful, they do not provide a total insight into microbial variations within an individual over time, as their power to observe phenomena such as succession and to factor out between-subject variation in is limited.. Such questions can only be addressed with innovative longitudinal study designs, thereby examining multiple time-points from the same individual. Microbial communities play a central role in fish physiology. Hence ecological succession of the gut microbial community is of particular interest in animal studies.. For this purpose, longitudinal study designs should be taken into account in future studies to accurately discriminate between the spatial and temporal distribution of the gut-associated bacterial community.

Studies in humans and other vertebrates have shown that the gut microbiota play a direct role in the etiopathogenesis of a number of systemic disorders (Llewellyn et al., 2014). While most of the studies in fish have mentioned that changing husbandry treatments (such as administration of plant-based diets) favours a shift of the fish gut bacterial community toward specific taxa (primarily lactic acid

bacteria), the functional impact of these bacteria on the fish intestine is unknown. Also, in our study Firmicutes phylum members constituted the prominent phyla of the mucosa-associated microbiota regardless of the fish gender. However, in luminal-associated microbiota, Fusobacteria phylum made up the most dominant group in both male and female respectively. This suggests that the abundance and diversity of bacterial populations in gut mucus is, in general, quite different from the microbiota in gut contents, indicating that some microbial species poorly colonise the gut mucosa layer. To address this issue, further research need to consider the application of metagenomics from a broader perspective and as complementary to other meta-omic approaches. While small-subunit ribosomal RNA (16S rRNA) has been widely used in fish gut microbiota studies to facilitate a general overview of the taxonomic profile of prokaryotes, metagenomic analyses actually make the entire genetic complement of a microbial community more accessible. Metagenomics is defined as the large-scale application of random shotgun sequencing to DNA extracted directly from environmental samples and resulting in at least 50 mega base pairs (Mbp) of sequence data (Warnecke & Hugenholtz, 2007). Transcriptomic and proteomic analyses have been focused on microbial isolates for several years to observe their expressed metabolic potential. These approaches have recently been applied in a high-throughput fashion to microbial communities, giving rise to the terms ‘metatranscriptomics’ and ‘metaproteomics’. Metatranscriptomics includes the retrieval and sequencing of mRNA from a complex microbial ecosystem to determine the active bacterial taxa in the gut and to assess what genes are expressed in that community, revealing responses to environmental fluctuations (Sorek & Cossart, 2010, Franzosa et al., 2015). Metaproteomics aims to identify the microbial proteins translated in a complex mixed population by matching their sequences with the available metagenome database and assigning putative functions to reflect the catalytic potential of the microbiota (Sorek & Cossart, 2010, Franzosa et al., 2015). In the near future, the challenge for the scientific community will be to integrate these complementary meta-omics data into an ecosystem-level approach to study the animal microbiome.

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CHAPTER 7

SUMMARY

This thesis focuses on the study of fish gut microbiome in terms of its characterization and potential applications. The first aspect is to show how fish gut associated bacteria may be an effective tool (bio-protective) to inhibit the growth of food-borne pathogens, food spoilage bacteria and contaminants in aquaculture.

The second aspect is to highlight the potential of next-generation sequencing (NGS) platforms for analysing fish gut microbiota. Furthermore, aspects of the biology of the fish gut microbiome are addressed by applying NGS technique. 1) What bacteria constitute the snow trout gut microbiome? 2) How variable is the composition in the snow trout intestinal mucosa and the intestinal lumen-associated microbiota? 3) How different are the gut microbiota of male and female Snow trout (*Schizothorax zarudnyi)* exemplars.

The antimicrobial spectrum and physico-chemical characteristics of bacteriocin-like inhibitory substances produced by lactobacilli isolated from the intestinal flora of Sturgeon fish were determined. Their inhibitory potential exerted against 42 food-borne and aquaculture-related bacterial pathogens as well as against food spoilage causing bacteria was investigated. There is some broad inhibitory effect against different Gram positive and Gram negative food- borne pathogens (like *Escherichia coli*, *Listeria* spp., *Salmonella* spp., *Staphylococcus aureus*, *Aeromonas hydrophila*, *Vibrio anguillarum*, and *Bacillus cereus)*. The technological properties, especially the stability (temperature stability (20 °C to 120 °C), pH tolerance (3-12) and chemical stability (SDS, EDTA, Tween 20, Tween 80) may lead to the assumption that the bacteriocins like inhibitory AP8 and H5 produced by *Lactobacillus casei* AP8 and *Lactobacillus plantarum* H5 may be applied as biopreservative agents to control pathogens and spoilage bacteria in different food products. Furthermore, their role as bioprotective agents in aquaculture systems is envisaged.

The results obtained from high-throughput parallel sequencing of the resident microbes (microbiota) in male and female Snow trout showed that Firmicutes phylum members represent the dominant phyla of mucosal-associated microbiota regardless of the fish gender, constituting 87.1% and 68.5% of total sequences in males and females, respectively. However, Fusobacteria phylum group make up the most dominant group in fish intestinal lumen of male and female with 75.9% and 66.6%, respectively. *In silico* functional analysis using PICRUSt revealed that the gut microbiome of Snow trout has pronounced activity in terms of metabolism of

carbohydrates, amino acids, vitamins etc. with comparatively similar gene ontology among the fish intestinal lumen and mucosa-associated microbiota. It is concluded that the community membership and structure of bacterial populations at the mucosal epithelium differs from the luminal-associated microbiota of Snow trout, indicating that some microbial species obviously poorly colonise gut mucosal layer and that host-related factors are of relevance to the gut ecosystem.