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THE DYNAMICS OF MICROBIAL COMMUNITIES AND
PROBIOTIC SUPPLEMENTS IN THE MARINE LARVICULTURE
FEED CHAIN

Thesis submitted in fulfillment of the requirements
for the degree of Doctor (PhD) in Applied Biological Sciences

Dutch translation of the title:

De dynamiek van microbiele gemeenschappen en probiotica supplementen in de voederketen van mariene larvenculturen

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Green water in turbot (*Psetta maxima* L.) larviculture

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List of abbreviations

AFDW	Ash-free dry weight
AFLP	Amplified fragment length polymorphism
AHL	Acyl homoserine lactone
ANOVA	Analysis of variance
ARC	<i>Artemia</i> reference center
ARDRA	Amplified ribosomal DNA restriction analysis
ARISA	Automated rRNA intergenic spacer analysis
BSE	Bacterial species evenness
CCA	Canonical correspondence analysis
CFU	Colony forming unit
DAPI	4', 6-diamidino-2-phenylindole
DGGE	Denaturing gradient gel electrophoresis
DHA	Docosahexaenoic acid
DHPLC	Denaturing high performance liquid chromatography
EC	Enrichment culture
ELISA	Enzyme-linked immunosorbent assay
EM	Effective microorganisms
EPA	Eicosapentaenoic acid
ERIC	Enterobacterial repetitive intergenic consensus
EUROSCARF	European <i>Saccharomyces cerevisiae</i> archive for functional analysis
FAO	Food and agriculture organization
FASW	Filtered and autoclaved seawater
FISH	Fluorescent <i>in situ</i> hybridization
GFP	Green fluorescent protein
GI	Gastrointestinal
GR	Growth rate
HNT	Hubbell neutral theory
HUFA	Highly unsaturated fatty acid
ICB	Immunocolony blot
LAC	Lorenz asymmetry coefficient
LB	Luria-Bertani
LMG	Laboratory of microbiology of the Ghent University

LPS	Lipopolysaccharide
MA	Marine agar
MANOVA	Multivariate analysis of variance
MB	Marine broth
MC	Microbial community
MCC	Microbial community composition
MDS	Multidimensional scaling
MOA	Mechanism of action
NCBI	National center for biotechnology information
NCM	Neutral community model
NOEC	Non-observable effect concentration
NSS	Nine salt solution
OD	Optical density
PCA	Principal component analysis
PCOA	Principal coordinate analysis
PCR	Polymerase chain reaction
PHB	Poly-beta-hydroxybutyrate
RAPD	Random amplification of polymorphic DNA
RPM	Rotation per minute
RST	ribosomal sequence tag
SARST-V6	Serial analysis of V6 ribosomal sequence tags
SD	Standard deviation
TCBS	Thiosulfate citrate bile salt sucrose
TGGE	Temperature gradient gel electrophoresis
T-RFLP	Terminal restriction fragment length polymorphism
WT	Wild type
YEPD	Yeast extract peptone dextrose

Introductory chapter

Introductory chapter

Aim of the study

With the increasing demand for aquaculture food, diseases are currently the main bottleneck for the further development of aquaculture. There is an urgent need for a better understanding of the roles of these microbial communities in aquaculture. Instead of using chemicals, such as disinfectants and antibiotics, probiotic bacteria are being suggested as sustainable alternatives for the control of microbiota in aquaculture. In order to effectively evaluate strategies for the application of probiotics in larval culture, molecular methods are needed to follow their fate.

The goal of this PhD research is to evaluate the putative beneficial effects of probionts in live feed cultures, such as rotifers, and in fish cultures aiming at improving rotifer performance and larval survival. Denaturing Gradient Gel Electrophoresis (DGGE) was used to characterize and better understand the role and dynamics of probiotics in microbial community composition in the larval culture. The objectives of the research are (1) to develop a methodology for the evaluation of the efficiency of microbial agents as pro/prebiotic feed additives in larviculture and (2) to develop a probiotic and prebiotic treatment to improve the rotifer cultures and the survival rate and health condition of fish larvae.

Introduction

The yearly aquaculture production is growing faster than livestock meat production (10% vs. 3%) (Rana and Immink 2001). The Food and Agriculture Organization (FAO) predicts a 36% increase of the world population, but only a 30% increase in production from aquaculture and fisheries in between 2000 and 2030 (Ye 1999). Because wild fish stocks have been heavily fished or overfished, only an increase in aquaculture production is believed to successfully cover the gap between supply and demand (FAO 2006).

Major improvements in aquaculture technology have been achieved through enhanced husbandry procedures, improved genetic traits, nutrition, disease diagnosis and control. With increasing demands for high quality aquaculture foods, more efficient and secure production system are needed.

Diseases are a significant constraint for the further development of aquaculture. The rapid growth of fish and shellfish culture was initiated by the technological breakthrough for seed production in 1980s. Since then it has been accompanied by increased disease problems. There is an urgent need for a better understanding of the roles of microbiota in hatchery operations. Disinfectants and antimicrobial drugs have had little effect in controlling diseases. Moreover, the massive use of antimicrobials increases the selective pressure on microbes and encourages the natural emergence of bacterial resistance. Resistant bacteria thrive after non-resistant strains have been

killed and can even pass on resistance genes to other bacteria that have not been exposed to antibiotics (Hernández Serrano 2005). The use of antibiotics in animal production has been seriously questioned and it becomes a more serious problem for sustainable aquaculture.

Instead of using chemicals and antibiotics, there is an increasing tendency to use probiotic bacteria in aquaculture. Probiotics in aquaculture are microorganisms with beneficial effect on the health of the host (Irianto and Austin 2002). The FAO has now designated the use of probiotics as a major mean for improving aquatic environmental quality. Initially, research in probiotics started with fish juveniles, but nowadays more attention is given to fish and shellfish larvae. Many publications about probiotics have emerged in aquaculture in the last decade (Verschuere *et al.* 2000). The results show that under certain environmental conditions probiotics can be promising, but *in vivo* mechanisms of action largely remain unravelled (Tinh *et al.* 2008).

Probiotic bacteria perform best when they find adequate environmental conditions. During their industrial production they should be protected against various stress factors. The beneficial bacteria often die during the manufacturing process or during the passage to the site of application. Temmerman *et al.* (2003) have reported that of 30 dried probiotic food supplements tested, 11 contained no viable bacteria. In aquaculture, the successful addition of probiotics to culture systems is hampered by

the customary sterilization, disinfection, washing and discontinuous culture cycles. To overcome those problems, pharmaceuticals and food industry constantly improve the technology for screening more resistance strains and via micro- and bioencapsulation. These techniques have readily been introduced into aquaculture. Applications of those techniques such as modification of yeast cell composition, *Bacillus* spores' germination and live feed encapsulations are showing positive results (Gatesoupe *et al.* 1991, 1997). Recently, interest has focused on the use of axenic rotifers and *Artemia*, whose intestinal microbiota were gradually implanted through controlled inoculation. Several studies could clearly demonstrate the value of the live bacteria added to the system (Rombaut *et al.* 1999; Marques *et al.* 2004; Tinh *et al.* 2006).

By monitoring probiotics and the microbial community structure and dynamics in the culture system, the functions and effects of probiotics can be documented in more detail. For this purpose, nucleic acid-based techniques have been used. There have been some studies in which the green fluorescent protein is expressed by target strains and this allows the probiotic strain to be tracked (Collins and Gibson 1999). Current techniques, including genetic fingerprinting, gene sequencing and oligonucleotide probes, discriminate closely related bacteria with varying degrees of success (McCartney 2002). Denaturing gradient gel electrophoresis (DGGE), fluorescent *in situ* hybridization (FISH) and microarray can be employed to demonstrate the roles of probiotic strains and their *in vivo* mechanism of action.

Overview of the different chapters

Chapter 1 provides an overview of current constraints for larviculture, the role of probiotics in larviculture and the methods used for *in vivo* evaluation. Chapter 2 presents the status of probiotics in aquaculture in China. Various types of commercial products and relevant research are described. Chapter 3 describes a 16S rRNA gene based PCR-DGGE approach to monitor microbial communities associated with turbot (*Psetta maxima* L.) larviculture in a commercial hatchery. In addition, Lorenz curve and Gini coefficient were used to assess the diversity of microbial communities and bacterial species distribution. In Chapter 4, the evolution of the composition of bacterial communities associated with 3 different strains belonging to different cryptic species of the rotifer *Brachionus plicatilis* was monitored during four subsequent cycles of batch cultures using DGGE, cluster analysis, multidimensional scaling and principal component analysis. In chapter 5, different feed types, different feeding regimes and mixtures of 3 probionts were evaluated for their effects on rotifer growth performance and for the composition of the microbial community associated with the rotifers. In chapter 6, the effects of a probiotic *Bacillus* mixture and yeast cell wall mutant mnn9 were evaluated in starving turbot larviculture. DGGE was used to monitor the bacterial community composition during larviculture, starting from the fertilized eggs and through the initial larval stages of turbot (*Psetta maxima* L.). In chapter 7, general discussion about studies of microbial communities and probiotics, final conclusion and future perspectives were made in light of obtained results.

Chapter

1

Literature review

Chapter 1

Literature review

1. Aquaculture for food security

According to UN World Population Prospects 2006 Revision, world population will grow significantly up to 9.2 billion by 2050. Asia will account for more than half of this growth. The FAO predicted that aquaculture production could contribute significantly to bridge the gap between demand and supply of aquatic food. Since farmed fish have the lowest feed conversion efficiency (kg of grain per kg of body weight) among intensively fed livestock animals, aquaculture has an important role to play in poverty reduction and food security. It can provide fish and other marine and freshwater products, which commonly are rich sources in protein, essential fatty acids, vitamins and minerals, and provide incomes and employment opportunities (FAO 2003). Today, aquaculture is the fastest growing food-producing sector in the world, with an average annual growth rate of 8.9% since 1970, compared to only 1.2% for capture fisheries and 2.8% for terrestrial farmed meat production systems over the same period. In 2002, Asia produced 91.2% (by volume) and 82.0% (by value) of global aquaculture production. Of the world total, China produced 71.2% of the total volume and 54.7% of the total value of aquaculture production (Crespi 2005).

Aquaculture expansion in China has been actively promoted by the government to provide food security.

2. The role of larval culture in aquaculture and current problems

In recent decades, aquaculture has shown great progress partly due to improvements in larval production technology. Reproductive biology makes it possible to induce differentiation and maturation of functional gametes for seed production in a large number of fish species, and as many as over 400 fish and shellfish species are now being cultured under artificially controlled conditions. Aquaculture species generally have much higher fecundity than terrestrial animal. Also the diversity of cultured species is much higher in aquatic animals with respect to terrestrial animals. Some genetically improved lines of fishes and shellfish species are being introduced in aquaculture. However the wide genetic pool from wild populations remains far from fully exploited. This suggests that in aquaculture there is still a large potential for breeding to enhance the productivity.

2.1. Nutritional improvements that contributed to the success of larviculture

Besides good broodstock and improved genetic management, it is well documented that important breakthroughs in aquaculture larval production were due to nutritional improvements. Progress in fish larviculture nutrition includes research on the biochemical composition of eggs, insight in the need for protein, essential fatty acids,

phospholipids, vitamins and the development of techniques for the enrichment of live feed, and formulated diets (Watanabe and Kiron 1994; Sorgeloos *et al.* 1994; Lavens and Sorgeloos 1996; Lee and Ostrowski 2001; Kanazawa 2003). The success of larval rearing is greatly influenced by first feeding regimes and the nutritional quality of starter diets, with dietary lipids being recognized as one of the most important nutritional factors that affect larval growth and survival (Watanabe *et al.* 1983). By simulating the natural food chain, the use of commodity live feed (microalgae, *Brachionus* sp., *Artemia* sp., nauplii) has been adopted as a standard diet in the commercial hatcheries of many fish and shellfish species. The modification of the nutritional value of these live food organisms was also investigated. The enrichment technique of live food with emulsified lipophilic products has allowed delivery of extra doses of essential nutrients, e.g. highly unsaturated fatty acids (HUFAs) and vitamins, to fish and crustacean larvae (Dhert *et al.* 1993). This has enabled the production of marine fish species that produce very small larvae, such as turbot, halibut and groupers, which could not be raised before. Aiming at more intensive larviculture, considerable progress has also been achieved the last years in the development of formulated artificial diets for the juveniles as well as start-feed (Zambonino infante and Cahu 2007).

2.2. System management and hygienic measures

Management of any type of larval rearing system (extensive, semi-intensive and intensive), requires the control over the biotic and abiotic factors such as stocking

density, food density and quality, the presence of phytoplankton, water flow (renewal), aeration, light (photoperiod) and different water parameters (temperature, salinity, dissolve oxygen, total ammonia nitrogen etc.) as well as the microbial environment. Due to the high density of living organisms and the heavy load of organic matter in an aquatic environment, the hygienic conditions in larval rearing tanks can deteriorate rapidly. This creates a favourable environment for bacterial growth, often harmful to the delicate larval fish. A complete control of the hatchery hygienic conditions should therefore be contemplated and duly enforced (Moretti *et al.* 1999). Currently, strict cleaning routines are applied in most of intensive hatcheries. Physical and chemical treatments including UV, ozone, skimmer, filtration, degassing and different disinfectants (hypochlorite, potassium permanganate, active iodine etc.) as well as antibiotics are commonly used to ensure an optimal health status of the larvae.

2.3. Current problems in larval culture

Although larval production operations have been standardized for many species, the consistent supply of good quality seed is still problematic. Hatcheries are expected to provide stable fry supply for farmers, but fry production is often highly variable due to low survival rates. The large variation occurs not only from hatchery to hatchery, season to season, but also within the same sibling group. This implies that some critical factors besides nutrition and egg quality are not under fully controlled (Skjermo *et al.* 1993). With the increasing intensification and commercialization of

larval production namely in the 90's, major disease problems occurred. Since then, the aquaculture industry has been increasingly confronted with its share of diseases and problems caused by viruses, bacteria, fungi, parasites and other undiagnosed and emerging pathogens. Obviously, intensive larviculture provides a suitable environment for the emergence, establishment and transmission of new pathogens. High rearing densities lead to rapid infection transmission and predisposes to clinical disease. The diseases problems require preventive measures to mitigate the risk of disease emergence, establishment and spread.

2.3.1. Live prey requirement

The intensive rearing of larvae of many species of fish and crustaceans is heavily dependent upon the availability of live food, both plant and animal species. The most widely used live food species are green microalgae, the rotifer *Brachionus* sp. and the brine shrimp *Artemia* sp. Microalgae are often at the basis of live feed production. Some mollusc species depend on algae throughout their life cycle, while fish and crustaceans require algae only during the larval stages. Also, microalgae are a major food source for other live animal prey. The filter feeding rotifers are essential for rearing many marine fish that produce upon hatching very small larvae. Rotifers can be cultured at relatively high densities, thus large numbers can be produced daily to supply the demand from hatcheries producing large numbers of fish. The brine shrimp, *Artemia*, has been intensively used as a live food for fish culture since the 1960s. Over 85% of all cultured marine animals utilize *Artemia* as a partial or sole

diet during their larval phase (Lavens and Sorgeloos 1996). The availability of the *Artemia* cysts, combined with long shelf life and easy procedures for hatching, make them a convenient live food for marine fish larvae. Despite above advantages, a couple of problems still remain in live feed productions. These include: variability in species traits such as unidentified genetic background in microalgae and rotifers, unsuitable size and nutritional deficiencies; cost-efficiency of production systems (especially for microalgae and rotifer culture which require large space and infrastructure), heavy labour, time and energy requirements, instability of production because of contaminations, sudden crashes (in case of rotifers) and shortage of natural cysts supply (in case of *Artemia*). The difficulty in supplying a constant flow of high quality live feed in sufficient quantity makes live food production a major bottleneck in the larviculture of different fish and shellfish species.

2.3.2. Importance of first feeding for successful larval culture

Considerable progress has been obtained during the last years in the development of formulated start-feed diets (Cahu and Zambonino Infante 2001). But live feed is still essential in juvenile production of most marine species. This is particularly true for larval quality in terms of deformities, malformations and disease resistance.

To reduce mortality at first feeding, it is important to identify the optimal live feed. Depending on the initial mouth size of fish larvae, copepod nauplii, rotifers, or brine shrimp are provided as the first feed to the larvae. Rotifers are commonly used as the

initial feed for many fish and shrimp species (Lee and Ostrowski 2001). Besides animal live feed, it is a traditional practice in Asia to introduce microalgae in the rearing tanks before initial zooplankton feed is supplied. This preconditioning of the water is often combined with adding fertilizers. It is thought that “matured water” can be obtained in this way; providing a stable water quality and a stable microbial community. Moreover the addition of algae has the dual benefits of providing nutrients to rotifers and maintaining better water quality in the rearing tanks (e.g. assimilation of ammonium). The nutritional value of the rotifers is decreasing if algae or other feed sources are not available to rotifers in the rearing tank. Thus, addition of algae to larval rearing tanks proved to be essential to improve survival of striped mullet (Tamaru *et al.* 1994), turbot (*Psetta maxima* L.), halibut (*Hippoglossus hippoglossus*) and summer flounder (Reitan *et al.* 1997; Alves *et al.* 1999). Skjermo *et al.* (1997) found that turbot larvae rearing in microbial-matured had a higher growth rate than in membrane-filtered water. Establishment of an intestinal microbiota in newly hatched larvae starts before exogenous feeding, and a rapidly increasing population of bacteria (from 10^2 colony-forming units (CFU) per larva to 10^4 CFU per larva) associated with the turbot larvae gut was observed after first feeding (Huys *et al.* 2001). These results indicate that quality first feeding not only requires proper size and nutrition optimisation, but also management of the incoming water, green water and the associated microbial communities (e.g. microbial matured water or green water).

2.3.3. Infectious disease problems

Just as for other farming sectors, the incidence of major disease problems increased as aquaculture activities intensified and expanded (Bondad-Reantaso *et al.* 2005). These problems appear more often in intensive larval culture. It is generally believed that larvae have a low resistance against diseases, as they only have the innate immune system operational. Under crowded culture conditions, their fragile bodies are frequently the victims of various diseases. Disease caused by viruses, bacteria, fungi, parasites and other undiagnosed and emerging pathogens is now a primary constraint to the culture of many aquatic species, impeding both economic and social development in many countries. Of the various pathogens, there is little doubt that viral pathogens constitute one of the most serious disease threats to the successful rearing of marine species and larvae (Muroga 2001). Viruses and bacteria are amongst the most serious disease threats for aquaculture, primarily because of their direct transmission and ability to proliferate rapidly in crowded conditions. Such conditions greatly facilitate the rapid spread of infectious agents. Over 500 potentially harmful bacteria for aquatic animals have been recorded (Buller 2004). They are taxonomic divers, being either Gram-positive or Gram-negative, or belong to each subdivision of α -, β - and γ -*proteobacteria*.

An infectious disease outbreak occurs under the following criteria. First the real pathogens must be present in both fish and culture water, and predominate in certain fish organs, blood and/or lesions Secondly, pathogens must be able to survive in terms

of number and longevity in the ambient culture environment with the presence of disease outbreak stress or cues. Thirdly the viable infection route must be present which leads to disease recurrence (Bondad-Reantaso *et al.* 2005). Very often the farmers are aware of disease only when clinical signs are occurring (i.e. significant mortalities, abnormal appearance/behaviour). However, once a pathogen or disease agent is introduced and becomes established into the culture environment, there is little or no possibility for either treatment or eradication. Not only because of the delay of pathogens diagnostics (time consuming reinfection test for fulfilling Koch's postulates), but also because the predisposing stress/environmental factors and infection routes are unknown. Thus, it is particularly important to prevent that all inputs into a culture tank become sources of infection. Natural seawater harbour millions of bacteria therefore treating the inlet seawater is the first line of defence against harmful bacterial contamination. Brood stocks are potential sources of fungal and bacterial infection. Algae and rotifers cultures carry a high load of bacteria, and *Artemia* is another potential carrier of infection. Different physical treatments and chemical prophylactic agents can be applied for input water, while disinfection of *Artemia* has been introduced successfully more than a decade ago (Dehasque *et al.* 1993). However, it is particular difficult to eliminate all the bacteria from microalgae and rotifer, simply because of the close associations of bacteria with microalgae/rotifer.

In the last century, the discovery of antibiotics revolutionized the treatment of infectious diseases, leading to a significant improvement in the health of the population in general. Aquaculture, as a young industry since 1970s, placed significant reliance on the use of antibiotics to combat a range of bacterial diseases. Antibiotics are defined as the drugs of natural or synthetic origin that have the capacity to kill or to inhibit the growth of micro-organisms. For decades antibiotics routinely used for treatment of human infections are also used for aquatic animals, for therapy, prophylactic reasons or growth promotion. Because of their dilution into the culture water, antibiotics may be used at sub-therapeutic doses. Also anticipating this phenomenon overdose of antibiotics can be used. Accumulating evidence shows that the widespread use of antibiotics for treating bacterial diseases has been associated with the development of antibiotic resistance. The contribution to the increased incidence in antimicrobial resistance (through the use of antibiotics in the aquaculture industry) has been reviewed by Hernández Serrano (2005). In 2006, the indiscriminate use of antibiotics for fish has increasingly become a matter of public concern in China, and a legal framework for their application is being enforced. However, the complex nature of aquatic culture system and the diversity of culture species and pathogens resulted in few antibiotics that could be licensed for efficient and safe usage. At present, there is an urgent need to discover new or alternative approaches for the antibiotics abuse.

3. The role of probiotics in larval culture

3.1. Impact of vaccination on larvae

Diseases can be controlled in a number of ways. Along with improved management, vaccination is the most powerful tool. By the beginning of the 1990s, a range of effective vaccines became available for salmon. Vaccines stimulate the immune response of fish to produce antibodies that help protect the fish against disease. However, only for a limited amount of pathogens the development of vaccines has been undertaken. However, for the fish or shellfish larvae, which are relying only on the innate immune system (the adaptive immune system only develops later; the time frame being species dependent) it is questionable that vaccines can stimulate the immune systems in those early life stages.

3.2. Strategies for aquaculture microbial management

It is important to note that good aquaculture management practices are essential to maintain a healthy environment for farmed finfish and crustaceans. It is now generally accepted that appropriate hygiene measures should not aim at removing all bacterial inputs in the tank but rather to reduce the chance of pathogen contamination, or the adverse activities of pathogens. Just as in human infants, larvae develop their own internal microbiota rapidly once the mouth is opened. It is not surprising that pathogens will become predominant if a normal microbial community has not been established in the larval gut. Based on this concept, beneficial bacteria (probiotics) are

being designated as living bacteria, which are able to inhabit a host, propagate, and act as antagonist towards pathogens or unidentified deleterious micro-organisms. For a broader application in aquaculture, Verschuere *et al.* (2000) extended the concept of probiotics as a live microbial adjunct which has a beneficial effect on the host by modifying the host-associated or ambient microbial community, by ensuring improved use of the feed or enhancing its nutritional value, by enhancing the host response towards disease, or by improving the quality of its ambient environment. An integrated approach (going back to 1970s), was suggested by a Japanese agronomist Teruo Higa who developed the “effective microorganisms” (EM), which is a combination of approximately 80 different microorganisms, capable of positively influencing the decomposition of organic matter. EM consists of three groups of microorganisms: regeneration microorganisms, decomposition microorganisms and opportunistic microorganisms. By combination at different ratios, Higa believed that it is possible to influence positively the given media. It is reported to include: lactic acid bacteria, photosynthetic bacteria, yeast, actinomycetes and fermenting fungi (Szymanski and Patterson 2003). EM technology was suggested to maintain sustainable practices such as farming and sustainable living, and to support human health and hygiene, animal husbandry, compost and waste management, disaster clean-up, and generally used to promote functions in natural communities.

With respect to larval culture system in aquaculture, it was first in 1993 that Skjermo and Vadstein *et al.* (1993) presented a strategy to obtain and maintain microbial

control during larval stages of marine fish. The strategy is based on three different elements that include non-selective reduction of bacteria, selective control of bacterial composition in water and the colonization of the larvae, and enhancement of the ability of larvae to sustain bacteria in the environment. This strategy of maintaining a stable fish culture is based on the development of a stable microbial community that is ready to withstand the colonization of pathogenic bacteria. From microbial ecological point of view, it provides the basic principles for applying efficiently probiotics, as well as a tool for manipulating bacterial populations during larval development of marine fish.

The use of microorganisms as a health aid is not new. People have asserted the health fortifying attributes of yogurt and fermented milk and soybean sauce for thousands of years. “However, with the advent of antibiotics as a cure for many ailments, the public interest in probiotics waned in 60s-70s. The emphasis shifted to the treatment of infections, as opposed to the prevention of infections. In the 1990s the interest in probiotics surged” (Lerner and Lerner 2003). Nowadays for human health, probiotics are being increasingly promoted worldwide, with suggestions that probiotics can play an important role in immunological, digestive, and respiratory functions and could have a significant effect in alleviating infectious disease in children. So far, a number of health effects were defined and measured, including reduction of acute diarrhoea caused by pathogens and viruses, diarrhoea caused by antibiotic therapy, reduction of infections and allergy, immune stimulation, treatment of inflammatory bowel diseases

and prevention of colon cancers (for summary and lists see Santosa *et al.* 2006; Pineiro *et al.* 2007). It was recognized that the intestinal microbiota plays a critical role in inflammatory conditions in the gut, and potentially, probiotics could remediate such conditions through modulation of the microbiota, particularly for infant.

3.3. Current progress and problems of probiotics for aquaculture

The use of probiotics became increasingly popular in aquaculture not only for disease prevention and improved nutrition but also because of an increasing demand for environment-friendly aquaculture. Dozens of scientific papers are published since late 1980s. For a comprehensive list, the reader is referred to Table 1-3 in the articles of Verschuere *et al.* (2000) and to Balcázar *et al.* (2006a). Most microorganisms proposed as biological control agents in aquaculture cover the authorized list of microorganisms of EU and FDA as probiotics in feedings stuffs, which include the lactic acid bacteria, *Bacillus*, yeast *Saccharomyces cerevisiae* etc.. More diverse putative probiotic species such as photosynthetic bacteria, nitrifying bacteria and vibrios are also applied in aquaculture. Gomez-Gil *et al.* (2000) reviewed the use and selection of probiotic bacteria for aquatic larviculture in commercial hatchery experiments. However, at that time the available information was inconclusive, since few experiments with sufficiently robust design had been conducted to permit critical evaluation. Verschuere *et al.* (2000) gave a detailed description of the possible modes of action and presented a rationale for the search for probiotics in aquaculture. More scientific evidence is accumulating in recent years. For instance, the improved

protease and haemocytes phagocytic activity of *Haliotis midae* (Macey and Coyne 2005), immunity enhancement in black tiger shrimp, gilthead sea bream and rainbow trout (Rengpipat *et al.* 2000; Ortuno *et al.* 2002; Irianto and Austin, 2002; Nikoskelainen *et al.* 2003), *in vivo* growth of probiotics (Huys *et al.* 2001), *in vivo* antagonism and inhibition of pathogenic vibrios (Spanggaard *et al.* 2001), enzymatic contribution in shrimp feed (Leonel Ochoa-Solano and Olmos-Soto 2006), significant higher digestive enzyme activity (Ziaei-Nejad *et al.* 2006), iron deprivation of probiont (Holmstrøm and Gram 2003) and disruption of quorum sensing (Defoirdt *et al.* 2006; Tinh *et al.* 2007) were reported.

However, despite recent progress, it is still worth to note that many questions remain unanswered regarding the use of probiotics in larviculture. Under complex larviculture conditions, it is unclear whether these beneficial effects are solely and directly due to the probiotics or partially due to their interactions with other microbes. Very little information is available in relation to their distribution, and how they are evolving and inhabiting larvae in ambient cultural environment. Neither is it known to what extent, probiotics can express their beneficial activities *in vivo* and how probiotics can cope with fluctuating and adverse culture condition. Many of these questions should be addressed during and after probiotics are being employed. It certainly asks for more intensive and integrated research on the use of probiotics, bacterial associations and their interactions under respective culture conditions.

FAO defined the development of affordable, efficient vaccines, the use of immunostimulants and non-specific immune enhancers, and the use of probiotics and bioaugmentation for the improvement of aquatic environmental quality as major areas for further research in disease control in aquaculture. Disease prevention by means of vaccination and immunostimulation has been shown to be effective for some species (Buchmann *et al.* 1997; Ronen *et al.* 2003). However, some major constraints in vaccination including new diseases and pathogens emerging from time to time, vaccination difficulty for small larvae and immature immune system of larvae are presented. Probiotics are proving very popular since with increasing evidence that they promote good health. Especially, in Asia and Latin America where the conditions for using live bacteria are relative flexible. Some hatcheries use routinely the conventional photosynthetic bacteria (Douillet 2000a; Azad 2002), while others prefer their own indigenous beneficial bacteria. It would be unfortunate to see this tendency of applying probiotics excluded from scientific guidelines in terms of validation and safety. It seems that the lack of commercial products of probiotics both in quantity and quality are obvious, partially because of strict safety evaluation and time consuming certification process, also because various species and culture systems. Needless to say, that the probiotics application procedures are far from standardized.

4. Methods of evaluations and characterizations of probiotics in larval culture

4.1. Quality standards and validation

In order to claim that a microorganism or a defined mixture of specific microorganisms has a probiotic effect, according to European Food & Feed Culture Association, quality standards for the evaluation of probiotics should be characterized in terms of strain identification, safety assessment, biological characterization, demonstration of beneficial effects through efficacy studies and confirmation to specification. The development of probiotics applicable to commercial use in aquaculture is a multidisciplinary process requiring both empirical and fundamental research, full-scale trials, and an economic assessment of its use. Verschuere *et al.* (2000) described the crucial phases and rationale for the research and development of probiotics as biological control agents in aquaculture. Vine *et al.* (2006) further proposed a research protocol for the selection of intestinal probiotics in marine larviculture. At first glance, these standards may seem overly stringent, especially for small companies and hatcheries in developing countries. Without sufficient data, the manufacturers may claim that their products are “bacterial-containing” not probiotics. It seems that there are no barriers for local hatcheries to develop their own “home-made” beneficial bacteria by relative simple methods, however lack of convincing scientific validation for the efficacy of any probiotics on health, has been a major

constraint for the development of the probiotics on larval health. It is often difficult to unravel what specific trait a desirable probiotic strain can express in the complex *in vivo* culture environment. Understanding of larviculture ecosystem and the functional role of specific bacteria are urgently needed. Traditional approaches for studying this ecosystem have provided a good foundation in this knowledge base. Complementation of the traditional approaches with the emerging sophisticated molecular tools shows enormous promise for obtaining the necessary insight into the composition and activity of intestinal microbiota (O'Sullivan 2000). In agreement with the definition and rationale of probiotic, *in vivo* validation plays a key role in the whole probiotic development process.

4.2. Conventional cultivation method

Conventional enrichment and cultivation of microorganisms contributed greatly and sustained microbiological research for centuries. As of 2003, over 4,800 bacterial species have been cultivated and published (Keller and Zengler 2004). The application of those isolates for the productions of cell protein, enzymes, organic acids, amino acids and antibiotics has been largely explored. Enrichment, isolation, and cultivation of bacteria are dependent on appropriate growth conditions. Bacteria that can grow on solid media may form visible colonies and can be applied for the determination of the number of bacteria.

4.3. Culture-independent molecular methods

From direct microscopic counts, it was reported that only 1% of the total microorganisms in oligotrophic environments were being cultivated using the viable plate count method (Ferguson *et al.* 1984; Lee and Fuhrman 1991). The use of molecular techniques over the past 20 years has proven the phenomenon that only an extremely small fraction of the microbial diversity has been cultivated from all habitats investigated (Keller and Zengler 2004). Molecular methods have several advantages for the enumeration of probiotics after production and during application. Unlike conventional culture-dependent methods, molecular methods recover and examine DNA or RNA directly from the microbial cells without cultivation. This eliminates both the time required for growth and biases associated with cultured growth. The recovered nucleic acids can be cloned and sequenced directly or amplified by using polymerase chain reaction (PCR), and subsequently the PCR products can be applied to a broader range of techniques for the analysis and differentiation of microbial DNA (Spiegelman *et al.* 2005). Molecular methods are highly sensitive and allow for a high degree of specificity, can precisely identify probiotics from very complex microbial communities. Some of these techniques are not only capable of producing a profile of the whole microbial community, but also provide information about individual members of the community (McCartney 2002). Assisted by biochemical methods, these molecular methods are very useful to

elucidate what traits probiotics harbour in complex microbial communities and what the interactions probiotics to the microbial communities and to the host.

Molecular biological methods that are based on nucleic acid techniques require a highly efficient and reproducible extraction method, which can deliver nucleic acids of high quality. The application of a proper DNA extraction protocol is crucial in applying quantitative molecular methods for the study of microbial community for environmental samples. Currently, many DNA/RNA extraction methods have been developed for different types of samples (water, sludge, soil, settlement etc.). Many optimization methods such as bead beating for the improving cell DNA recovery (De Liphay *et al.* 2004), using propidium monoazide (PMA) selective removal of DNA from dead cells (Nocker and Camper 2006), simultaneous recovery of RNA and DNA from soils and sediments (Hurt *et al.* 2001) are available. Addition of a coprecipitant (yeast tRNA) improved the precipitation of low-concentration DNA as well as optimized sampling size/volume were reported (Boström *et al.* 2004). It is also important to select good sample preservation and storage methods to prevent degradation of DNA/RNA if they cannot be analyzed instantly. Neglecting these aspects may result in failure for *in situ* study and for metabolic status analysis of bacterial and microbial communities (Lam and Cowen 2003).

Apparently, many methods for bacterial and microbial communities characterisation can be applied for the study of probiotics in aquaculture. Spiegelman *et al.* (2005)

categorized three major methods: molecular, biochemical, and microbiological for the characterization of microbial communities, and examined the advantages and disadvantages in details of the various methodologies in terms of technical, economic (time and cost), and regulatory aspects. Dozens of methods including ‘direct cloning and sequencing’; ‘Functional PCR’; ‘DNA microarrays’; ‘randomly amplified polymorphic DNA (RAPD)’; ‘denaturing gradient gel electrophoresis (DGGE)’; ‘terminal-restriction fragment length polymorphism (T-RFLP)’ etc. have been discussed. The following Table 1.1 is not intended to list all of these methods, but rather to summarize most commonly used and newly emerged methods, evaluating and prospecting those molecular methods that are or can be used for probiotic studies in aquaculture.

1 Table 1.1. Comparison of commonly used molecular methods and new methods suitable for probiotic studies.

Methods	Principles	Advantages	Disadvantages	Applications	Prospects for probiotics study
Amplified Ribosomal DNA Restriction Analysis (ARDRA)	PCR-amplified rRNA gene analysis by restriction digestion	Simple, rapid and cost-effective can combine probe hybridization	Pre-selection of digestion enzymes Produce only a community profile without specific phylogenetic groups information	Broad applications, used also for grouping colony library, over hundred reports on probiotics, still popular today (Wu <i>et al.</i> 2007, Carmen Collado and Hernández 2007).	Can be performed in most laboratories, will contribute more on probiotics application
Denaturing/Temperature Gradient Gel Electrophoresis (DGGE/TGGE)	Separation of PCR-amplified rRNA gene sequences based on each sequence has different mobility through a chemical denaturants/temperature gradient	Sensitive to complex microbial community Suitable for spatial and temporal differences analysis a single band representing a single microbial species gel profiles are readily to apply for standard statistical analysis therefore link to environmental variables can combine others techniques such as hybridization	Only works fine below 500bps Only detection predominance species (above 1%) Multiple bands for a single species	Most common used methods Numerous reports on probiotics recent more reports in aquaculture (Schulze <i>et al.</i> 2006; Romero and Navarrete 2006)	Will gain more emphasis Comparison of DGGE data would be possible with accumulated results
Terminal Restriction Fragment Length Polymorphism (TRFLP)	Describe a simplified community restriction pattern using fluorescent PCR primers and automated scanners	Simpler and more sensitive than ARDRA Can detect as low as 0.5% dominance species in a community High-throughput, automated methods	Similar as ARDRA Require costly equipment	Broad applications, also many use in aquaculture (Pond <i>et al.</i> 2006)	May not spread over because no access to equipment

Methods	Principles	Advantages	Disadvantages	Applications	Prospects for probiotics study
Automated rRNA Intergenic Spacer Analysis (ARISA)	Using fluorescence-labeled PCR primers to amplify prokaryotic ribosomal intergenic region, produce the species-specific length polymorphisms	Very simple, rapid and high-throughput, automated methods More sensitive than others fingerprinting methods	Database of intergenic spacer has less information for species identification	Some applications of using RISA and ARISA (Danovaro <i>et al.</i> 2006), no probiotics study report	RISA can be applied if no equipment available
Enterobacterial Repetitive Intergenic Consensus (ERIC)-PCR	PCR amplification of bacterial genomic DNA with primer pair which based on entire conserved central core inverted repeat, results in highly reproducible and unique banding patterns for different genomes.	Simple and high reproducibility Working well for some G+ bacteria Can combine hybridization to achieve unique genome fragment as biomarker	Limited band patterns and resolution Less comparable information of acquired genome fragment information	Some applications for fingerprinting bacteria mixtures and natural microbial communities, also for fingerprinting and tracking microbial population gut microbiota, used for detection of pathogens in seafood (Wei <i>et al.</i> 2004; Shabarinath <i>et al.</i> 2007)	Can be perform in many laboratories Similar to Repetitive extragenic palindromic (rep)-PCR Expect more applications on probiotics study
Serial Analysis of V6 Ribosomal Sequence Tags (SARST-V6)	Produces sequences of large concatemers of PCR amplified ribosomal sequence tags (RSTs) targeting the 16S rRNA genes V6 hypervariable region	Efficient and reproducible analysis provide direct taxonomic information and more phylotype information Less PCR pitfalls due to the small target size	Require labeled primers and magnetic-coated beads for purification Difficult to identify sequences from poorly described taxa from database	Few similar techniques such as SARST, single-point genome signature tags (SP-GSTs) applied to microbial communities composition analysis (Kysela <i>et al.</i> 2005; Neufeld and Mohn 2005; van der Lelie <i>et al.</i> 2006)	Can be used to discriminate closed related probiotics strains or a member in microbial communities

Methods	Principles	Advantages	Disadvantages	Applications	Prospects for probiotics study
Denaturing High Performance Liquid Chromatography (DHPLC)	Separation of PCR products using an ion-pair reversed-phase high-performance liquid chromatography method.	<p>Very sensitive can discriminate one base pair difference</p> <p>Works fine until 1.5 kb</p> <p>Rapid (few minutes), high through-put automated methods</p> <p>Separated fragments can be collected individually for further analysis</p>	<p>Costly equipment</p> <p>Need to optimize running conditions for different samples</p>	Few applications for identifying bacteria, monitoring of microbial population and intestinal biota (Hurtle <i>et al.</i> 2002; Barlaan <i>et al.</i> 2005; Goldenberg <i>et al.</i> 2007)	<p>Very good technique but luxury equipment</p> <p>not widely available</p>
Fluorescence <i>in situ</i> Hybridization (FISH)	Fluorescence labeled oligonucleotides are hybridized to complementary nucleic acid sequences from community samples	<p>Detection of various taxonomic groups by different probes</p> <p>Provide relative abundance of specific taxa and information about spatial distribution</p> <p>Rapid and low cost, matured techniques and protocol can be easily adapted to different sample</p> <p>Combine many other techniques: microautoradiography, flow cytometry and mass spectrometry</p>	<p>Unspecific probe hybridization</p> <p>Probe for rare microbes can be difficult</p> <p>Need equipment to detect fluorescence</p>	Numerous applications for <i>in situ</i> characterization of bacteria communities, many applications on probiotics study and also some on aquaculture (Asfie <i>et al.</i> 2003; Tanaka <i>et al.</i> 2004; Jorquera <i>et al.</i> 2004)	Essential and powerful technique for probiotics and environmental microbial communities study, it becomes more important with more probes available and techniques improvement
Microarray	A collection of microscopic DNA/protein/antibody spots, commonly representing single genes/enzymes, arrayed on a solid surface by covalent attachment to a chemical matrix	<p>Contain a very large number of genes or protein</p> <p>Quick and high throughputs</p> <p>Combine different experiments in a single database</p>	<p>Produce extremely large amount data, require effective data analysis</p> <p>Very expensive</p>	A number of applications for probiotic study (Dubois <i>et al.</i> 2004; Callanan 2005; Marco <i>et al.</i> 2006)	Probably can apply soon to some gnotobiotically grown model animals such as <i>Artemia</i> and zebra-fish for the test of probiotics

Despite of a wide range of available molecular tools, these current methods are often inadequate because they are too slow, require too much effort to obtain data, yield biases, are too expensive, offer insufficient quantification, and lack coverage over the ranges of structure and function that are important in relevant microbial communities (Rittmann *et al.* 2006). To overcome these problems, the choice of right methods that are suitable for the study of probiotics in aquaculture appears very important. As listed in Table 1.1, some methods are powerful for pure cultures e.g. ARDRA, while others can provide rich data both in species and community levels. These methods can be used to analysis microbial communities and target specific probiotics in an environmental sample. By choosing different genes, microarray can provide phylogenetic or functional information of a microbial community (Zhou 2003). To the hosts, microarrays are also very useful for the study of host responses to microbes. High-density microarrays containing hundreds of thousands of distinct oligomers have been developed for zebrafish, salmon and catfish (Vanya *et al.* 2005; Douglas 2006; Li and Waldbieser 2006). Probing techniques such as FISH and microarray can obtain sufficient quantitative data in few minutes or hours. At present, DGGE fingerprinting and FISH are the most used methods in many microbial systems since not only they can detect and monitor specific microbes *in vivo*, but also provide high throughput data of environmental microbial communities profiles. DGGE combined with FISH techniques have been used in many microbial ecosystems. Taking the

advantage of both DGGE and FISH, a protocol is proposed and can be used for the study of probiotics in aquaculture (Fig. 1.1).

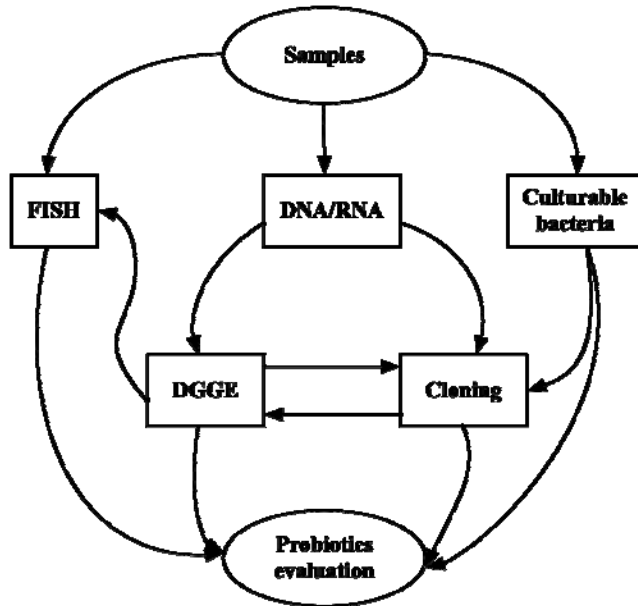


Fig. 1.1. Diagram of DGGE-FISH and clone libraries to study probiotics in aquaculture. FISH offers semi-quantitative data and spatial view of probiotics in microbial community; DGGE can provide microbial community composition profile and can be used for screening clones; both colony library and culturable bacteria can be used to verify the effect of probiotics and the interactions with other community members. After DGGE analysis, the sequencing information can develop new probes for FISH analysis.

4.4. Molecular fingerprinting and data processing

Molecular fingerprinting techniques such as DGGE generate large amounts of data.

While the first application of DGGE in microbiology dates from 1993, a single search on ISI Web of Knowledge results over 2000 articles in the last 5 years. The DGGE fingerprinting method is also adopted in the field of probiotics study where it enables the simultaneous analysis of vast numbers of samples. Fromin *et al.* (2002) listed several features that are important before performing analysis: the assigning a single

band to a single bacterial population, the band intensity related to the density of corresponding bacterial phylotypes and the reproducibility of DGGE. Based on these criteria, single band and whole profile analysis can take advantage of ecology studies, where several diversity indices and models can be applied. Shannon-Weaver and Evenness indices are most commonly calculated to describe possible changes of dominant species, recently Mertens *et al.* (2005) presented the Lorenz curve as an alternative diversity measurement for PCR-DGGE data. “It provides a non ambiguous graphical presentation of species evenness which can be numerically measured with the Gini coefficient. Together with the number of bands as a value for species richness, this method describes diversity in a way that both species evenness and richness can be considered separately” (Mertens *et al.* 2005). To compare banding patterns in pairs, similarities coefficients can be calculated either based on band presence/absence (such as Jaccard or Dice coefficient), or based on band intensity profile (such as Pearson correlation coefficient). The similarities can serve for further clustering and ordination analysis or for moving windows analysis for consecutive data. Ramette (2007) considers the cluster analysis, principal component analysis (PCA), multidimensional scaling (MDS) and principal coordinate analysis (PCOA) as exploratory analysis which are often used; hypothesis-driven techniques such as redundancy analysis, canonical correspondence analysis (CCA), or Mantel tests are more rarely used but with great potential in microbial ecology. The author also pointed out that multivariate analyses could reduce data set complexity and identify

major patterns and putative causal factors. In the study of Gafan *et al.* (2005), logistic regression successfully differentiated between the profiles and specified the individual operational taxonomic units associated with these differences. At present, evidence supports that the age for hypothesis-driven studies for which new models and analysis applied in the field of microbial community, has been reached. Together with environmental data, these analysis can help to clarify the influences of probiotics to the microbial community composition and the contributions of probiotics to the functionality of microbial communities, and finally, to predict a shift or consistence in a microbial community composition in aquaculture.

It is true that no single tool and analysis method can answer the questions of probiotics function in a complex microbial community. Even the most powerful technique ‘shotgun cloning of whole community DNA’, which directly produces a large amount of information, is still very time-consuming and costly. To clarify the roles of probiotics in larviculture, a clear combined approach of different methods can provide information not only establishing the presence of the probiotics in the whole microbial community, but also the possible linkages to their function. In a study of Kim and Austin (2006), DGGE was used with a view not only to determine the bacterial diversity but also more specifically to determine the colonization of *Carnobacterium maltaromaticum* and *Carnobacterium divergens* in rainbow trout. Combined with immune analysis, it showed that the two carnobacterial cultures were beneficial for rainbow trout in terms of resisting challenge to *A. salmonicida* and *Y.*

ruckeri, and enhancing cellular and hormonal immune responses, such as, phagocytic and respiratory burst activity of head kidney macrophages and lysozyme activity of serum and gut mucus. Lipid class and fatty acid composition were used as biochemical indicators of health in haddock (*Melanogrammus aeglefinus*) larvae (Plante *et al.* 2007). In combination with DGGE as monitoring tool of probiotics and microbiota, it was found larvae exposed to a candidate probiotic bacterium *Arthrobacter* sp. showed a superior rate of increase in dry mass and lipid accumulation compared to control groups. DGGE analysis revealed that this bacterium enhanced the general health of haddock larvae by modifying their bacterial microbial environment.

5. Studying probiotic effects through microbial community analysis in larval culture feed chain

Successful larviculture relies on a better insight into the complex interactions between the cultured organisms and the bacterial communities that develop in the rearing systems. In aquaculture, the term ‘probiotics’ is often loosely used to describe a microbial formulation responsible for biocontrol or bioremediation (Vine *et al.* 2006).

5.1. Eggs

Probiotic control in tank water and colonization of larvae can both be achieved by direct addition of selected bacteria strains to the tanks, or by incorporation of the

bacteria into live or formulated feeds. The fish egg is the first stage of a fish life-cycle that is exposed to bacteria. Presently, egg disinfection is the very first effective barrier against transmission of fish diseases, and is therefore highly recommended for all batches of eggs, both those produced in the hatchery and those brought from other hatcheries.

Due to the undesirable side-effects and the risks to induce bacterial resistance, antibiotics are rejected as the preferred disinfectant. However, most commonly used disinfectants, such as active iodine, unselectively kill (almost) microorganisms on the surface of eggs, making them vulnerable to opportunistic colonization by bacteria. This disinfection approach may disturb the balance between microbial communities, or favour proliferation of opportunistic bacteria or unpredictable development of bacterial communities (Olafsen 2001). According to Hansen and Olafsen (1999), in aquaculture, eggs are kept in incubators with a microbiota that differs considerably from that in the sea, and become heavily overgrown with bacteria within hours after fertilization. Using conventional microbial methods, the epibiota of fish eggs have been described in a number of papers, reviewed by Hansen and Olafsen (1999).

In a natural environment, Míguez and Combarro (2003), using plating methods, studied the influence of bacterial epibiota on egg hatching of the sardine (*Sardina pilchardus*). Total bacteria, viable bacteria or the presence of specific potential pathogens for eggs, such as *Pseudoalteromonas piscicida* and *Tenacibaculum*

(Flexibacter) ovolyticus, did not affect the viability of sardine eggs. Additionally, no relationship was observed between the presence of *Vibrio* spp., pathogenic for fish larvae, and the egg hatching. The authors concluded that the epiphytic bacteria did not affect the wild sardine eggs. This was probably because the amount of bacteria associated with the eggs was between 10^2 and 10^4 times lower than those found so far on the eggs of different fish species in rearing systems. In some aquatic invertebrates, strong experimental evidence indicates an essential role for specific bacteria in host egg protection (McFall-Ngai 2002). A recent study by Verner-Jeffreys *et al.* (2006), characterized the microbiota of rinsed eggs of Pacific threadfin, *Polydactylus sexfilis*, and amberjack, *Seriola rivoliana*, using a combination of culture based and non-culture PCR clone-RFLP methods, which the bacterial 16S rRNA genes were PCR amplified from the egg homogenate and directly cloned into *E. coli*. Insert containing clones were screened by PCR-RFLP and representatives from each RFLP pattern sequenced. The adherent microbiota of eggs, examined within a few hours of release, varied both qualitatively and quantitatively. A highly virulent *Vibrio* sp. was also found.

It is recommended that measures should be taken to treat the eggs to remove, or otherwise control, the development of possibly harmful microorganisms. Therefore, a relatively dense, non-pathogenic, and diverse adherent microbiota present on the eggs would probably be an effective barrier against the formation of a colony by pathogens on fish eggs (Farzanfar 2006). It can be hypothesized that inoculation of selected

bacteria to the incubator would not only reduce the chances of opportunistic bacteria becoming dominant, but also have a beneficial effect on the further first colonization in the gut of the fish larvae. With the support of advanced techniques, the structure and function of epimicrobiota of eggs, the effects and influence of probiotics and disinfectants, could be revealed.

5.2. Microalgae

Microalgae are an essential food source in the rearing of all stages of marine bivalve molluscs (clams, oysters, and scallops), the larval stages of some marine gastropods (abalone, conch), larvae of several marine fish species and penaeid shrimp, and zooplankton (Lavens and Sorgeloos 1996). Several microalgae such as *Isochrysis* sp., *Chlorella* sp., *Nannochloropsis* sp., *Dunaliella* sp., *Chaetoceros gracilis*, and *Tetraselmis* sp. are often used directly in the tanks for rearing marine fish larvae. This “green water” technique is part of the commonly applied techniques for rearing larvae of gilthead sea bream *Sparus aurata*, milkfish *Chanos chanos*, Mahimahi *Coryphaena hippurus*, halibut *Hippoglossus hippoglossus*, and turbot *Psetta maxima* L. (Lavens and Sorgeloos 1996).

There is no doubt that microalgae play a vital role in nutritional values for larvae and live feed rotifer and *Artemia*. Microalgae are also believed to play a role in stabilizing the water and microbial control. In aquaculture, mass cultures of microalgae in open condition are commonly accompanied by heterotrophic bacteria. Most bacteria

probably originate from stock cultures or from ambient environment. It seems that growth of bacteria in algal cultures is inevitably a function of and closely follows algal growth (Ward and Moyer 1966). For the stabilization of mass microalgae culture, Suminto and Hirayama (1997) tested the effect of a marine bacterium, *Flavobacterium* sp., which was found to promote growth of a marine diatom *Chaetoceros gracilis* in the axenic culture condition, on the mass cultures of three marine microalgae (*C. gracilis*, *Isochrysis galbana*, and *Pavlova lutheri*). The specific growth rate of *C. gracilis* in treated cultures was significantly higher than that of control cultures, and the stationary growth phase in the treated cultures lasted longer till the end of the culture period. However, the bacterium had no apparent effect on the exponential growth phase of two phytoflagellates, *I. galbana* and *P. lutheri*, but extended the high cell density in the stationary growth phases. The added bacterial strain (*Flavobacterium* sp.) was the dominant species (more than 45%) among the bacterial biota during the culture period. Fukami *et al.* (1997) investigated several examples of stimulating and inhibitory effects of bacteria on microalgae growth and the importance of bacteria in algal mass culture. A benthic diatom, *Nitzschia* sp., was stimulated by a bacterial film of *Alcaligenes* on the surface of the substratum. On the other hand, a strain of *Flavobacterium* sp. isolated from natural seawater during the decline period of an algal bloom had a strong algaecide effect on the red tide plankton, *Gymnodinium mikimotoi*.

On a community level, specific associations of bacteria with microalgae have recently been reported. In a study by Sapp *et al.* (2007a), diversity and succession of bacterial communities was analyzed. Members of α -proteobacteria and γ -proteobacteria and the *Flavobacteria-Sphingobacteria* group within the *Bacteroidetes* phylum predominated in the cultures. Shifts in the bacterial communities could not be correlated to changes of nutrient levels or algal growth phases. The authors could not conclude that the compositions of the bacterial communities were strictly species specific for microalgae. The importance of factors like the composition of exudates is apparent. In another study by Sapp *et al.* (2007b), the composition of bacterial communities associated with four diatom species was monitored during isolation and cultivation of algal cells. Strong shifts in the associated communities, linked with an increased number of phylotypes belonging to members of the γ -proteobacteria, were observed during cultivation. Similarly, Grossart *et al.* (2005) described two marine diatoms species harbouring distinct bacterial communities. There is increasing evidence that there are specific bacterial taxa associated with phytoplankton, suggesting the presence of specific selective mechanisms, and implying that the bacteria have some function to the benefit of the alga (Jasti *et al.* 2005).

It is well known that many algal strains require exogenous vitamin-B12. Croft *et al.* (2005) provided genetic evidence that a bacterium, *Halomonas*, upregulates the biosynthesis of vitamin B12 when in the presence of algal exudates, and demonstrated algae acquire vitamin B12 through a symbiotic relationship with bacteria. In the field

of aquaculture, Avendano and Riquelme (1999) demonstrated the use microalgal cultures as vectors for the introduction of bacterial antagonists against bacterial pathogens in mollusc larval culture. Inhibition of luminous *Vibrio harveyi* by “green water” was investigated by Huervana *et al.* (2006).

5.3. Rotifers

During the last 30 years, the increase in marine fish larvae rearing around the world has been due partly to the availability of rotifers (*Brachionus* spp.), as live feeds for first-feeding fish larvae (Yoshimura *et al.* 1996). Today, more than 60 marine fish species and 18 crustacean species cultures require adequate and reliable production of high quality, nutritious rotifers. The quality of rotifer cultures is evaluated not only by reproduction rate and density, but also nutrients essential and associated microbiota for the larval predators (Dhert 1996). Bacteria are always associated with mass production of rotifers and may cause unexpected mortality or suppressed growth to rotifers. In some other cases, no harm is caused to the rotifers but infected rotifers cause a detrimental effect on fish larvae. Although most bacteria are not pathogenic for rotifers, their proliferation must be avoided since their potential accumulation and transfer via the food chain can cause detrimental effects to the predator (Dhert 2001).

In a study by Dhert *et al.* (2001), rotifers were examined as vector of bacteria and the effect of associated microbiota on rotifer cultures were discussed. The authors indicated that the present disinfection procedures are not sufficient to guarantee

complete sterile conditions, and probiotics could be used to control the microbiota associated with the rotifers. The success of this method depends on the microbiota present in the system at the moment of inoculation. Therefore, the start-conditions are of big importance and axenic start-cultures may improve the subsequent cultures (Rombaut *et al.* 1999). Recently, terrestrial lactic acid bacteria were tested for the enhancement of rotifer cultures (Planas *et al.* 2004). Autochthonous potential probiotic bacteria were also selected and identified from rotifer cultures (Hjelm *et al.* 2004).

To evaluate the effect of different microbial communities, Tinh *et al.* (2006) developed a technique to obtain axenic rotifer cultures from amictic eggs using disinfectants of non-antibiotic nature. The experiments with heat killed microbial communities yielded no increase in rotifer growth rate, suggesting that the observed enhancement in rotifer growth rate was truly a probiotic effect rather than a nutritional effect.

However, it should be pointed out that recent results have demonstrated that the most commonly cultured rotifer strain, *Brachionus plicatilis*, is actually a species complex (Papakostas *et al.* 2005; Dooms *et al.* 2007). The results of those studies show that many hatcheries are growing rotifers strains that are different from their presumed identity. This has important implications for rotifer cultures, with respect to the different temperature and salinity optima of each biotype. Their specific associations

with microbial communities will be worth to identify. Interestingly, our study shows considerable strain specific associations in continuous batch cultures of three rotifer biotype (*B. plicatilis* ‘sensu strictu’, *B. plicatilis*. ‘Cayman’ and *B. plicatilis* ‘Nevada’). These results could help us to find distinct bacterial species links to different rotifer species. *Brachionus* strain specific microbial communities could be part of an integrated microbial management in hatcheries.

5.4. *Artemia*

The brine shrimp *Artemia* is the main organism used as ‘live food’ in the later stages of larviculture. Over 85% of all marine animals now utilize *Artemia* as a partial or sole diet during their larval phase (Sorgeloos *et al.* 1994). It is well documented that during the hatching and enrichment processes, the numbers of bacteria in *Artemia* increase significantly (Olsen *et al.* 2000; Eddy and Jones 2002; Ritar *et al.* 2004; Makridis *et al.* 2006). The bacterial microbiota associated with enriched *Artemia* nauplii may harbour bacteria deleterious to the fish larvae. Results of Eddy and Jones (2002) showed a shift towards the microbiota with higher levels of *Vibrio* and *Enterobacter*. Those studies have established a direct link between bacterial biota composition in *Artemia* and the microbial community of larval species to which they were fed.

During enrichment of *Artemia*, interesting results from Bourne *et al.* (2004) show the presence of Gram-negative γ -proteobacteria: *Alcanivorax* species retrieved from the

DGGE, which are common in seawater and often associated with degradation processes in oil-contaminated nutrient-supplemented environments (Kasai *et al.* 2002). This group of bacteria may be favoured by the high lipid content in the enrichment diet. This study can help to understand the microbiology associated with larval-rearing technology such as live feed enrichment.

In another report, the microbial conditions and antimicrobial activity were determined in batch cultures of two microalgae species, *Tetraselmis chuii* and *Chlorella minutissima* (Makridis *et al.* 2006). Incubation of enriched *Artemia* in cultures of the two microalgae for 30 min resulted in a significant decrease of the bacterial load in *Artemia*, and a significant decrease of the level of *Vibrio* in *Artemia* homogenates. The results of this study demonstrate a simple and practical approach to decrease the microbial load and at the same time reduce the percentage of *Vibrio* among the bacteria associated with enriched *Artemia* (Makridis *et al.* 2006).

However many of those studies are conducted in open xenic cultures, it does not separate the effect of supplements from any contribution of *Artemia* native microbiota. Needless to say, those extra supplements may interact with microbes, shifting *Artemia* associated bacteria communities composition, resulting directly and indirectly in indistinguishable effects. To define the functioning of the host in the absence of bacteria and then to evaluate the effects of adding a single or defined population of microbes, or certain compounds (i.e., under gnotobiotic conditions),

Marques *et al.* (2004) have recently developed and validated the usefulness of an *Artemia* gnotobiotic test system allowing to study the effect of host-microbe interaction and to evaluate potential probiotic bacteria before testing in target-organisms. Based on this test model, a bundle of intensive studies were carried out by Marques *et al.* (2004, 2005, 2006a, 2006b), Defoirdt *et al.* (2006), and Soltanian *et al.* (2007a, 2007b). Different yeast cell wall mutants, bacteria, microalgae as well as short-chain fatty acids and β -glucans were tested against different pathogens. A similar rotifer gnotobiotic test system was developed by Tinh *et al.* (2006) for the evaluation of microbial functions and the nutritional value of different food types.

5.5. Prebiotics

Beside the analysis of all the possible bacterial input to the larviculture system and the examination of every entry point for efficient application of probiotics, there is an additional approach based on the use of prebiotics aiming at improving the survivability and the implantation of probiotics (Bomba *et al.* 2002). Dietary application of probiotics, which are live microbial organisms, may be restricted due to technical constraints such as heat inactivation during feed manufacturing, and harsh conditions of stomach and guts may hinder exogenous probiotics survival and propagation. Examples of *Bacillus* spores germinators such as inosine or L-alanine (Barlass *et al.* 2002), pH mediator such as metabolisable sugars (Corcoran *et al.* 2004) can be good candidates of prebiotics. This does not exclude the possibility that various tested health-promoting (to the host) products (beta glucans, chitins etc),

known as immunonutrients and immunostimulants, could also act as probiotic enhancing or diverting positively the associated microbial community. Therefore, tests using gnotobiotic systems and intensive evaluations *in vivo* are required. In our study by DGGE monitoring, autoclaved yeast mutants, acting as prebiotics, are able to support the growth of probiotics against a *Vibrio* pathogen.

After many attempts of probiotics applications, it became clear that a single addition of a single probiotic species can hardly be expected to achieve constant good results. However, combinations of several probiotics or mixture of as much as possible ‘good’ bacteria do not mean that they can work together. Recent studies provided some insight in this, demonstrating the improved adhesion to intestinal mucus by probiotics mixes (Collado *et al.* 2007a), enhanced *in vivo* probiotic effects of co-immobilized *Lactobacillus acidophilus* and *Bifidobacterium bifidum* (Kushal *et al.* 2006) and more effectiveness in the inhibition of pathogen adhesion (Collado *et al.* 2007b).

6. Recent highlight research and guiding principle

Over the past two decades, new discoveries and breakthroughs in microbiology provide great opportunities for probiotic technology. Figure 1.2 presents various strategies to improve probiotic effects in fish larviculture. Recent studies have revealed diverse chemical signals that enable intra-specific and prokaryote-eukaryote communications. Discovery of the chemical signals disruptors and manipulators from bacteria and algae has not only provided a promising means to control bacterial infections, but also presents new challenges to investigate their roles in host organisms and their potential impacts on ecosystems (Joint *et al.* 2002; Defoirdt *et al.* 2004; Dong and Zhang 2005). As a number of bacteria, yeasts, algae, fungi and actinomycetes in the biosynthesis of metal nanoparticles have been described (Sastry *et al.* 2003). Studies of antimicrobial effects of silver nanoparticles by bacterial absorption and reduction (Chen *et al.* 2003; Mandal *et al.* 2006) may provide excellent 'arms' for probiotics to fight pathogens.

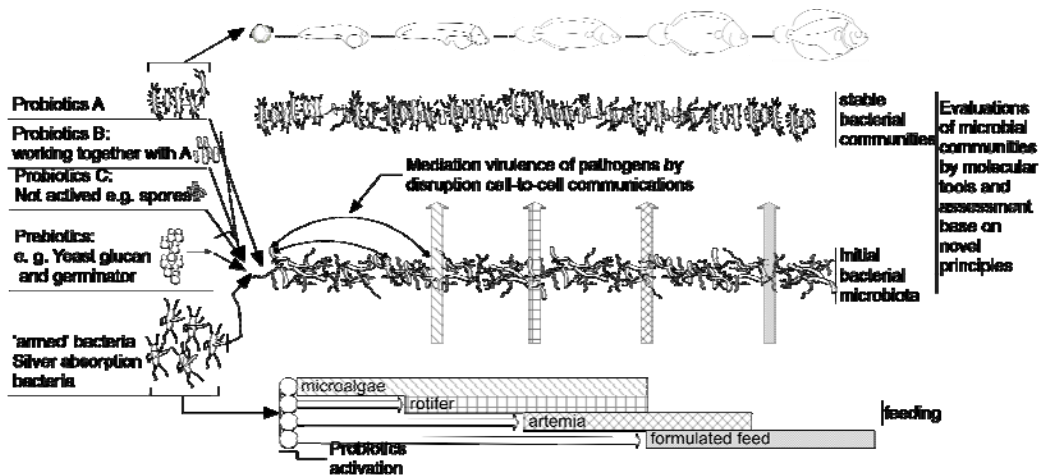


Fig. 1.2. Diagrams of strategies to improve probiotics effects in fish larviculture. Probiotics can be applied immediately after eggs disinfection, to limit the possible of colonization of potential pathogenic bacteria (probiotics A). Probiotic products can consist two or more probiotic strains, each has its own beneficial trait (probiotics A + B). Spores forming probiotics are able to resistant harsh conditions during the production cycle, and they are excellent for transportation and storage (probiotics C). Some additives such as spores germinators and yeast cell wall composition glucan can be used as prebiotics to improve probiotic effects or benefit directly the hosts. Some microbes can accumulate metal nanoparticles such as silver; their antimicrobial effects can be utilized to fight pathogens. Probiotics also can be selected to disrupt chemical signal communication of pathogens. The probiotic applications should fuse into larviculture procedures and their interactions with live and formulated feed must be evaluated. Using proper monitoring tools, the fate of probiotics and dynamic changes of microbial community can be evaluated. The final goal is to achieve the stable and balanced microbial community for a health larviculture.

To properly manage complex microbial systems, engineers need well-documented concepts and principles, such as the Hubbell neutral theory (HNT), and the macroeconomic Pareto 80/20 principle. Ecological equivalence or functional redundancy is the cornerstone of Hubbell neutral theory (Hubbell 2001, 2005); it is based on the idea that there is equality of fitness of individuals over all environmental conditions in spatial scales and time frames. “In particular, strong support for neutral community models (NCMs) would be indicated if closed replicate interconnected

microbial communities maintained different species compositions and diversities for substantial amounts of time, and if these patterns matched those seen in a NCM (and nature). Alternatively, rapid convergence of different (closed) microbial communities to dominance by the same species in all replicates would be more consistent with non-neutral views” (Holyoak and Loreau 2006). Due to the fact that bacterial communities also exhibit diversity–energy relationships (Prosser *et al.* 2007), Pareto's law can be tested, i.e. 20% of the species govern 80% of the energy flux of the ecosystem (Dejonghe 2001), therefore, the contributions of species richness and evenness to the functional stable microbial community can be determined. Those concepts would be useful to interpret the normal, respectively, distorted functioning of microbial communities in open ecosystems (Verstraete 2007). This of course can help us to design and define probiotics to be used in aquaculture system, in order to obtain a ‘stable’ and ‘mature’ microbial community in a larviculture system.

Chapter

2

Current status and prospect of probiotics in aquaculture of China

Abstract

Aquaculture is now the fastest growing food-producing sector in the world, and nearly 70% of the aquacultural production is produced in China. With the development of aquaculture, disease problems inevitably emerged. The development of non-antibiotic and environmental friendly agents is one of the key factors for health management in aquaculture. The application of probiotics in aquaculture of China could be traced back in 1980s, with photosynthetic bacteria being used in penaeid shrimp aquaculture as probiotics. In the middle of 1990s, commercial probiotic products from USA, Japan and United Kingdom were introduced into China, and trial experiments were conducted in penaeid shrimp hatcheries. In the mean time, scientists in China started to screen new probiotics strains from local aquaculture rearing unit to suit the specific requirement in China. Also the mode of action of probiotics was studied. Currently, products containing probiotic bacteria are gaining popularity in aquaculture of China, and the application areas have been greatly expanded. Manufacturers claim high numbers of viable counts and multifunctional. However, very few or no studies substantiates these claims. The quality control of probiotics in aquaculture has become an important issue in China; therefore, several types of effective evaluation methods are developing. In conclusion, research on probiotics in aquaculture of China is still in an early stage of development and much work still needs to be done.

Keywords : probiotics; aquaculture; China; quality control

1. Introduction

Today, aquaculture is the fastest growing food-producing sector in the world, with an average annual growth rate of 8.9% since 1970, compared to only 1.2% for capture fisheries and 2.8% for terrestrial farmed meat production systems over the same period (Subasinghe 2005). World aquaculture has grown tremendously during the last fifty years from a production of less than a million metric tons in the early 1950's to 59.4 million metric tons by 2004. This level of production had a value of US\$70.3 billion. Of this production, 41.3 million metric tons, or 69.6 %, was produced in China and 21.9 percent in the rest of Asia and the Pacific region (FAO 2006).

China is one of the most important contributors to world aquaculture production. Marine capture fisheries were most important until the 1980s, when the fisheries authority of China, being aware that many major marine fishery resources were being depleted, shifted its priority from wild fishing to aquaculture. As a result of this significant shift, aquaculture development has accelerated throughout the country. Since 1993, aquaculture has accounted for more than half of the country's fisheries production, making China the first major fisheries country in the world whose cultured fisheries produce more than its capture fisheries. Mariculture in China may date back to the Song Dynasty about 1000 years ago, involving cultivation of a kind of seaweed called glueweed, *Gloiopeltis furcata*, by a simple rock-cleaning method in Jinmen, Fujian Province (Tseng 1993). The intense development of mariculture started only 3 decades ago. Thus, over the last 30 years, the production of mariculture has been increasing rapidly in China from 184,000 metric tons in 1970, to 444,000 metric tons in 1980, to 1.6 million metric tons in 1990, to 10.6 million metric tons in

2000, and to 13.5 million metric tons in 2005 (Zhong and Power 1997; FAO 2006).

Globally, aquaculture is expanding into new directions, intensifying and diversifying. With the increasing intensification and commercialization of aquaculture production, disease problems inevitably emerged. Disease is now a primary constraint to the culture of many aquatic species, impeding both economic and social development in many countries (Bondad-Reantaso *et al.* 2005). Infectious disease may emerge within a country in a number of ways, for example by the introduction of known exotic diseases, by sudden changes in the pattern of existing endemic diseases, or by the appearance of previously unrecognized diseases. Contingency planning, early warning and early response are critical to the effective management of such disease emergencies (FAO 2006). Shrimp diseases in China caused US\$ 420 million loss in 1993 (Wei 2002). The disease is believed to have been caused by baculovirus (hypodermal and hematopoietic necrosis baculovirus; HHNBV) which became epizootic because of the deteriorating ecological conditions (Wang *et al.* 1997). Fish diseases in China are mainly caused by bacterial pathogens, including *Edwardsiella tarda*, *Vibrio anguillarum*, *V. harveyi*, *Photobacterium damsela* subsp. *piscicida*, etc. (Xiao *et al.* 1999; Wang *et al.* 2002; Wang *et al.* 2007; Lan *et al.* 2008).

For decades, antibiotics routinely used for treatment of human infections were also used for aquatic animals, for therapy, prophylactic reasons or growth promotion. The accumulated evidence shows that the widespread use of antibiotics for treating bacterial diseases has been associated with the development of antibiotic resistance (Hernández Serrano 2005). In 2006, the indiscriminate use of antibiotics for fish has increasingly become a matter of public concern in China, and a legal framework is being enforced. Currently, a limited number of government-approved antibiotics and

chemotherapeutic agents are used for prevention and treatment of infectious aquatic diseases. However, because of the complex nature of aquatic culture systems and the diversity of culture species and pathogens, few antibiotics can be licensed for efficient and safe use. At present, there is an urgent need to discover new alternatives or approaches for the abuse of antibiotics.

The use of probiotics, which control pathogens through a variety of mechanisms, is increasingly viewed as an alternative to antibiotic treatment (Verschuere *et al.* 2000). Recently, the need for probiotics in aquaculture, the principles and mechanisms of action were summarized (Kesarcodi-Watson *et al.* 2008). Particular emphasis was placed on *in vivo* challenge test needs at the initial probiotic screening stage. The goal of this review is to summarize the studies on probiotics quality control in aquaculture, the current status of using probiotic products in aquaculture in China and to outline the molecular microbial ecological tools for the enumeration of probiotics in a complex microbial community.

2. Studies on probiotics quality control in aquaculture

Probiotics, which are microorganisms or their products with beneficial effect on the health of the host, have found use in aquaculture as a means of disease control, supplementing or even in some case replacing the use of antimicrobial compounds (Irianto and Austin 2002). FAO has now designated the use of probiotics as a major means for the improvement of aquatic environmental quality (Subasinghe *et al.* 2003). Many publications about probiotics have emerged in aquaculture in the last decade (Gatesoupe 1999; Gomez-Gil *et al.* 2000; Vine *et al.* 2006). A lot of studies claim beneficial effects, while most of them lack proof for the mode of action *in vivo*

(Verschuere *et al.* 2000). A wide range of yeasts, Gram-positive and Gram-negative bacteria have been evaluated as putative probiotics (Balcázar *et al.* 2006b). Most studies on the effects of probiotics on cultured aquatic animals have emphasized a reduction in mortality or the improved resistance against putative pathogens (Irianto and Austin 2002). However, the beneficial effects are sometimes temporal, depending on the time of exposure (Verschuere *et al.* 2000). As most fish contain a specific intestinal microbiota established at the juvenile stage (Olafsen 2001), the colonization of probiotics to fish intestines requires adequate probiotics presented in ambient microbial community (MC) and their interaction with MC should not be neglected.

Commercial probiotic production should take into account beneficial traits of strain useful during industrial processing. To overcome the problem of inactivation during the manufacturing process, aquaculture industries try to improve the technology by screening for more resistant strains or alternatively by protecting the probiont through micro-bioencapsulation. Frequently, rotifer and *Artemia* are being used as live carrier of probiotics in larviculture (Gomez-Gil *et al.* 1998; 2000; Patra and Mohamed 2003). However, few studies demonstrated the colonization properties of probiotics and the microbial community evolution in those bioencapsulation processing (Makridis *et al.* 2000a; Martínez-Díaz *et al.* 2003).

By monitoring probiotics and the microbial community structure and dynamics in the manufacture process and *in vivo* culture system, the viability and effects of probiotics can be documented in detail. For this purpose, nucleic acid-based techniques have been used. For instance, in some laboratorial studies the green fluorescent protein is expressed by target strains; allowing the probiotic strain to be tracked (Collins and Gibson 1999). Alternatively, by group- or species-specific gene sequences, different

groups or closely related bacteria can be discriminated by fluorescent *in situ* hybridization (FISH). Fingerprinting techniques such as denaturing gradient gel electrophoresis (DGGE) allows us to determine the constituents of complex microbial communities in aquaculture systems; with FISH and microarray tests probionts can be enumerated rapidly (McCartney 2002). Therefore, the characters of probiotic strains *in vivo* can be demonstrated not only with regard to their presence or absence, but also their influences in microbial community in the host. Highly discriminative molecular methods such as 16S rRNA gene sequencing and oligonucleotide probes can also be used for accurate probiotic species labeling, which is important for responsible quality control efforts, to build consumer confidence in product labeling, and for safety considerations (Yeung *et al.* 2002). The reliable identification of probiotics requires molecular methods with a high taxonomic resolution that are linked to up-to-date identification libraries.

3. Current status of using probiotic products in aquaculture of China

Modern application of probiotics in Chinese aquaculture could be traced back to the 1980's following the popularity of using antibiotics. In the last 10 years, there has been an exponentially growing application of probiotics in aquaculture: at present more than hundred companies are producing many types of probiotics for aquaculture, and probably over 50,000 metric tons of commercial probiotic products are sold annually with a market value estimated at 50 million euro. The probiotics used in Chinese aquaculture are mainly photosynthetic bacteria (PSB), antagonistic bacteria (*Pseudoaltermonas* sp., *Flavobacterium* sp., *Altermonas* sp., *Phaeobacter* sp., *Bacillus* sp., etc.), microorganisms for nutrition and enzymatic contribution to digestion (lactic acid bacteria, yeast, etc.), bacteria for improving water quality

(nitrifying bacteria, denitrifiers, etc.), *Bdellovibrio*, and commercial probiotics.

3.1. The photosynthetic bacteria

Currently, photosynthetic bacteria are found in five bacterial phyla, i.e. *Chlorobi*, Cyanobacteria, *Chloroflexi* (filamentous anoxygenic phototrophs), *Firmicutes* (heliobacteria) and *Proteobacteria* (purple sulfur and purple non-sulfur bacteria) (Bryant and Frigaard 2006). Traditionally in Chinese aquaculture, the photosynthetic bacteria refer to the photosynthetic bacteria in the *proteobacteria* alpha subdivision, i.e. the purple non-sulfur bacteria. Purple non-sulfur bacteria are widely distributed in freshwater, marine, soil and hot-spring environments. They have various metabolic pathways for the degradation of organic wastes. They also have a more digestible bacterial cell wall, and are rich in proteins, carotenoids, biological cofactors, and vitamins (Kobayashi and Kurata 1978). The species currently used in Chinese aquaculture are *Rhodopseudomonas palustris*, *Rubrivivax gelatinosa*, *Rhodobacter capsulata*, *Rhodobacter spheroides*, *Phaeospirillum fulvum*, etc (Fig. 2.1). Those are probably the earliest and the most widely used probiotics in China since the 1980's. It was reported that the addition of photosynthetic bacteria as food additives stimulated the growth of shrimp and fish (Zhang *et al.* 1988), enhanced the survival rate of fish larvae, and improved the production of scallop seed (Huang *et al.* 1990; Wang *et al.* 1994). They were also found to increase the population growth rate of live food such as *Brachionus plicatilis* (Xu *et al.* 1992). Furthermore, some plant and herb extracts were identified as growth promoter for the mass cultivation of photosynthetic bacteria (Xu *et al.* 1994). Nowadays, using photosynthetic bacteria as probiotics is common practice in many fish or shellfish hatcheries and farms in China. Instead of using home-made photosynthetic bacterial products, many farmers today are using

concentrated and encapsulated commercial photosynthetic bacterial products. Many commercial photosynthetic bacterial products are labeled as either single or multiple species at concentrations higher than 10^9 mL⁻¹, and are often combined with growth promoters or conditioners, and are claimed to have multi-functional effects such as improvement of water quality, enhancement of growth rate and prevention of disease.



Fig. 2.1. Photosynthetic bacteria used in aquaculture of China

3.2. Antagonistic bacteria

Bacterial antagonism is a common phenomenon in nature; therefore, microbial interactions play a major role in the equilibrium between competing beneficial and potentially pathogenic microorganisms. The microbiota in the gastrointestinal tract of aquatic animals can be modified, for example, by ingestion of other microorganisms; therefore, microbial manipulation constitutes a viable tool to reduce or eliminate the incidence of opportunist pathogens (Balcázar *et al.* 2006a). In aquaculture, *Pseudoalteromonas fluorescens* has shown inhibitory effects against *Vibrio anguillarum*. Mo *et al.* (2001) screened a *Vibrio*-antagonistic strain QJ2, which could inhibit 33 out of 37 *Vibrio* strains, from 602 bacterial strains isolated from the aquaculture environment. Strain QJ2 was identified as *Flavobacterium odoratum*. Li

et al. (2001) isolated five bacterial strains, from healthy penaeid shrimp and shrimp culture ponds, which could inhibit the bacterial pathogens of aquaculture animals. All these five strains belong to *Alteromonas*, and one strain A18 was identified as *A. aurantia* (now renamed as *Pseudoalteromonas*). It was found that A18 can produce an antimicrobial peptide *in vitro* and a reduction of the number of vibrios was achieved by applying the probiotic A18 to larvae culture of bay scallop (*Argopecten irradians*) (Wang *et al.* 2002). Dong *et al.* (2007) isolated a bacterial strain DL2 from biofilm in seawater, and found it could inhibit various pathogenic *Vibrio* species, including *V. anguillarum*, *V. harveyi*, *V. parahaemolyticus*, etc. The strain DL2 was identified as *Phaeobacter inhibens*. Some autochthonous isolated *Bacillus* strains such as *Bacillus subtilis* are also being applied in aquaculture. They were isolated from fish gut or shrimp ponds, and antagonistic effects to pathogen *Aeromonas hydrophila* or decreased ammonia levels in culture water were found (Hu and Yang 2006). Other *Bacillus* isolated from terrestrial livestock were used more frequently, and are the major source of probiotic species (Liu *et al.* 2002).

3.3. Microorganisms for nutrition and enzymatic contribution to digestion

Taking benefit from the experiences of non-aquaculture industries and also for safety reasons, some lactic acid bacteria and yeasts have been quickly accepted as probiotics in aquaculture. The most commonly used organisms in probiotic preparations are the lactic acid bacteria; these are found in large numbers in the gut of healthy animals and are, in the words of the American Food and Drug Administration (FDA), generally regarded as safe (GRAS status) (Parvez *et al.* 2006). In aquaculture in China, probiotic products using lactic acid bacteria *Lactobacillus* and *Bifidobacterium* are frequently mentioned. However, the exact species or strain names were rarely found

both in commercial products and in literature.

3.4. Bacteria for improving water quality

The organic enrichment and nitrogenous waste are serious concerns in aquaculture. Biological processes are the most important ones with respect to aquaculture wastewater treatment and the major biological process is nitrification (Van Rijn 1996). Nitrification is a process of transfer ammonia to nitrate by two groups of bacteria, i.e. ammonia-oxidizing bacteria (oxidize ammonia to nitrite) and nitrite-oxidizing bacteria (oxidize nitrite to nitrate). Commercial nitrifying bacteria are performing weak especially in marine culture, and large amounts of commercial nitrifying bacteria are not widely available on the Chinese market. Aerobic denitrifiers are also considered to be good candidates to reduce nitrate and/or nitrite to N₂ under aerobic conditions in aquaculture water. Liao *et al.* (2006) isolated a new aerobic denitrifying strain X0412 from shrimp ponds, which was identified as *Stenotrophomonas maltophilia*. This denitrifying strain was found to contain the nitrite reductase gene *nirs*. Later on, 27 denitrifying bacterial strains were isolated from a shrimp pond. 16S rRNA gene sequence analysis revealed that the 27 bacterial strains belonged to 11 genera, including *Pseudomonas*, *Holomonas*, *Acinetobacter*, *Paracoccus*, *Arthrobacter*, *Microbacterium*, *Cellulosimicrobium*, *Bacillus*, *Stenotrophom* and *Sphingobacterium* (Wang *et al.* 2007). For organic waste, it is believed that Gram-positive bacteria convert of organic matter back to CO₂ than Gram-negative bacteria. *Bacillus* sp. are very common species for improving water quality. Currently there is strong tendency to combine the above-mentioned species, therefore the probiotic compounds are often labeled as multifunctional and can be applied to different culture species in various culture conditions.

3.5. *Bdellovibrio*

Bdellovibrio spp. are motile Gram-negative δ -*proteobacteria* that attack other Gram-negative cells, penetrate their periplasm, multiply in their cytoplasm, and finally burst their cell envelopes to start a new cycle (Jurkevitch 2007). While *Micavibrio*, *Vampirovibrio*, and *Vampirococcus* have been reported to be able to grow upon microorganisms of only a single genus, *Bdellovibrios* as a group can utilize any of a wide variety of Gram-negative bacteria as a substrate cell. For example, *Bdellovibrio bacteriovorus* 109J can be grown on certain strains of *Escherichia*, *Pseudomonas*, *Rhizobium*, *Chromatium*, *Spirillum*, as well as other genera (Ruby 1991). In mixed prey cell populations, *B. bacteriovorus* 109J does not randomly infect prey cells but infects and kills some prey more readily than others (Rogosky *et al.* 2006). *Bdellovibrio* were intensively studied in the 1960s and 1970s with the developmental stages observed microscopically and partially assayed biochemically (Lambert *et al.* 2006). The characteristic life-cycle of *Bdellovibrios* makes them attractive candidates for a number of applications concerning reducing or modulating bacterial populations, i.e. biological control of pathogens, water purification, and biofilm control (Yair *et al.* 2003). Worldwide little emphasis has been put on studying the possible role of *Bdellovibrio* in agricultural applications, except in China, where the use of *Bdellovibrio* for animal production was authorized by the Ministry of Agriculture in 1994. Especially in recent years, it was reported that using *Bdellovibrios* in aquaculture contributed to improved performance of fish, shrimp, crab and sea cucumber (Zhao 2002; Cai and Zhao 2006). It is believed that using *Bdellovibrio* as bio-control agent has a bright prospect in the future.

3.6. Commercial probiotics

In the middle of 1990s, commercial probiotics products from USA, Japan and United Kingdom were introduced into China. The first three commercial probiotics that were introduced were “Allelopathy” from Araya in Japan (antagonistic bacteria), probiotics from Alken-Murray Corp, USA (for degrading organic waste) and immunostimulants from AGA Group, UK. The trial experiments were conducted in penaeid shrimp hatcheries in Dahua Aquaculture Company, Laizhou, Shandong Province, and were found to produce beneficial effects to penaeid shrimp. Since then, commercial probiotics became popular in China. Currently, the most popular one in China is Effective Microorganisms (EM) from Dr. Teruo Higa's EM Technology, Japan. The original developer of the technology that combines microorganisms for various beneficial uses, the Japanese agronomist Higa named his discovery "Effective MicroorganismsTM" or "EM" and started to use the term internationally in 1986 (<http://www.emrochina.com/doc>). EM consists of a group of beneficial and non-pathogenic microorganisms, such as lactic acid bacteria, photosynthetic bacteria, yeasts and actinomyces. EM has been stated to have the capacity to degrade the environmental and culture wastes, and produce various beneficial effects (such as health effects) contributing to more sustainable technologies at a low cost (EM Technology Network, <http://www.emtech.org/>). After many years of applications, EM appears to play a great role in the agriculture sector in China. Annual EM production probably reaches up to 10,000 metric tons. In aquaculture, EM was used in both freshwater and marine culture for many species such as shrimp, water tortoise, carp, eel, abalone etc (Li and Ni 1995; Liu *et al.* 2006). A number of studies support the beneficial effects of EM. It was reported that EM could increase significantly the

amino acid and vitamin B family contents of culture water, decrease the COD level in ponds and improve disease resistances. Evidence for a higher food conversion ratio and digestive enzymes activities in the gut, for increased hemoglobin levels and numbers of red blood cells was also found (Huang *et al.* 1999; Li *et al.* 2002; Wu *et al.* 2004; Ye *et al.* 2004).

The EM technology was also developed in China based on autochthonous microorganisms isolated from different regions in China by the China National Microorganism Conservation Center. In their formula, no genetically engineered species were used, and no microbes have been imported from other countries. Currently the demand for EM in aquaculture is high, and commercial EM products and similar products for aquaculture are widely available on the market. However, the exact composition of different EM products specifically for aquaculture has not been described.

With the increased public concern on the use of antibiotics in aquaculture, it is not surprising to see a rapid growth of the probiotic industry for aquaculture. In 1999, the Ministry of Agriculture of China noticed that only ten bacterial species and two yeasts species were permitted to be used directly as feed. However, the microbial species currently used in probiotic products are rarely been indicated and the existing species in probiotic products appear much more numerous than those permitted. Clearly, the government policy development lags behind the dynamic evolutions in the probiotic market and the lack of criteria to assure quality is obvious. In 2006, over 100 aquacultural microbial agent producers gathered in Shanghai to start defining their own industrial criteria and 90 companies signed the self-discipline agreement for quality control. At present, consideration of the safety of probiotics for aquaculture is

urgently needed. In food and pharmaceutical industries, the factors that must be addressed in the evaluation of safety of probiotics include pathogenicity, infectivity, toxicity, metabolic activity, and the intrinsic properties of the microbes (Ishibashi and Yamazaki 2001). However, rigorous safety assessment for novel commercial probiotics and especially mixed probiotic productions for aquaculture in China still needs to be established.

4. Molecular techniques for evaluation of commercial probiotics

Currently the consumer, the industry as well as legislative authorities urgently require sensitive and reliable methods to identify and characterize the microbial content of probiotics (McCartney 2002). Conventional methods rely on phenotypic characterization, growth requirements and characteristics, fermentation profiles, and serology studies have been proved useful but with inherited deficiency. To date, various molecular fingerprinting techniques, as well as genetic marking, have proven useful in subspecies discrimination or strain differentiation. Molecular techniques have been employed in studies of probiotics for quantitative and quantitative monitoring of specific organisms or populations. A number of studies evaluated the bacterial composition of commercial probiotic products for human consumption (Fasoli *et al.* 2002; Temmerman *et al.* 2003; Huys *et al.* 2006). Despite the availability of various molecular techniques, reports on the evaluation of the composition of probiotic products in China are rare. Recent Chinese reports of using ERIC-PCR and PCR-DGGE/TGGE to analyze microbial fertilizers (Zhao *et al.* 2005; Hu and Yang 2006), showed these methods to be fast and accurate.

4.1. PCR-DGGE/TGGE techniques

Of the many molecular methods for the investigation of specific groups or microbial community analysis, PCR-DGGE/TGGE is the most widely used. Profiling the 16S rRNA population by DGGE and TGGE enables the rapid estimation of the presence and relative abundance of microorganisms in a sample (Muyzer *et al.* 1993). The general principle of DGGE/TGGE is the separation of fragments of the individual rRNA genes based on differences in chemical stability or melting temperature of these genes. Polyacrylamide gels consisting of a linear denaturing gradient, formed by urea and formamide are employed for DGGE, whereas a linear temperature gradient is used during TGGE. GC-rich sequences can be incorporated into one of the primers to modify the melting behavior of the fragment of interest to the extent that close to 100% of all possible sequence variations can be detected” (Sheffield *et al.* 1989). After more than a decade of application in microbial population studies, the DGGE/TGGE technique gradually reaches maturity.

To allow comparative analysis of various probiotic products and to evaluate the composition changes with increasing shelf life, as well as to investigate the specific probiotics in actual culture samples, the method must be carefully assessed and standardized. The sample size/volume, DNA extraction efficiency, PCR biases and interference of DNA from dead bacterial cells should be carefully examined and optimized (Boström *et al.* 2004; Nocker *et al.* 2006). To examine reproducibility and reduce experimental errors, internal standards could be used throughout the DNA extraction and PCR-DGGE (Petersen and Dahllöf 2005). The use of internal standards significantly reduced variation among replicate samples and was shown to accurately describe changes in relative abundance in an environmental sample using PCR-

DGGE. In our study, we quantitatively added viable *Escherichia coli* cell and plasmid DNA as control spiking DNA in commercial probiotic products which consisted of *Bacillus* spores; the results in Fig. 2.2 show that the optimal sample size, detection limitation of number of viable cells and the reproducibility of PCR-DGGE can be demonstrated.

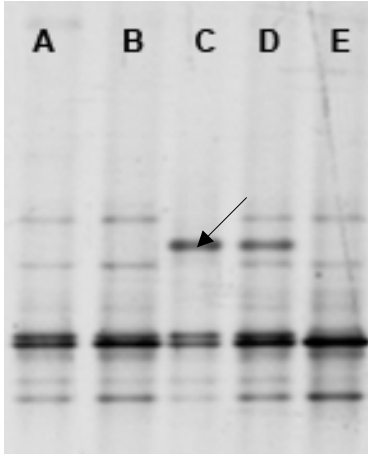


Fig. 2.2. DGGE gel patterns of analysis of probiotic samples (mainly *Bacillus* spores) with addition of *E. coli* cells. Lane A: 0.1 mg probiotic samples used for DNA extraction; Lane B: 1 mg probiotic samples used for DNA extraction; Lane C: 0.1 mg probiotic samples with the addition of 10^9 viable *E. coli* used for DNA extraction; Lane D: 1 mg probiotic samples with the addition of 10^9 viable *E. coli* used for DNA extraction; Lane E: 10 mg probiotics samples with 10^9 viable *E. coli* cells used for DNA extraction; Arrow indicates band representing *E. coli*.

4.2. FISH technique

Although PCR-based fingerprinting is the most sensitive technique to detect sequences that are present in very low concentrations in the samples, many factors can influence the amplification reaction and the fingerprinting techniques alone often do not provide sufficient quantitative data (Von Wintzingerode *et al.* 1997). FISH with rRNA target probes has been developed for the *in situ* identification of single microbial cells and is the most commonly applied among the ‘non-PCR-based’ molecular techniques (Amann *et al.* 1990). This method is based on the hybridization

of synthetic oligonucleotide probes to specific regions within the bacterial ribosome and does not require cultivation. FISH has been applied to the detection of pathogens, the study of microbial symbiosis, the identification of nitrifying bacteria and the monitoring of fecal microbiota (Schramm *et al.* 1996; Jansen *et al.* 1999; Schwiertz *et al.* 2000). The FISH technique can also be applied for the *in situ* detection of probiotic *Latobacillus* cells in fecal and biopsy samples. The potential of flow cytometry in combination with FISH has recently been demonstrated for bifidobacteria in fecal samples (Vaughan *et al.* 1999). Due to its speed and sensitivity, this technique is considered a powerful tool for phylogenetic, ecological, diagnostic and environmental studies in microbiology (Bottari *et al.* 2006). In our study indicated in Fig. 2.3, we applied the FISH technique to characterize a probiotic photosynthetic bacteria mixture used in aquaculture. Through the use of group or species-specific probes, it is possible to identify different bacterial groups in complex probiotics mixtures, thus providing quantitative information for the understanding of the probiotics mixture and the possible interspecies interaction.

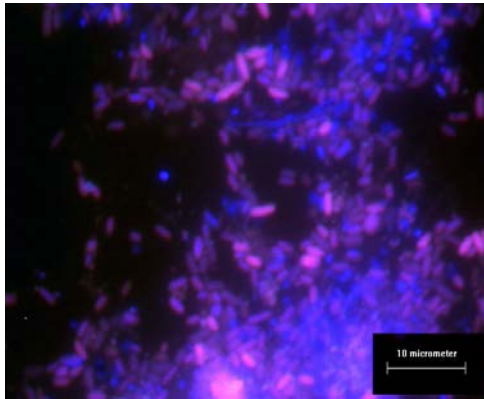


Fig. 2.3. Epifluorescence picture of a mixture of photosynthetic bacteria in probiotic products. Bacterial cells (pink) were detected by *in situ* hybridization with TAMRA-labeled oligonucleotide probe for *proteobacteria* α subdivision. Blue bacterial cells are DAPI stained (Bar = 10 μ m).

Although other advanced methods such as terminal restriction fragment length

polymorphism (T-RFLP), multilocus sequence typing (MLST) and Fluorescence amplified fragment length polymorphism (F-AFLP) are available, currently PCR-DGGE with FISH technique are proven effective, sensitive and inexpensive and therefore can widely be applied in probiotics studies. Because various phylogenetic and functional genes can be selected for PCR-DGGE and FISH analysis, both methods possess great potential and flexibility to study various probiotic products. Nowadays, plenty of primers and probes sources are available for distinction of *Bacillus*, lactic bacteria and *Bifdobacteria* species. Primers or probes for 16S rRNA gene region or 16S-23S rRNA gene intergenic spacer region as well as functional genes can readily be applied to describe specific strains and group function within a sample.

5. Prospects

Overall, the research of probiotics in aquaculture of China is still in its early stage, and not much of commercial probiotics products were licensed in China so far. Scientists in China will continue to screen new probiotics strains from local aquaculture rearing unit to suit the specific requirement in China. In the near future, probiotics will gain more popularity in aquaculture of China, and the application areas will be expanded. Also, quality control of probiotics in aquaculture of China will become an important issue. With the increased use of molecular methods for the definitive analysis of the bacterial components of probiotic products and for *in vivo* validation, it is expected that both the probiotics quality and functional properties can significantly be improved in China. This type of research can aid for the development of adequate technology for the evaluation of the efficiency of microbial agents as probiotics in aquaculture.

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Chapter

3

Characterization of microbial communities in the larval culture of turbot (*Psetta maxima* L.)

Abstract

In this study, a 16S rRNA gene based PCR-DGGE approach was used to monitor microbial communities associated with turbot (*Psetta maxima* L.) larviculture in a commercial hatchery. In addition, the colour changes of the green water as well as the larval health status were monitored. The DGGE profiles revealed bacterial community changes in the culture water before and after first feeding. It was found that the DGGE patterns are plant-specific, suggesting that different aquaculture plants harbour their own specific microbial community. Based on the DGGE profiles, Lorenz curves and Gini coefficients were calculated for the description of species evenness. In general, for the different tanks the evenness was similar at the same culture stage and that the communities were more even during later culture stages.

Key words: bacterial community, turbot, larval culture, green water

1. Introduction

The turbot, *Psetta maxima* L., is a local flatfish species in Europe. The well-established turbot aquaculture in Europe yields several thousands of metric tons annually (FAO 2007). After it was introduced into China in 1992, great success of turbot aquaculture was achieved, especially during the last five years. The annual production is now estimated at 50,000 metric tons with a market value of up to 450 million Euros (Lei 2007).

For most cultured species, seed production and larval rearing are one of main constraints for the industrial expansion. Despite many years' efforts, the overall survival rate of turbot larvae is only 10% in the best commercial hatcheries (Olsen 1997). The frequent occurrence of disease and high mortality of the larvae resulted in massive use of disinfectants and antibiotics, and this hindered greatly the sustainable development of aquaculture (Shields 2001).

In order to make the aquaculture industry more sustainable, alternative methods to control bacterial diseases (without using antibiotics) are needed and the “green water” technique is one of the environmental-friendly rearing approaches getting more attention (Defoirdt *et al.* 2007). In the intensive culture of marine fish larvae, microalgae are often added to the water along with microzooplankton or microparticulate food for the improvement of the survival and growth rate of larvae

(Reitan *et al.* 1997; Papandroulakis *et al.* 2002). However, the interactions between microalgae and microbial communities in the culture systems are almost not explored (Nicolas *et al.* 2004; Grossart *et al.* 2005; Nakase and Eguchi 2007).

Numerous bacterial genera have been isolated from the intestinal tract of turbot (Blanch *et al.* 1997; Ringø and Birkbeck 1999; Cerda-Cuellar and Blanch 2004).

Despite the accumulation of data on the microbiota that are associated with turbot, the exact causes of bacterial diseases, or the factors that lead to pathogens to grow and spread, are still unknown. It is general accepted that the establishment of the gut microbiota in fish larvae is determined by the microbiota of the eggs, the live feed and the bacteria present in the tank water (Olafsen 2001). However, differences in bacterial abundance and in microbial population composition make it difficult to characterize those bacterial communities. In order to understand the bacterial roles in complex, dynamic larval culture system, a number of reliable tools and well-documented concepts are needed. Recently, a new approach based on molecular fingerprinting and pragmatic analysis by Pareto-Lorenz curve analysis was suggested to link the microbial community structure with the functioning in open ecosystems (Dejonghe *et al.* 2001; Verstraete *et al.* 2007)

In this study, we used a PCR–DGGE approach to characterize the bacterial community associated with the early life stages of turbot and to describe the bacterial community changes during unhealthy and recovered periods. Based on DGGE

patterns, species evenness was calculated by Pareto-Lorenz curves.

2. Materials and methods

2.1. Turbot larval rearing

Turbot larval rearing was performed at the intensive hatchery of Huaxinhaida Marine Biology Co. Ltd, Weihai, China. Briefly, fertilized turbot eggs were transferred in 10 m³ volume cylindrical fibreglass tanks with a slightly conical bottom. Deep-well seawater was used. Water exchange was started on day 4 and the exchange rate was gradually increased from 20% to 200% per day in 5 days. The cultures were moderately aerated. The green algae *Chlorella* sp. and *Nannochloropsis* sp., and the diatom *Phaeodactylum* sp. were added and maintained at a density 0.5×10^6 cells L⁻¹ in the larval rearing tank after hatching of the larvae (day 3). The initial density of the larvae was 10-30 individual L⁻¹. The feeding and rearing of the larvae were performed following the rearing procedure described by (Lei 2003).

2.2. Growth performance record

The growth performance of the larvae was monitored daily; dead larvae or larvae with disease symptoms were removed from the culture tanks and the larval health status in the tanks was recorded. The visually distinguishable colour changes of culture water were also recorded as the light green, the dark green or the pale.

2.3. Sampling

According to the larval growth performance, volumes of 500 - 750 mL water from the culture tanks in normal culture or disease occurred plants were collected and filtered through a sterile cellulose acetate membrane filter (Whatman, 47 mm diameter, 0.22 μm pore size) by vacuum filtration. The filters were stocked at $-20\text{ }^{\circ}\text{C}$ until further DNA extraction. The sampling scheme is listed in Table 3.1.

2.4. DNA extraction

Total DNA from the filter was obtained by a DNA extraction method described previously by Rombaut *et al.* (2001). To obtain bacterial DNA, the filter was put into 10 mL centrifuge tube with 4 mL of 10 mM Tris-HCl (pH 9) and 1 g of beads (0.10–0.11 mm diameter). This mixture was homogenized three times for 30 s using a homogenizer at maximum speed. After this, 160 μL of 50 mg mL^{-1} lysozyme was added, and then the suspension was incubated at $37\text{ }^{\circ}\text{C}$ for 15 min on a shaker (200 RPM). Chemical lysis of the bacterial cells was achieved by adding 300 μL of 20% SDS and the suspension was slowly mixed for 5–10 min. Subsequently, 1 mL of 8 M ammonium acetate was added. DNA was obtained from the lysates using standard phenol–chloroform extraction and isopropanol precipitation procedures (Boon *et al.* 2000). The quality of the extracted DNA was verified by electrophoresis on a 1% (wt/vol) agarose gel.

2.5. Amplification of 16S rRNA genes

DNA extracted from the samples was amplified with primers GC338f and 518r spanning the V3 region of the 16S rRNA gene (Muyzer *et al.* 1993) using a 9600 thermal cycler (Perkin–Elmer, Norwalk, CT, USA). A “touchdown” PCR (TD PCR) was used (Roux 2002): the initial annealing temperature used was 10 °C above the expected annealing temperature, and the temperature was decreased by 1 °C every cycle until the touchdown temperature (53 °C) was reached; then, an additional 20 cycles were carried out at 53 °C. Primer extension was carried out at 72 °C for 2 min. Finally, the samples were incubated for 10 min at 72 °C (final extension).

All PCR products (5 µL volumes) were analyzed by electrophoresis in 1% (w/v) agarose gels before DGGE analysis was performed.

2.6. Analysis of PCR products by DGGE

DGGE of the PCR products was performed as previously described (Boon *et al.* 2002), using the Bio-Rad D Gene System (Hercules, CA, USA) with 8% (w/v) polyacrylamide gels in 1 × TAE (20 mM Tris, 10 mM acetate, 0.5 mM EDTA, pH 7.4), containing a linear chemical gradient ranging from 40% to 60% denaturant (where 100% denaturant contains 7 M urea and 40% formamide). The electrophoresis was run for 16 h at 60 °C, at 38 V.

After completion of the electrophoresis, the gels were stained for 20 min in SYBR GreenI nucleic acid gel stain solution (1:10,000 dilution in 1 × TAE; FMC

BioProducts, Rockland, ME, USA). The stained gel was immediately photographed on a UV transillumination table with a video camera module (Vilbert Lourmat, Marne-la vallée, France).

2.7. DGGE pattern analysis

The obtained DGGE patterns were analyzed using Bionumerics software version 5.0 (Applied Maths, Sint-Martens-Latem, Belgium). A matrix of similarities for the densitometric curves of the band patterns was calculated using the Pearson coefficient and dendrograms were created using on the UPGMA method. Species richness was evaluated by the number of bands in the DGGE gel. Bacterial community evenness analysis was based on the method described by Mertens *et al.* (2005). In order to have good estimators of species evenness, Lorenz curves were used to describe the equality of distribution within a population. A Lorenz curve is obtained as follows: abundances of the species are ranked from high to low. The cumulative proportion of species is used as x-axis and the y-axis is presented by the cumulative proportion of abundances. This yields a concave curve. The Gini coefficient is the numerical value of equality and is calculated as twice the area above the Lorenz curve, using the following formula:

$$G = 1 - [(N + 1) / N] - [(2 / \mu N^2) * \sum_{i=1}^N iX_i]$$

with N = number of species; X_i = abundance of species I ; μ = mean abundance. The Gini coefficient is a number between 0 and 1, with 1 representing total evenness. Due

to theoretically the same MC resources presented in larviculture system, the number of species was determined by the maximum number of bands in one of the DGGE gel lanes. For the lanes that the number of bands was below the maximum number, more species were added until maximum number was reached. So all the samples had the same species richness and the abundance of the added species was assigned the value '0'. These approaches of species evenness and richness were used to assess the microbial species composition.

Table 3.1. Water sampling scheme, health status of turbot larvae and water color in the turbot culture tanks, Gini coefficients of the microbial community was calculated based on the DGGE gel patterns.

Sample No.	Sampling date	Plant No.	Tank No.	Filtered volume	Larval age	Health Status	Colour of green water	Gini coefficient
1	2004.5.13	5	A1	500 mL	Day 12 after hatching	Swollen abdomen	Light green	0.53
3	2004.5.13	8	A8	750 mL	Day 3 after hatching	Normal (microalgae added)	Dark green	0.35
5	2004.5.14	5	A1	500 mL	Day 13 after hatching	Ingestion OK	Light green	0.52
7	2004.5.14	8	A8	600 mL	Day4 after hatching	Normal	Dark green	0.38
11	2004.5.15	5	A2	560 mL	Day 14 after hatching	Ingestion bad (suspected)	Pale	0.48
21	2004.5.17	5	A1	500 mL	Day 16 after hatching	Unhealthy, ulcer at tail	Light green	0.68
22	2004.5.17	8	C6	400 mL	Day 13 after hatching	Unhealthy, ascites	Light green	0.48
25	2004.5.18	5	A1	500 mL	Day 17 after hatching	Ulcer at tail, increased larval mortality	Light green	0.85
31	2004.5.20	5	A1	500 mL	Day 19 after hatching	Less ulcer, recovering	Light green	0.76

3. Results

The larval survival rate was approximately 60-70% at the onset of first feeding (day 3 after hatching). The health status of the larvae is shown in Table 3.1. Disease symptoms such as ulcer and ascites were observed in several tanks. Visually distinguishable colour changes of the green water (dark green to light green, or pale) were also observed. This seemed to accompany changes of larval health status indicating by the visible features of sickness such as no ingestion (pale colour), ulcers or ascites, and the increased mortality (dark green colour). With the appearance of disease symptoms, higher numbers of dead larvae were observed at day 17 after hatching, and the recovery period started on day 20 after hatching.

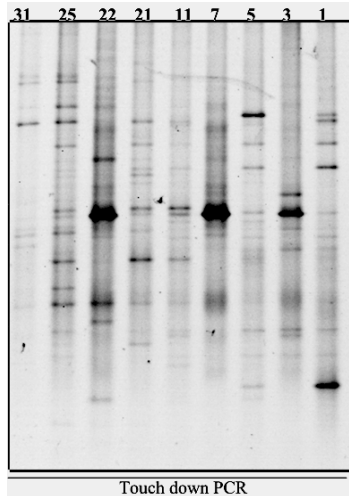


Fig. 3.1. PCR-DGGE band patterns of water samples from turbot larval culture tanks (lane numbers correspond to sample numbers in Table 3.1). DGGE patterns obtained from the samples are shown in Fig. 3.1. Band profiles of samples from tanks with sick fish (lanes 1, 5, 21, 22 and 25) seemed to be more complex than band profiles of samples from tanks with healthy animals (lanes 3, 7) (Fig. 3.1). There was a clear difference between patterns obtained before and after first feeding (lane 3 versus lane 7) and there were also large differences between different hatchery plants (plant 5 and 8), whereas DGGE band patterns obtained for different tanks of the same plant (lanes 3, 7 and 22 were from plant 8; lanes 1, 5, 21 and 25 were from plant 5) were more similar. The “plant-specificity” of the DGGE banding patterns were also reflected in the clustering analysis, where patterns obtained from samples from the same plant clustered together, whereas patterns obtained from samples of different plants were separated (Fig. 3.2). In general, the clustering did separate the patterns obtained for samples from cultures with healthy animals and those obtained for samples from cultures with diseased animals (Fig. 3.2 sample No. 22).

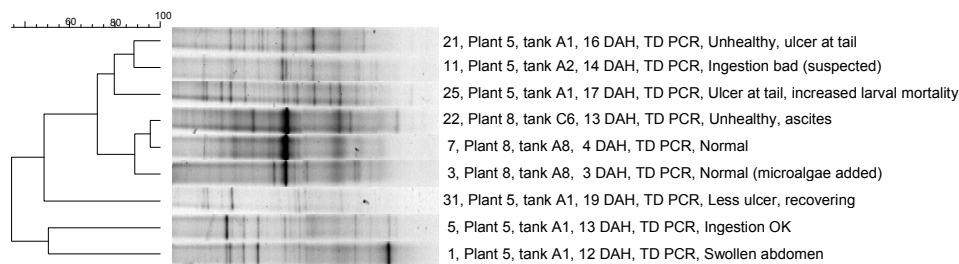


Fig. 3.2. TD PCR-DGGE profiles of the microbial community of water samples from turbot larval cultures. Clustering of the patterns (using the UPGMA method based on Pearson coefficients) is shown on the left (Scale 0–100 correspond to similarity levels ranging from 0% to 100%; DAH = days after hatching).

Lorenz curves and Gini coefficients were calculated based on the data obtained with PCR-DGGE analysis. These curves (Fig. 3.3) showed that profiles were tank specific. The samples from tank A1 (No. 21, 25, 31) generally showed a more evenly distributed community when compared to the samples from other tanks. The Gini coefficients showed that the bacterial communities in tank A1 were changing to more evenly distributed from DAH 12 to DAH 17 (Table 3.1, Gini coefficient increasing from 0.53 to 0.85) and bacterial communities from samples at the closed larval age (DAH 12-14) appeared to have similar evenness (Gini coefficient 0.48-0.53) even there were from different plants and contained different microbial composition (plant 5 or 8).

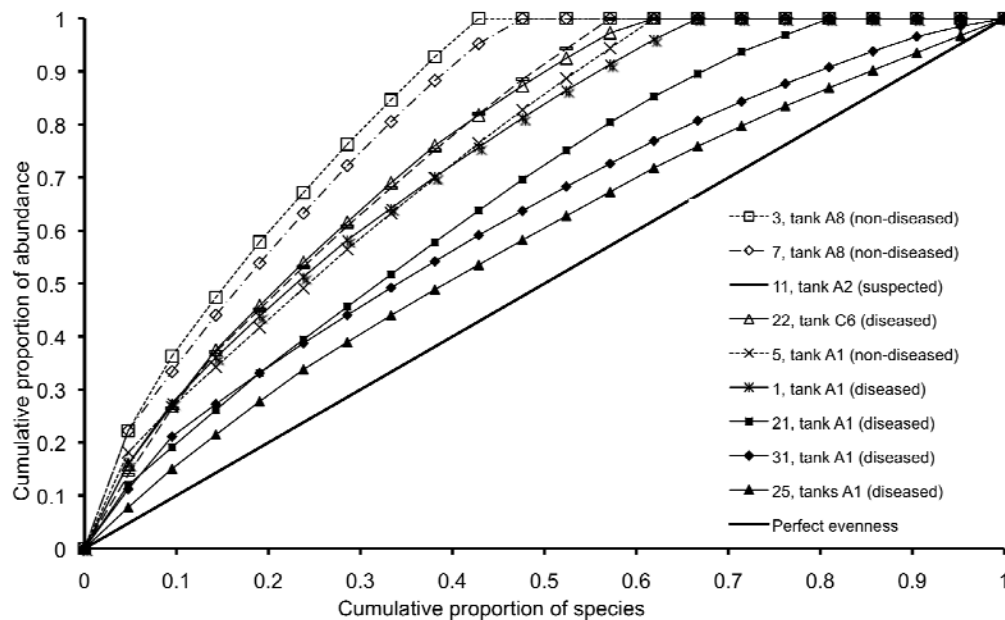


Fig. 3.3. Lorenz curves based on PCR-DGGE analysis of bacterial communities in turbot larval culture tanks.

4. Discussion

It is important to provide fish larvae with a healthy environment that includes a beneficial microbial community (Gomez-Gil *et al.* 2000). However, the intensive larval rearing systems operated with today's technology entail a high load of organic matter and bacteria, as well as substantial variation in the environmental conditions (Skjermo and Vadstein 1993). It is generally recognized that opportunistic bacterial pathogens are a major cause of infectious disease in cultured larvae. These bacteria normally co-exist with the host without causing problems, but under sub-optimal and stressful hatchery conditions they can take advantage of the resulting immunosuppression of the animals and cause disease (Bourne *et al.* 2004). Unfortunately, in a complex and dynamic larval culture system, such stress conditions and ecological changes are rather difficult to determine and if they can be determined, it is often economically not feasible to improve the culture conditions. It is of particular importance to characterize the bacterial community that is present in such a dynamic system. The characterization of the microbial community by molecular techniques might reveal specific correlations between the microbial community composition and the environmental factors, or disease cues, and this knowledge might ultimately enable farmers to predict problems and allow steering the microbial community before disease starts to occur. In our study, DGGE analysis revealed that there were dynamic changes in the bacterial community composition associated with turbot

larvicultures.

The use of culture-independent DGGE to study the microbial ecology is well established. Culture-dependent techniques revealed only 0.1 to 1% of the total bacterial community under standard laboratory conditions (Amann *et al.* 1995) and the actual diversity will probably be much larger. However, with the accumulation of complex fingerprinting data, the analysis of DGGE data can be important in that it can transform a complex band pattern into a characteristic that is easier to interpret. In this work, Lorenz curves and Gini coefficients were used for this purpose. Together with the number of bands as a value for species richness, this method describes diversity in a way that both species evenness and richness can be considered separately (Mertens *et al.* 2005). Interestingly, the bacterial communities tended to be similar in the samples at the same culture stage even from different culture tanks in different plants. This possible implied that the present culture procedures (feeding regimes, water exchanges, etc) or larvae ages were related to that bacterial evenness. The changes of Lorenz curves and Gini coefficients might indicate that the bacterial communities are still in strong evolving and resulted an insufficient organization in the bacterial community. The studies of microbial communities in fish and shellfish hatcheries are revealing a vast microbial diversity with various functions (Schulze *et al.* 2006). The accumulation of this kind of data requires the formulation of theories that link organization and structure of microbial communities to mechanistic insights and, that would ultimately result in some kind of predictive power that is of practical value

(Prosser *et al.* 2007). Our results suggest that a change in evenness, expressed by the Gini, can be linked to a microbial community associated with different cultures stages. However, further research studying microbial communities associated with different host animals in different aquaculture plants at different time points will be necessary in order to verify whether this is a general rule.

In a study of the bacterial community associated with the early stages of great scallop (*Pecten maximus*) using DGGE, Sandaa *et al.* (2003) found high similarities in the community composition between different water and larvae samples collected at different time periods, indicating a high stability in the bacterial community composition. In another study of the microbial community dynamics in a larval aquaculture system of the tropical rock lobster (*Panulirus ornatus*), DGGE profiles of water, biofilm and phyllosoma environments were more different, which suggests the presence of distinct niches sustaining different microbial populations (Bourne *et al.* 2004). In our study, microbial populations appeared dynamic since there were daily shifts of DGGE profiles. Nevertheless, the DGGE profiles appeared to be plant-specific, with patterns obtained from samples of different tanks of the same plant being more similar than patterns from samples of different plants. This suggests that the microbial community in each plant was evolving in its own specific way with different species compositions in spite of similar species distribution was found by Gini coefficients index.

In a recently study, Schulze *et al.* (2006) demonstrated that aquatic hatcheries contain diverse microbial communities including pathogenic, innocuous and beneficial bacteria, and pointed out that the ability to maintain a proper balance of this microbiota may be the key to a successful culture. Instead of using clear water in which particles are controlled, transparent and no additions of microalgae, green water techniques are applied in many hatcheries. Especially in outdoor soil bottom ponds for shrimp and crab larval culture, microalgae are one of the important factors and are maintained by fertilization. The green water technique used in the hatcheries that were sampled in this study was similar to the “pseudo-green water” technique described by Papandroulakis *et al.* (2002). The main difference from the conventional green water technique is that phytoplankton is not produced within the rearing tank but its concentration is kept constant through daily addition of algae grown outside the culture tank. The “pseudo-green water” method is applied during the most critical stages of the rearing process, i.e. at the beginning of larval rearing (until the 25th to 30th day post hatching), when the larvae are still extremely weak and sensitive to alterations in the rearing environment (Papandroulakis *et al.* 2002). The colour changes of green water can indicate the growth of specific microalgae, especially when mixtures of microalgae containing different chromatophores are used. In larval culture tanks, microalgae may play an important role in stabilizing the water and in microbial control.

In conclusion, in this study, we investigated the microbial communities associated with larval cultures of turbot by DGGE and found that the microbial species composition is plant-specific, suggesting that different aquaculture plants harbour their own specific microbial community. Furthermore, Lorenz curves and Gini coefficients suggested that the bacterial communities present in samples at the early larviculture stage were less evenly distributed than samples coming from later culture stage. Based on our results, we suggest that a correlation between culture procedures, fish health and bacterial evenness could be present. Among the various factors of culture procedures, the further determinations of the effects of input bacteria via live feed, as well as the effects of feeding regimes and probiotics on microbial community, could be necessary.

Chapter

4

Analysis of the evolution of microbial communities associated with different cultures of rotifer strains belonging to different cryptic species of the *Brachionus plicatilis* species complex

Abstract

The evolution of the composition of microbial communities associated with cultures of 3 different strains belonging to different cryptic species of the rotifer *Brachionus plicatilis* was monitored during four subsequent cycles of batch cultivation using Denaturing Gradient Gel Electrophoresis, cluster analysis, Multidimensional Scaling and Principal Component Analysis. The data suggest that the evolving microbial communities are different with different *Brachionus plicatilis* strain cultures. Moreover, large changes in rotifer growth rate were found to be associated with large changes in the microbial community composition, suggesting that there might be a causal link. Finally, Lorenz curves and Gini coefficient analysis revealed that good performing *B. plicatilis* cultures showed a more even microbial community structure.

Keywords: microbial community; rotifer; Denaturing gradient gel electrophoresis;

1. Introduction

Rotifers (*Brachionus* spp.) have been used as a live food for feeding larval marine fishes for over 30 years (Yúfera 2001). Today, more than 60 marine fish species and 18 crustacean species require adequate and reliable production of high-quality, nutritious rotifers. The success of rotifer mass cultures is determined not only by reproduction rate and density, but also their nutritional composition and their associated microbiota (Dhert 1996; Dhert *et al.* 2001).

Batch cultivation, due to its simplicity and reliability, is probably the most common type of rotifer production in marine fish hatcheries (Lubzens *et al.* 2001). Initially, rotifers are introduced at low density into tanks or ponds. The culture strategy consists of either the maintenance of a constant culture volume with an increasing rotifer density or the maintenance of a constant rotifer density by increasing the culture volume. Rotifers are fed with microalgae, bakers' yeast or an artificial diet. A total harvest of the rotifers is applied with part of the rotifers used as food for fish larvae and part used as inoculum for the next culture (Lubzens *et al.* 1987). Using an artificial diet (e.g. Culture Selco[®]), the density at harvest time is about 600 rotifers mL⁻¹ after 4 days of culture starting from 200 to 250 rotifers mL⁻¹ (Suantika *et al.* 2000).

Although they are frequently used, batch culture systems generate highly variable conditions that can have an influence on growth performance, and that also affects the composition of associated microbial communities (Rombaut *et al.* 2001). Bacteria are always associated with mass production of rotifers and may cause unexpected mortality or suppressed growth of rotifers. Moreover, since they are used as the first

food of larvae, rotifers are often suspected as vectors of potential harmful bacteria to the cultured animals (Dhert *et al.* 2001). Using conventional culture-based methods, the dominant bacterial groups in rotifer cultures were classified as *Pseudomonas*, *Vibrio* and *Aeromonas* spp. (Nicolas *et al.* 1989; Skjermo and Vadstein 1993; Verdonck *et al.* 1997). Large variations in the number of rotifer-associated ($1.8 - 7.6 \times 10^3$ CFU rotifer⁻¹) and free-living bacteria ($0.6 - 25 \times 10^7$ CFU mL⁻¹) have been observed (Skjermo and Vadstein 1993). Rombaut *et al.* (2001) described the evolving microbial community present in rotifer batch and recirculation systems by means of the molecular biological technique DGGE. The authors found that in a recirculation system, subsequent to a high-variable period, a climax community was established, which remained more or less stable and was characterized by the dominance of one bacterial genus, i.e. *Marinomonas*. In contrast to the recirculation system, the profiles of the microbial community present in the batch system were more susceptible to variation. Shifts in dominant bands could be observed on a daily basis.

Especially in relation to microbial communities associated with live food, it is important to study microbial communities associated with close related hosts. A recent study found that different cryptic *B. plicatilis* strains exist within aquaculture hatcheries (Papakostas *et al.* 2006). It is likely that many previous studies were done with unknown and different strains of the *B. plicatilis* complex or even mixtures of species. Therefore the characterization of microbial communities of rotifer cultures needs to be done with clear knowledge of the rotifer species.

To address the questions whether cultures of different cryptic *B. plicatilis* strains have different associated microbial communities, and how the microbial communities are evolving in consecutive batch cultures, DGGE fingerprints of PCR-amplified 16S

rRNA gene fragments were made and analyzed with a series of ecological tools.

2. Materials and methods

2.1. Rotifer strains

Experiments were performed with 3 different cryptic *B. plicatilis* strains, namely *B. plicatilis* sensu strictu, *B. plicatilis* Cayman and *B. plicatilis* Nevada. Before the start of the experiment, the rotifer strains were kept in non-sterile cultures at the Laboratory of Aquaculture and *Artemia* Reference Center (Gent, Belgium). The rotifer stocks were regularly examined by microscopy for the presence of ciliates and other protists and maintained at controlled culture conditions: 28 °C, light intensity 2000 lux, salinity 25 g L⁻¹, and fed with yeast-based Culture Selco[®] following the culture procedure described by Dhert (1996).

2.2. Preparation of microbial communities (MCs)

MCs were prepared following the methods described by Tinh *et al.* (2006). Briefly, MCs were isolated from normal performing rotifer *B. plicatilis* sensu strictu cultures. For isolation of MCs, the culture water collected from rotifer culture was filtered through 250 µm and 60 µm meshes to remove big food particles and all the rotifers, respectively, and was subsequently centrifuged at 1600 g for 5 min to remove the algal cells, thus only retaining the MCs were retained in the supernatant. These MCs were preserved for further experiments in 1 mL eppendorfs containing 20% glycerol and 80% bacterial suspension and kept at – 80 °C. Before starting each experiment, the eppendorfs were thawed. Subsequently, 50 µL of the MC suspension was spread plated on Marine Agar (MA). After 24 h of incubation at 28 °C, the bacteria were harvested by swabbing the MA plate and suspending in autoclaved Nine Salt Solution

(NSS). Cell density of the suspensions was calculated according to the McFarland standard (BioMerieux, Marcy L'Etoile, France), based on optical density measurements ($OD_{550} = 1.000$ corresponds to 1.2×10^9 cells mL^{-1}). Subsequently, the appropriate volume to be added to each treatment was calculated in order to have a density of 10^6 cells mL^{-1} in the rotifer culture water at the start of each experiment independent of the *Brachionus* strains.

2.3. Experimental set-up of rotifer batch cultures

The separate runs of experiments of each rotifer strains were performed in 50 mL sterile falcon tubes (TRP[®], γ -irradiated) with four replicates for rotifer *B. plicatilis* Nevada and three replicates for *B. plicatilis* sensu strictu and *B. plicatilis* Cayman. Rotifers were harvested by filtration from stock cultures, rinsed 3 times with 0.22 μ m-filtered and autoclaved natural seawater (FASW) to remove most of the bacterial load, and distributed into falcon tubes containing 32.5 mL of 25 g L^{-1} FASW, to have a density of 30 rotifers mL^{-1} at the start of each experiment. The MCs were added once to each tube to have a density of 10^6 cells mL^{-1} at the start of experiment. The falcon tubes were put on a rotor (4 RPM) which was placed inside a temperature-controlled room (28 °C, light intensity 2000 lux). Each experiment was run for 4 batch cycles and each cycle consisted of a 3-day culture period. At the end of each batch, rotifers were harvested, rinsed and re-distributed at a density of around 30 rotifers mL^{-1} in fresh FASW before starting the next batch culture.

2.4. Rotifer diet

The rotifer diet consisted of a commercial diet Culture Selco 3000[®], CS 3000 (INVE), Belgium. The rotifers were fed daily following a standard feeding regime for Culture Selco 3000[®] according to manufacturer's instructions below:.

$$CS\ 3000 = 0.0168 * D^{0.415} * V$$

Where: CS 3000 = the weight of experimental diet (g); D = rotifer density (individuals mL⁻¹); V = culture water volume (L).

Sampling, counting and growth data analysis

Three samples of 0.5 mL were taken from the rotifer cultures. The rotifers in each sample were killed by adding three drops of lugol, and were counted. Empty and transparent lorica belonging to dead rotifers were not taken into consideration.

The specific growth rate was calculated using the following equation described by Rombaut et al. (2001): $\mu = (\ln N_t - \ln N_0) * t^{-1}$, where: μ = specific growth rate; N_t = rotifer density after culture period t (individuals mL⁻¹); N_0 = initial rotifer density (individuals mL⁻¹); t = culture period (day).

Data of the growth rates on day 3 were evaluated using Levene's test for homogeneity of variances and Shapiro–Wilk's test for normality. As data were normal-distributed and homoscedastic, the growth rates on day 3 for batch 1 and batch 4 were compared between experiments using one-way ANOVA, followed by Tukey test. All the tests were performed using the computer program SPSS release 12.0 (SPSS, USA).

2.5. Microbial analyses

2.5.1. Sampling procedure

One-milliliter culture water with rotifers were collected from the rotifer culture in sterile endpordf tubes after inoculation at the start of experiment and at the end of each batch culture. Samples were centrifuged at 5000 g for 5 min and stored at –20 °C.

2.5.2. DNA extraction

Total DNA from the samples was obtained by a modified DNA extraction method as described previously by Rombaut *et al.* (2001). To obtain bacterial DNA, the samples were centrifuged for 30 min at 5000 g. The pellet was dissolved in 0.2 mL Milli-Q water, transferred to 0.4 mL of 10 mM Tris-HCl (pH 9) and 0.3 g of glass beads (0.10 – 0.11 mm diameter) were added. This mixture was homogenised three times for 30 s using a bead beater at 2000 RPM (B. Braun Biotech International, Melsungen, Germany). After this, 16 μL of 50 mg mL^{-1} lysozyme was added, and then the suspension was incubated at 37 °C for 15 min on a shaker (200 RPM). Chemical lysis of the bacterial cells was achieved by adding 30 μL of 20% SDS after which the suspension was slowly mixed for 5 – 10 min. Subsequently, 0.1 mL of 8 M ammonium acetate was added. DNA was obtained from the lysates using standard phenol–chloroform extraction and isopropanol precipitation procedures (Boon *et al.* 2000). The total DNA extracted was quantified by a spectrophotometer ND-1000 at 280 nm (NanoDrop Technologies, Wilmington, USA).

2.5.3. Amplification of 16S rRNA genes

DNA extracted from samples was amplified with primers gc338f and 518r spanning the V3 region of the 16S rRNA gene (Muyzer *et al.* 1993) using a 9600 thermal cycler (Perkin–Elmer, Norwalk, CT, USA). PCR amplification was carried out in 24 μL reaction volumes to which 1 μL of DNA extract was added.

The PCR master mix contained 0.5 μM of each primer, 200 μM of each deoxynucleoside triphosphate, 1.5 mM MgCl_2 , 10 μL of thermophilic DNA polymerase 10 \times reaction buffer (MgCl_2 -free), 2.5 U of Taq DNA polymerase (Promega, Madison, WI, USA), 400 ng μL^{-1} of bovine serum albumin (Boehringer)

and sterile water, to a final volume of 100 μ L.

PCR was performed in a 9600 thermal cycler as follows: 95 °C for 10 min, followed by 30 cycles at 95 °C for 1 min, 53 °C for 1 min, and 72 °C for 2 min.

2.5.4. Analysis of PCR products by DGGE

DGGE was performed as described previously (Boon *et al.* 2002), using the Bio-Rad D Gene System (Hercules, CA, USA) with 8% (w/v) polyacrylamide gels in 1 \times TAE (20 mM Tris, 10 mM acetate, 0.5 mM EDTA, pH 7.4). The gels contained a linear gradient ranging from 40% to 60% denaturant (where 100% denaturant contains 7 M urea and 40% formamide). PCR products (10 μ L) obtained from total DNA of samples were used for separation in denaturing gradient gels. The electrophoresis was run for 16 h at 60 °C, at 38 V.

After completion of electrophoresis, the gels were stained for 20 min in SYBR Green I nucleic acid gel stain solution (1:10,000 dilution in 1 \times TAE; FMC BioProducts, Rockland, ME, USA). The stained gel was immediately photographed on a UV transillumination table with a video camera module (Vilbert Lourmat, Marne-la vallée, France).

2.5.5. Analysis of DGGE patterns

The obtained DGGE patterns were analyzed using Bionumerics software version 5.0 (Applied Maths, Sint-Martens-Latem, Belgium). DGGE gels were normalized by the assigned markers. A matrix of similarities for the densitometric curves of the band patterns was calculated using the band-based Dice coefficient and dendrograms were created using on the Ward method (Ampe and Miambi 2000). Multidimensional scaling (MDS) analysis was used for a three dimensional space view of clusters

distribution (Boon *et al.* 2002). For further analysis of the bacterial community described by DGGE banding patterns, principal component analysis (PCA) was used to generate a few linear variables that served as indicators of the successive changes in the community composition (Hori *et al.* 2006; Alonso-Saez *et al.* 2007). For the PCA analysis, the bacterial DGGE fingerprints were converted to band match table and the quantitative values were used for PCA analysis by Bionumerics software version 5.0 (Applied Maths, Sint-Martens-Latem, Belgium). In order to evaluate the bacterial community stabilities of rotifer batch cultures, the correlation values from matrix of similarities between two consecutive rotifer batches were used for moving window analysis (Wittebolle *et al.* 2005). Lorenz curves and Gini coefficients analysis were performed to each DGGE banding patterns for the description of microbial species distributions (Mertens *et al.* 2005).

3. Results

3.1. Rotifer performance

Rotifers were cultured in 50 mL sterile falcon tubes, undergoing 4 subsequent 3-day batch culture cycles. Starting from an initial density of around 30 rotifers mL⁻¹, the culture reached a maximum density of 400 rotifers mL⁻¹ after 3 days culture when fed with CS 3000. Figure 4.1 shows the population density changes of 3 different rotifer strains during the 4 batch cycles. The maximum population densities were observed at the end of batch 1 for *B. plicatilis* sensu strictu and *B. plicatilis* Cayman, while for *B. plicatilis* Nevada the lowest population density was observed.

The rotifer growth rates of each batch culture are presented in Table 4.1. The highest growth rates were found for *B. plicatilis* Cayman and *B. plicatilis* sensu strictu in batch 1, being significantly different from the growth rate of *B. plicatilis* Nevada ($p < 0.05$). Considering the 4 batches together, the lowest growth rates of *B. plicatilis* Nevada were found compared to other two species (Table 4.1).

Table 4.1. Growth rate over 3 days during sequential batch cultivation of 3 different strains belonging to 3 different cryptic species of the rotifer *B. plicatilis* (mean \pm SD; n = 3 for *B. plicatilis* sensu strictu and *B. plicatilis* Cayman and n = 4 for *B. plicatilis* Nevada).

Rotifer species	Batch				
	1	2	3	4	Average
<i>B. plicatilis</i> sensu strictu	0.81 \pm 0.06 ^a	0.53 \pm 0.04	0.38 \pm 0.03	0.31 \pm 0.06 ^a	0.51 \pm 0.20 ^a
<i>B. plicatilis</i> Cayman	0.84 \pm 0.06 ^a	0.56 \pm 0.03	0.54 \pm 0.02	0.61 \pm 0.06 ^b	0.63 \pm 0.13 ^b
<i>B. plicatilis</i> Nevada	0.51 \pm 0.08 ^b	0.48 \pm 0.07	0.42 \pm 0.11	0.42 \pm 0.11 ^a	0.46 \pm 0.10 ^a

Treatments with different superscripts in each batch are significantly different from each other (Tukey test, p < 0.05).

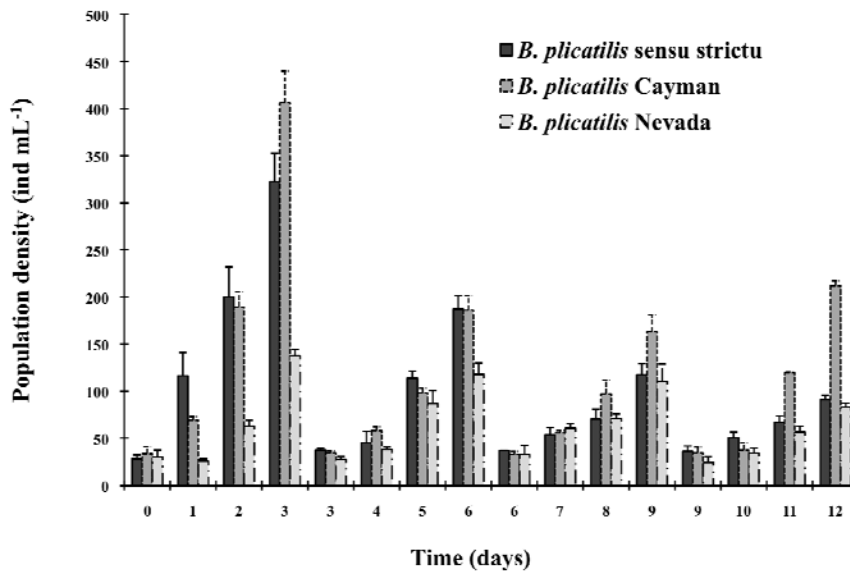


Fig. 4.1. Culture performance during 4 cycles of sequential batch cultures of 3 strains belonging to 3 different cryptic species of the rotifer *B. plicatilis* fed with Culture Selco 3000[®]. The error bars represent the standard deviation (n=3 for *B. plicatilis* sensu strictu and *B. plicatilis* Cayman; n=4 for *B. plicatilis* Nevada).

3.2. Evolution of the microbial community composition

To analyze the variability of the microbial community associated with the different rotifer cultures, samples collected at the beginning of experiments and at the end of each batch culture were subjected to PCR-DGGE analysis of 16S rRNA gene fragments. The analysis yielded highly reproducible profiles for different replicates of the same rotifer strain (Fig. 4.2). The DGGE patterns showed dominant bands that were apparently specific for the communities associated with *B. plicatilis* Nevada and sensu strictu (Fig. 4.2, arrows). In terms of presence or absence of dominant bands, large changes occurred during batch 1 for the Cayman strain and the sensu strictu strain (Fig. 4.2). After the first batch, the dominant bands of the mixed microbial culture that was used as inoculum were still present in the DGGE pattern of the MC associated with the Nevada strain, although extra bands appeared. The DGGE profile of the community associated with the Nevada culture underwent relatively large changes during the second batch, after which the pattern was relatively stable during further batches.

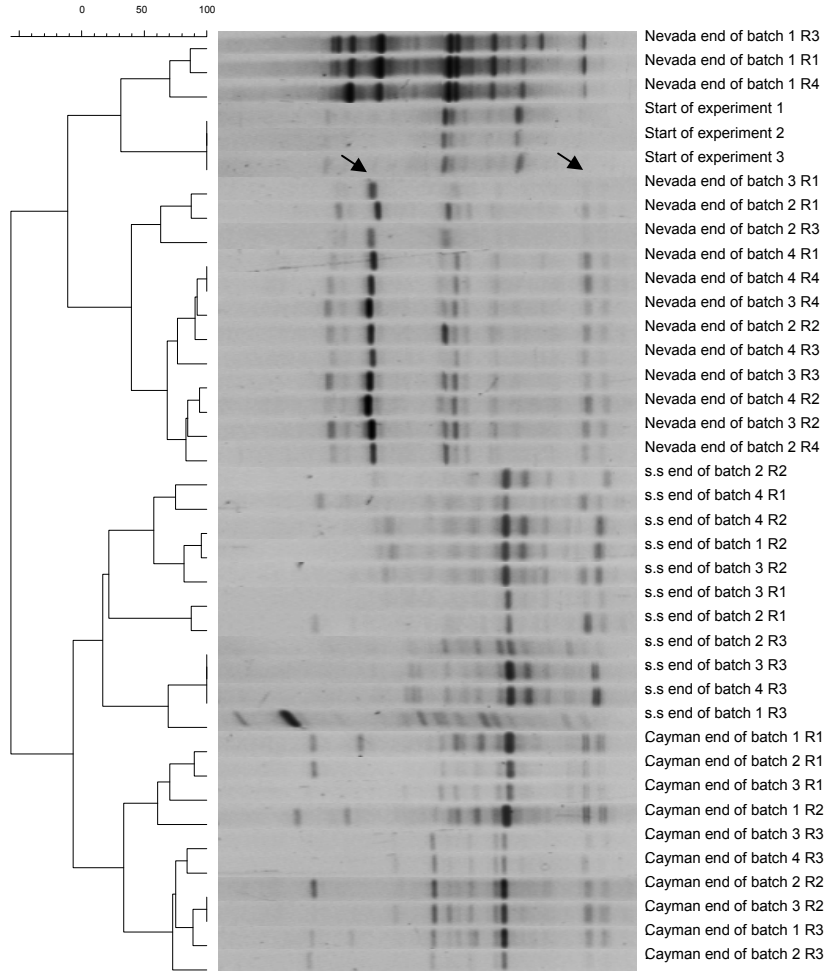


Fig. 4.2. DGGE profiles and clustering analysis of the bacterial community associated with rotifer cultures based on total DNA extracts from sequential batches of 3 different strains belonging to 3 different cryptic species of the rotifer *B. plicatilis* (s.s = *B. plicatilis* sensu strictu; Nevada = *B. plicatilis* Nevada; Cayman = *B. plicatilis* Cayman; R1, R2, R3 = replicate 1, 2 and 3; Start of experiment = samples collected after inoculations). The arrows indicate dominant bands that are specific for the communities associated with *B. plicatilis* Nevada and sensu strictu and that were not present in the patterns of the inoculum. Clustering of the patterns (using the Ward method based on Dice coefficients) is shown on the left (Scale 0–100 correspond to similarity levels ranging from 0% to 100%). Few data (Nevada batch 1 R2, s.s. batch 1 R1, Cayman batch 4 R1 & R2) are not presented due to no PCR results.

Cluster analysis of the DGGE profiles showed 3 major clusters (Fig. 4.2). Interestingly, each cluster corresponded to a different rotifer *B. plicatilis* cryptic species. MDS analysis showed a similar division in 3 major clusters (Fig. 4.3a). This

observation suggests that the microbial community associated with rotifers can evolve differently depending on the rotifer strains.

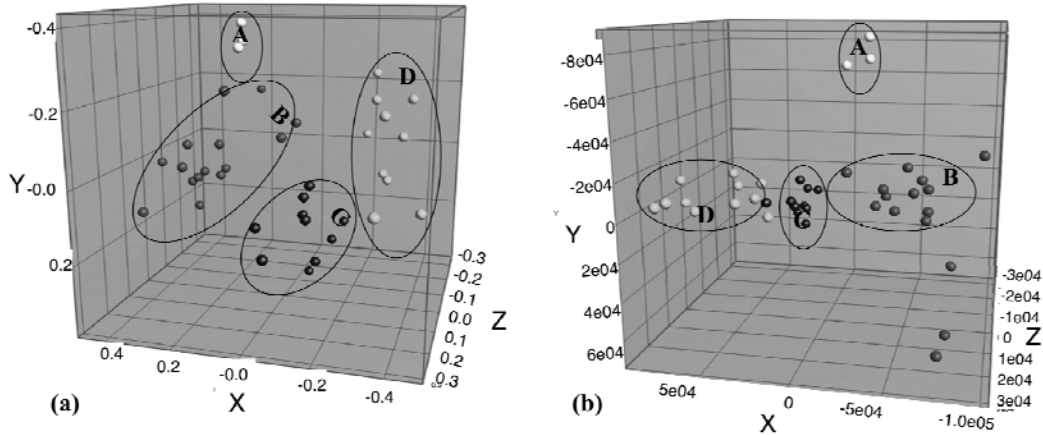


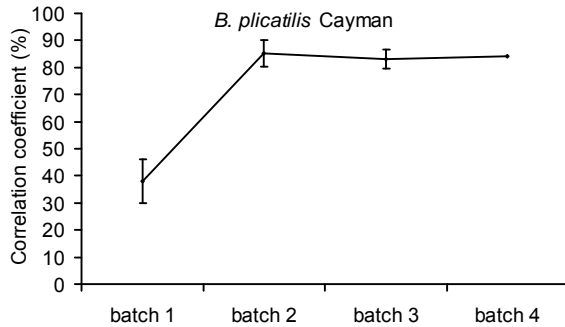
Fig. 4.3. Multidimensional scaling (a) and Principal component analysis (b) of the DGGE profiles of the bacterial communities associated with 3 different strains belonging to 3 different cryptic species of the rotifer *B. plicatilis*. Circle A corresponds to samples of the start of the experiment, Circle B corresponds to samples from *Brachionus plicatilis* Nevada cultures, circle C corresponds to samples from *Brachionus plicatilis* Cayman cultures and circle D corresponds to samples from *Brachionus plicatilis* sensu strictu cultures.

In order to reduce the number of variables of these profiles, principal component analysis (PCA) was applied. PCA simplified the DGGE patterns into 3 new linear variables that encompassed 58.8% of the variability present in the original data. Fig. 4.3b shows that the ordination of samples by PCA, based on the DGGE band patterns, agreed with the clustering results. Indeed, 3 different clusters were obtained, which correspond to the 3 different rotifer strains.

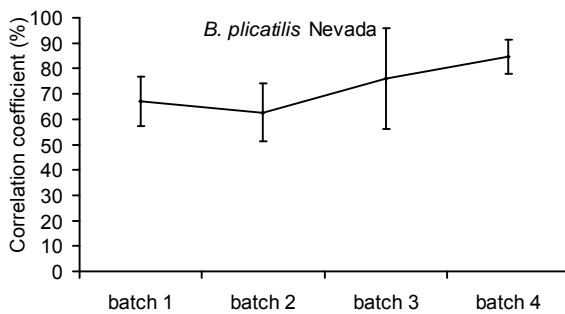
Finally, in order to track the successive dynamic changes within the MC of each species from batch 1 to batch 4, moving windows analysis was applied on the DGGE profiles. The analysis resulted in different curves for the 3 rotifer species (Fig. 4.4). For the species *B. plicatilis* sensu strictu and Cayman, relatively large changes of DGGE patterns occurred during batch 1, with the similarity between the patterns obtained before batch 1 and after batch 1 being lower than 50%. DGGE patterns of

the Nevada strain in batch 1 also changed, but to a smaller extent (70% similarity between patterns obtained before and after batch 1). For all rotifer strains, the similarity between subsequent DGGE analyses increased with increasing batch cultures to 80% or more similarity between subsequent profiles. This suggests that the microbial communities associated with the rotifers became relatively stable after several subsequent batches and only minor shifts in the community composition occurred during the last batches.

(A)



(B)



(C)

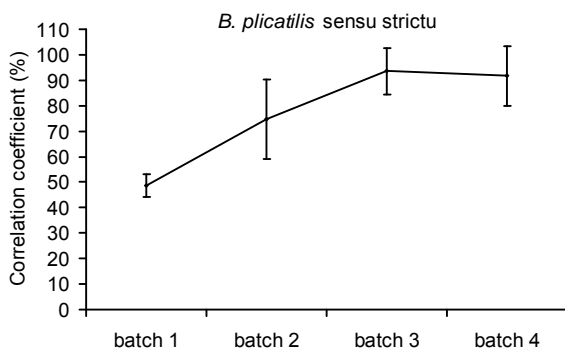


Fig. 4.4. Moving window analysis of the DGGE patterns of the bacterial communities associated with 3 different strains belonging to 3 different cryptic species of the rotifer *B. plicatilis*. The variability between two consecutive batches was calculated based on the Dice coefficients. For each batch, the similarity between the profiles of the batch of interest and the previous batch is plotted (for the first batch, this is the similarity between the profile after the batch and the initial community that was inoculated). Bar = SD.

3.3. Microbial species distribution evenness and variations

The microbial species distribution analysis was based on the method of Lorenz curves. Lorenz curves were plotted according to the numbers of bands and their intensities present in DGGE pattern. Figure 4.5 shows graphs of changing Lorenz curves of each culture unit in subsequent batch cultures. For MC in rotifer strain *B. plicatilis* sensu strictu cultures, it shows an overall tendency of less bending curves with increasing batch number compared to the curve of the initial MC. The flattest curve for *B. plicatilis* sensu strictu indicates the nearly even distribution of microbial species at the end of batch 3 (Fig. 4.5 s.s end of batch 3 R1). This decreasing tendency in the bending of curves is also present for the rotifer strain *B. plicatilis* Cayman, but to a lesser extend, since the curves are closer and cross each other (Fig. 4.5 Cayman R1, R2 and R3). On the contrary, overall highly bended Lorenz curves are found in the rotifer strain *B. plicatilis* Nevada cultures, indicating an uneven distribution.

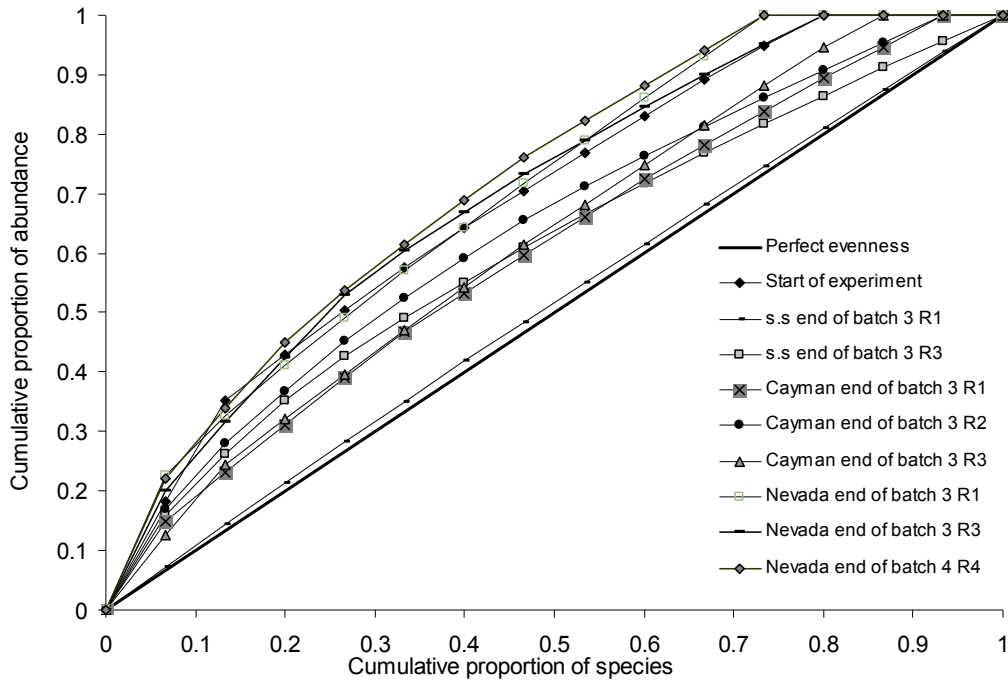


Fig. 4.5. Lorenz curves of the microbial community associated with rotifer cultures. (s.s = *B. plicatilis* sensu strictu; Nevada = *B. plicatilis* Nevada; Cayman = *B. plicatilis* Cayman; R1, R2, R3 = replicate 1, 2 and 3; Start of experiment = samples collected after inoculations. To assess changes in evenness of microbial species distribution, Gini coefficients were calculated (Fig. 4.6) and results show the different change tendencies of microbial species distributions among the three rotifer strains cultures. In the first batch, an increasing Gini coefficients are present in all 3 rotifer strains cultures which indicated the more evenly distributions of microbial species. However during the consequent 3 batches, The 3 rotifer strain cultures were ending in different ways in terms of microbial species distribution as reflected by Gini coefficient changes. In general, high Gini coefficients are found in the cultures of rotifer strains *B. plicatilis* sensu strictu and *B. plicatilis* Cayman and low Gini coefficients corresponds to rotifer strain *B. plicatilis* Nevada (Perfect evenness: reference line, in this case all species have equal population size, Gini coefficient = 1).

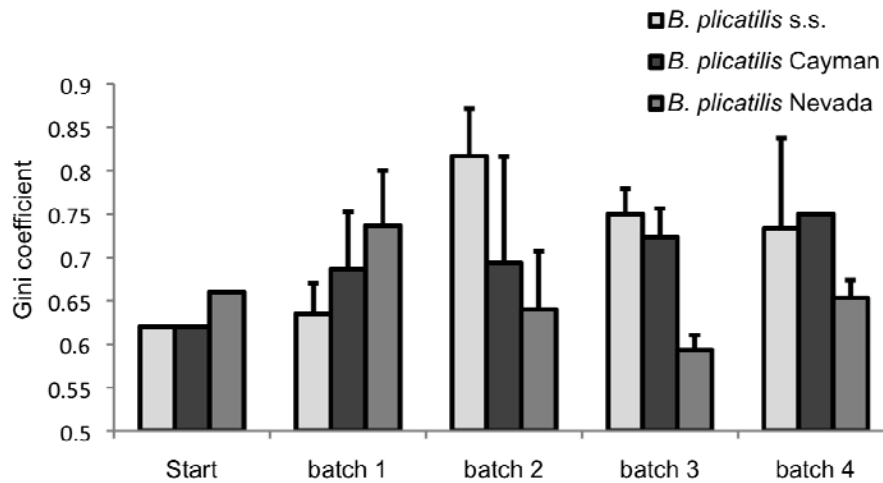


Fig. 4.6. Gini-coefficients of rotifer cultures samples at the end of each batch based on Lorenz curves (Start = samples collected after inoculations; Bar = SD, n=3 for *B. plicatilis* sensu strictu and *B. plicatilis* Cayman; n=4 for *B. plicatilis* Nevada).

4. Discussion

Despite technological improvements of batch culture of *Brachionus* including turbidity regulation and optimization of diets (Dhert *et al.* 2001; Yoshimura *et al.* 2003), the problem of “bad performances” is still common in hatcheries. As a consequence, in practice, hatcheries have to set up many tanks running batch cultures simultaneously in order to obtain sufficient amounts of live feed. Such practices increase overall cost and decrease efficiency in terms of labor and utilization of infrastructure (Dhert *et al.* 2001). Many factors have been linked to rotifer growth performance, such as nutrition, the presence of microalgae, cryptic rotifer species and different microorganisms. The bacterial communities associated with rotifers might be one of the most important factors. High rotifer densities demanding high feeding levels and producing high concentrations of waste products thus create a high load of organic material that is utilized as feed source by bacteria. These intensive rearing conditions allow heterotrophic bacteria to grow fast (Skjermo *et al.* 1997; Verschuere *et al.* 1997; Skjermo and Vadstein 1999). Conventional microbial techniques have

revealed high loads of potentially pathogenic bacteria, such as vibrios, in conventional rotifer batch cultures. Also highly variable microbial communities are found after rotifer disinfection (Romabut *et al.* 1999). Consequently, there is currently an interest in manipulating the composition of the microbial community aiming at more reliable rotifer culture performances (Dhert *et al.* 2001). In this study, we analyzed the correlation between the culture performances of different *Brachionus plicatilis* strains and the microbial communities that were associated with the rotifers. Our results indicate that microbial community composition shows large differences between different rotifer strains although all were inoculated with the same mixed bacterial community. Smaller differences in the microbial community composition were observed between replicates of the same rotifer strain.

From our results, we can conclude that shifts occur in the microbial communities associated with rotifers during batch cultivation, and the changes in the microbial community composition were found to be different for the different rotifer cryptic species. Interestingly, these changes were also associated with different culture performances. By comparing rotifer growth performances and stability of the microbial community, we found that large changes in growth rate were associated with large changes in the MC composition (Fig. 4.1, batch 1 to 2; Fig 4.4 batch 1). The rotifer growth rate of the Cayman strain reached stability from batch 2 onwards and this was accompanied by the stabilization of the microbial community (Fig. 4.4A). This was also manifested in the Moving Window Analysis (Fig. 4.4A), where low similarities were observed in the patterns obtained before and after the first batch of Cayman strain. Also, the smaller decrease in culture performances observed in later batches was accompanied by smaller changes in the microbial community composition. Culture performances of the Nevada strain, on the other hand, were

more stable as was the composition of the microbial community stability. Hence, our results suggest that the performance of rotifer cultures is strongly influenced by the associated microbial community. This conclusion corroborates previous reports mentioning that the introduction of microorganisms can significantly change the performance of rotifer cultures (Rombaut *et al.* 1999).

Skjermo and Vadstein (1993) found that the use of enrichment diets caused a shift in the bacterial composition to a microbial community totally different from the initial community and reasoned that this was partly due to a bloom of fast-growing, opportunistic bacteria, which were favored by the high substrate levels in the culture. Also in our study, significant changes were observed in the microbial community associated with rotifers upon feeding them an artificial diet. However, the shifts we observed were unlikely determined solely by the diets. Indeed, although the microbial communities changed during culturing of the rotifers, the dominant bands that appeared and/or disappeared were different for the different rotifer strains. This implies that the rotifer strain could have strong effects on the determination of the composition of the microbial community.

Recently, axenically grown *B. plicatilis* sensu strictu were used by Tinh *et al.* (2006) as a test model to reveal the role of “endogenous microbiota” which were isolated from cultures of the same rotifer species. When yeast strains were given as food, rotifer growth performance was dependent on the origin of the MCs. When a MC isolated from a crashed rotifer culture was added, however, it did not have any negative effect in the rotifer growth test, suggesting that the MC associated with the crashed rotifer culture was not responsible for the crash. Hino (1993) suggested that changes in the composition of the microbial community, and not the microbiota that

are present at that specific moment, are the cause of the collapse of rotifer cultures. In accordance to this, in this study, we found that the cultures showing the largest changes in microbial community composition also had the largest decreases in growth performance.

Most of the microbial community studies concentrate on comparing species richness (i.e. the number of distinct species), However, community diversity is more than richness. It also includes evenness and dynamics (Marzorati *et al.* 2008). Lorenz curves and Gini coefficient describe the distribution of the different species within a community and has been shown to be a good estimator of microbial species evenness (Mertens *et al.* 2005; Halet *et al.* 2006; Wittebolle *et al.* 2008). In our study, differences in the shapes of Lorenz curves and Gini coefficient were found among 3 different strains of rotifer *B. plicatilis* batch cultures. The lower evenness (0.6-0.65) from batch 2 to 4 in *B. plicatilis* Nevada culture indicated that a smaller fraction of the different microbial species present was dominating the community. Interestingly, this few dominant microbial species were presented with a stable microbial community composition and rotifer growth performance. On the contrary, an increasing trend of evenness of microbial species was found with the strain *B. plicatilis* Cayman when the culture performance was stabilizing. Nevertheless, the large shifts in microbial community composition at the end of batch 1 seemed to result always an increasing of evenness of microbial species distribution in all cultures. Thus it seems that for a *B. plicatilis* culture with high population densities, a high evenness is required.

The DGGE fingerprinting technique has been shown to be an interesting tool for the study of microbial communities in aquaculture systems. This technique, combined

with other physiological/chemical analyses, can be useful in unraveling the ecological functions of bacterial communities in rotifer culture systems. It has been shown that cryptic species differ in ecological preferences and life cycle parameters (Serra *et al.* 1998; Ortells *et al.* 2003) and the same might be true for the microbiota that are associated with the rotifers. In this study, we found that different cultures of rotifer strains belonging to 3 different cryptic species of the rotifer *B. plicatilis* were carried different microbial communities. As microbial communities associated with the rotifers are transferred with the rotifers to the larval fish cultures, it would be interesting to verify if the larval fish performance (which is highly influenced by microorganisms) can be modulated by choosing the proper rotifer strain cultures.

In our study, the inoculation of the same mixed bacteria community which resulted an identical DGGE banding patterns, suggested the inoculated bacteria dominated in numbers in initial MCs. However, this dominance was non-substantial as the occurrences of different dominant DGGE bands in consecutive batch cultures. It was likely some bacterial members of inoculation were favored in the rotifer batch cultures. However, bacteria from others sources, for example, the accompanying bacteria which were still presented after washing, may re-established during the cultures. Therefore, our results also indicated that manipulation of the microbial community of rotifer cultures (e.g. by inoculation with a culturable beneficial microbial culture) should be evaluated carefully with the monitoring of microbial community changes.

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Chapter

5

Effects of feeding regime and probionts on the diverting microbial communities in rotifer *Brachionus* culture

ABSTRACT

Rotifer growth performance and microbial community changes associated with rotifer cultures were monitored while different feed types (*Nannochloropsis oculata* paste and the commercial yeast based feed CS-3000), different regimes (daily changes, changes per batch and no changes) and mixtures of 3 probionts (*Phenylobacterium* sp.; *Gluconobacter* sp. and *Paracoccus denitrificans*) were provided. It was shown that the dominant bacterial species in the cultures receiving either *N. oculata* or CS-3000 were different. However, in cultures receiving both feeds (either switching between feeds on a daily basis or on a batch basis), a high similarity in microbial community fingerprint was found. The presence of probionts was detected by the end of 4 batch culture cycles in spite of strong shifts of the bacterial community. By group discriminant analysis, it was found that *Phenylobacterium* sp. and *Paracoccus* sp. contributed positively to the CS-3000 fed group, while *Gluconobacter* sp. contributed positively to the *N. oculata* fed group although they did not appear as very dominant species. By Lorenz curve and Gini coefficient analysis, in the absence of probionts, a higher uneven bacterial community was found when *N. oculata* was included in the feeding regimes.

Key words: DGGE, feeding regime, microbial community, probiont, rotifer

1. Introduction

Rotifer cultures are one of the key factors for successful marine larval culture. Cultivation of brachionid rotifers has been successful using microalgae such as *Chlorella* sp., *Nannochloropsis* sp., *Tetraselmis* sp. and *Isochrysis* sp. (Lavens and Sorgeloos 1996). Since the cultivation of algae under controlled conditions is time consuming and expensive, alternative feed types, such as yeast-based artificial diets, have been used for rotifer mass cultures (Castell *et al.* 2003).

Batch culture is the most commonly used system for rotifer production in hatcheries. The high number of rotifers in a culture, and their accompanying feed inevitably creates a high load of organic material, which is rapidly used as a nutrient source by bacteria (Skjermo and Vadstein 1993). The accumulation of metabolic products and uneaten feed are both common in rotifer cultures and many other aquaculture systems, and the response of microbial communities to these high organic loads can be very important. Indeed, the growth of heterotrophic bacteria can lead to deterioration of the water quality and the bacteria that are able to proliferate rapidly under these conditions can be pathogenic to the cultured animals (Verschuere *et al.* 2000).

Intensive rearing conditions can change the normal interaction between bacteria and rotifers to one that is detrimental to the cultured organisms (Skjermo and Vadstein 1993). Microbial communities associated with rotifer cultures are gaining attention,

because they potentially consist of many opportunistic pathogenic bacteria. Indeed, *Vibrio* spp. appeared to be the dominant species in many studies (Verdonck *et al.* 1997; Gomez-Gil *et al.* 2003). Attempts at microbial control, such as the use of physical disinfection equipment and chemical disinfectants, can reduce the bacterial load, but only temporarily and in a non-selective way (Skjermo *et al.* 1997). Moreover, a non-selective kill-off of bacteria results in a high colonization potential for micro organisms that subsequently proliferate in the cultures. As the composition of the remaining microbial community and the subsequent shifts in community composition are not reproducible, culture performances might also be highly variable (depending on whether the most competitive micro organisms are beneficial, neutral or pathogenic) (Verschuere *et al.* 2000).

Several studies evaluated the effects of added putative probiotics for the improvement of rotifer cultures (Makridis *et al.* 2000b; Rombaut *et al.* 2003). However, few studies have demonstrated the fate of probiotics in rotifer culture systems and have reported on the effect of bacterial supplements on the microbial community composition. To study the microbial community composition, culture dependent methods are considered inadequate because more than 99% of all bacteria cannot be cultivated (Amann *et al.* 1995). Direct molecular detection of specific DNA or RNA targets avoids the biases associated with traditional culture-based methods. Denaturing Gradient Gel Electrophoresis (DGGE) is based on sequence-specific separation of PCR-derived rRNA gene amplicons in polyacrylamide gels containing a linearly

increasing concentration of denaturant (Muyzer and Smalla 1998). The molecular fingerprinting method DGGE allows more rapid comparison of samples and is generally used to detect shifts in populations over time and/or under different environmental conditions (McCaig *et al.* 2001)

In this study, we present the dynamic shifts in microbial community composition in response to different diets as well as different feeding regimes. In addition, putative probiotic strains are tested for their ability to introduce temporal or permanent changes in microbial community composition. To address the question whether different feeding regimes and the addition of probiotics have different impact on bacterial species evenness (BSE), Lorenz curve and Gini coefficient analysis were performed for two consecutive batch cultures of three days each.

2. Materials and methods

2.1. Rotifer strain

Experiments were performed with a mixture of 3 different *B. plicatilis* strains belonging to 3 different cryptic species: *B. plicatilis* sensu strictu, *B. plicatilis* ‘Cayman’ and *B. plicatilis* ‘Nevada’. Before the start of the experiment, the rotifer strains were kept at the Laboratory of Aquaculture & Artemia Reference Center. The rotifer stocks were maintained in 2 L batch cultures under controlled conditions: 28 °C, salinity 25 g L⁻¹ seawater, and preconditioned by feeding Culture Selco 3000®

(CS-3000; INVE, Belgium) (50%) and *Nannochloropsis oculata* paste (50%) (Reed Mariculture, Campbell, CA).

2.2. Preparation of microbial communities (MCs)

MCs were prepared following the methods described by Tinh *et al.* (2006). Briefly, MCs were isolated from normal-performing rotifer *B. plicatilis* sensu strictu cultures. These MCs were preserved for further experiments in 1 mL eppendorfs containing 20% glycerol and 80% bacterial suspension and kept at $-80\text{ }^{\circ}\text{C}$. Before starting each experiment, the eppendorfs were thawed; subsequently, 50 μL of the corresponding MC suspension was spread plated on Marine Agar (MA) (Difco, Detroit, MI, USA). After 24 h of incubation at $28\text{ }^{\circ}\text{C}$, the bacteria were harvested by swabbing the MA plate and suspending them in autoclaved Nine Salt Solution (NSS) (Olsson *et al.* 1996). The cell density of the suspensions was calculated according to the McFarland standard (BioMerieux, Marcy L'Etoile, France), based on optical density measurements ($\text{OD}_{550} = 1.000$ corresponds to 1.2×10^9 cells mL^{-1}). Subsequently, the appropriate volume to be added to each treatment was calculated in order to obtain an initial density of 10^6 CFU mL^{-1} in the rotifer culture water.

2.3. Preparation of mixture of probiotics

Three probiotics isolated from well-performing rotifer cultures which showed increased rotifer population growth rates, were chosen for the experiment. They were identified as *Phenylobacterium* sp., *Gluconobacter* sp. and *Paracoccus denitrificans*

(Rombaut 2001). The probionts were cultured in Marine broth for 24 h at 28 °C, harvested by centrifugation and suspended in autoclaved NSS. The bacterial density was measured as mentioned above. The three probionts were mixed in 1:1:1 ratio. The appropriate volume to be added to each treatment was calculated in order to obtain an initial density of 10^6 CFU mL⁻¹.

2.4. Experimental set-up of rotifer batch cultures

The two experiments were run to test for the effects of different feeding regimes (experiment 1) and probionts (experiment 2), respectively. The experiment was performed in 2 L autoclaved glass bottles and each experiment was repeated three times. Rotifers were harvested from the stock cultures, rinsed with 0.22 µm-filtered, autoclaved natural seawater (FASW) three times to remove most of the bacterial loading, and 3 rotifer biotypes were mixed at 1:1:1 ratio and distributed to the autoclavable bottles containing 1.4 L of 25 g L⁻¹ FASW, at an initial density of approximately 100 rotifers mL⁻¹. The MCs and the mixture of probionts were added once to each bottle at an initial density of 10^6 CFU mL⁻¹ at the start of experiment 2. The bottles were put in a water bath at 28 °C, and supplied with continuous light (intensity 2000 Lux) and 0.2 µm filtered aeration. Each experiment was run for 4 batch cycles. One cycle consisted of a 3-day culture period. At the end of each batch, rotifers were harvested, rinsed and re-distributed at a density of approximately 100 rotifer mL⁻¹ in fresh FASW before starting the next batch culture.

2.5. Rotifer diet and feeding regimes

In experiment 1, the different feeding regimes were: 1) daily CS-3000 feeding, 2) daily *N. oculata* feeding, 3) a switch between CS-3000 and *N. oculata* after each batch, 4) a daily switch between those two feed types. In experiment 2, the rotifers were fed solely CS-3000 or *N. oculata* paste. Rotifers were fed following the manufacturer's instructions at equal dry weight basis.

$$\text{Feed} = 0.0168 * D^{0.415} * V.$$

Where: Feed = the weight of experimental diet (g); D = rotifer density (individuals mL⁻¹); V = culture water volume (L).

2.6. Sampling, counting and growth data analysis

Three samples of 0.5 mL were taken from the rotifer cultures using an automatic micropipette. The rotifers in each sample were killed by adding three drops of lugol and were counted. Empty, transparent loricae belonging to dead rotifers were not counted.

The specific growth rate was calculated using the following equation described previously (Rombaut *et al.* 2001): $\mu = (\ln N_t - \ln N_0) * t^{-1}$, where: μ = specific growth rate; N_t = rotifer density after culture period t (individuals mL⁻¹); N_0 = initial rotifer density (individuals mL⁻¹); t = culture period (day).

Data of the growth rates on day 3 were evaluated using Levene's test for homogeneity of variances and Shapiro–Wilk's test for normality. As data were normal distributed and homoscedastic, the growth rates on day 3 for batch 1 and batch 4 were compared between experiments using one-way ANOVA, followed by Tukey test. All the tests were performed using the computer program SPSS release 12.0 (SPSS, USA).

2.7. Microbiological analyses

2.7.1. Sampling method

One mL culture water with rotifers was collected in sterile eppendorf 1.5 mL tubes at the experiment start and at the end of each batch culture. Samples were centrifuged at 5000 g for 5 min and stored at -20 °C.

2.7.2. DNA extraction

Total DNA extraction from the samples was obtained by a modified bacterial cell extraction method described previously (Rombaut *et al.* 2001). To obtain bacterial DNA, the samples were centrifuged for 30 min at 5000 g. The pellet was dissolved in 0.2 mL Milli-Q water, transferred to 0.4 mL of 10 mM Tris-HCl (pH 9) and 0.3 g of beads (0.10–0.11 mm diameter). This mixture was homogenised three times for 30 s using a bead beater at 2000 RPM (B. Braun Biotech International, Melsungen, Germany). After this, 16 μL of 50 mg mL^{-1} lysozyme was added, and then the suspension was incubated at 37 °C for 15 min on a shaker (200 RPM). Chemical lysis of the bacterial cells was achieved by adding 30 μL of 20% SDS and the suspension

was slowly mixed for 5–10 min. Subsequently, 0.1 mL of 8 M ammonium acetate was added. DNA was obtained from the lysates using standard phenol–chloroform extraction and isopropanol precipitation procedures (Boon *et al.* 2000). The total DNA extracted was quantified by a spectrophotometer ND-1000 (NanoDrop Technologies, Wilmington, USA).

2.7.3. Amplification of 16S rRNA genes

The extracted DNA was amplified with primers GC338f and 518r spanning the V3 region of the 16S rRNA gene (Muyzer *et al.* 1993) using a 9600 thermal cycler (Perkin–Elmer, Norwalk, CT, USA). PCR amplification was carried out in 24 μL reactions, and 1 μL of extracted total DNA (containing equal amount of DNA) was added. The PCR master mix contained 0.5 μM of each primer, 200 μM of each deoxynucleoside triphosphate, 1.5 mM MgCl_2 , 10 μL of thermophilic DNA polymerase 10 \times reaction buffer (MgCl_2 -free), 2.5 U of Taq DNA polymerase (Promega, Madison, WI, USA), 400 ng μL^{-1} of bovine serum albumin (Boehringer) and sterile water, to obtain a final volume of 100 μL .

PCR was performed in a 9600 thermal cycler as follows: 95 °C for 10 min, followed by 30 cycles at 95 °C for 1 min, 53 °C for 1 min, and 72 °C for 2 min.

All PCR products (5 μL volumes) were analyzed by electrophoresis in 1% (w/v) agarose gels before DGGE analysis was performed.

2.7.4. Analysis of PCR products by DGGE

DGGE was performed, as previously described (Boon *et al.* 2002), using the Bio-Rad D Gene System (Hercules, CA, USA) with 8% (w/v) polyacrylamide gels in 1 × TAE (20 mM Tris, 10 mM acetate, 0.5 mM EDTA, pH 7.4), containing a linear chemical gradient ranging from 40% to 60% denaturant (where 100% denaturant contains 7 M urea and 40% formamide). Equal amounts of PCR products were used for separation in denaturing gradient gels. The electrophoresis was run for 16 h at 60 °C, at 38 V.

After completion of electrophoresis, the gels were stained for 20 min in SYBR GreenI nucleic acid gel stain solution (1:10,000 dilution in 1 × TAE; FMC BioProducts, Rockland, ME, USA). The stained gel was immediately photographed on a UV transillumination table with a video camera module (Vilbert Lourmat, Marne-la vallée, France).

2.7.5. DGGE pattern analysis

The obtained DGGE patterns were analyzed using Bionumerics software version 5.0 (Applied Maths, Sint-Martens-Latem, Belgium). A matrix of similarities for the densitometric curves of the band patterns was calculated using the band-based Dice coefficient and dendrograms were created using the Ward method. For the group discriminant analysis, the automatic band searching facility of the software was used. Any band that represented more than 1% of the total surface under the densitometric curve was scored. Bands were then automatically assigned to band classes by band

matching using 1% position tolerance. Subsequently, multivariate analysis of variances was performed based on different band classes and the two defined groups (CS-3000 and *N. oculata*). For the Lorenz curve and Gini coefficient analysis, the data of numbers of bands and their intensities present in DGGE patterns were used (Mertens *et al.* 2005).

3. Results

3.1. Effect of different feeding regimes on rotifer growth rate (Experiment 1).

Starting from an initial density of around 100 rotifers mL⁻¹, the culture reached a density of 200-400 rotifers mL⁻¹ after 3 days. The highest population densities were observed at the end of batch 4 when *N. oculata* was provided as the sole feed.

Table 5.1 shows that the best growth was found when only *N. oculata* was provided as feed. In contrast to other treatments, *Brachionus* spp. fed with *N. oculata* did not display a deprivation in growth rate during batch 2. *Brachionus* spp. fed CS-3000 also suffered a loss in growth rate in batch 2 and no full recovery was achieved. The addition of 50% of *N. oculata* in feeding regime (a daily or batch bath basis) resulted in similar growth rates compared to *N. oculata* fed cultures, after two batch cultures. The frequency of shifting between the feed types had no significant effect on the rotifer growth rate.

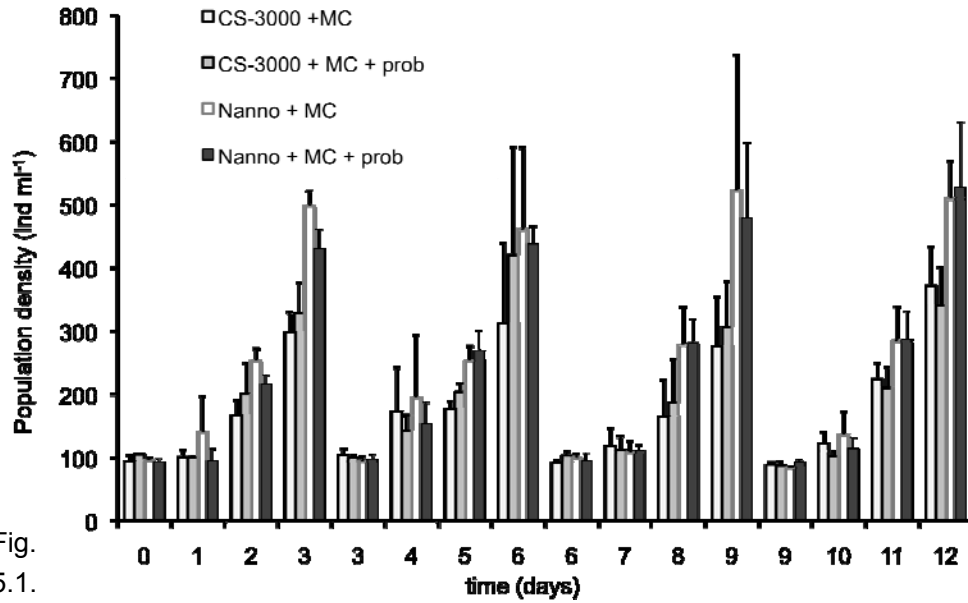


Fig. 5.1.

Culture performance of 4 consecutive batch cultures of rotifers with the addition of microbial communities and mixtures of probiotics (CS-3000 + MC = CS-3000 as diet with the addition of microbial communities (MC); prob = mixture of probiotics; Nanno = *N. oculata*; n=3; bars = SD).

Table 5.1. Growth rate (day⁻¹) of each batch over 3 days (mean ± SD, n = 3) of rotifer batch culture from experiment 1.

Feeding regime	Batch			
	1	2	3	4
CS-3000	0.41 ± 0.18 ^a	0.02 ± 0.39 ^a	0.24 ± 0.39 ^a	0.20 ± 0.18 ^a
<i>N. oculata</i> paste	0.63 ± 0.08 ^a	0.45 ± 0.11 ^b	0.61 ± 0.05 ^b	0.67 ± 0.08 ^b
<i>N. oculata</i> /CS-3000 per batch	0.70 ± 0.14 ^a	0.12 ± 0.29 ^a	0.49 ± 0.05 ^b	0.52 ± 0.12 ^b
<i>N. oculata</i> /CS-3000 per day	0.63 ± 0.13 ^a	0.19 ± 0.37 ^a	0.55 ± 0.02 ^b	0.52 ± 0.19 ^b

Treatments with different superscripts in each batch are significantly different from each other (Tukey test, p < 0.05).

3.2. Effect of the probiont mixture on the rotifer density in batch cultures

(Experiment 2).

Rotifers were cultured in the same conditions as in experiment 1. Figure 5.1 shows the changes in the rotifer population density with different feed in four consecutive batch cycles. The growth rate remained good during four batch culture cycles ($\mu > 0.36 \pm 0.07 \text{ day}^{-1}$) and a stable rotifer production was achieved (Table 5.2). The rotifer reproduction was significant higher ($p < 0.05$) when *N. oculata* was provided (except in batch 2). In terms of the rotifer growth rate, no significant difference between probiont-treated groups and untreated groups under the same feed type was found.

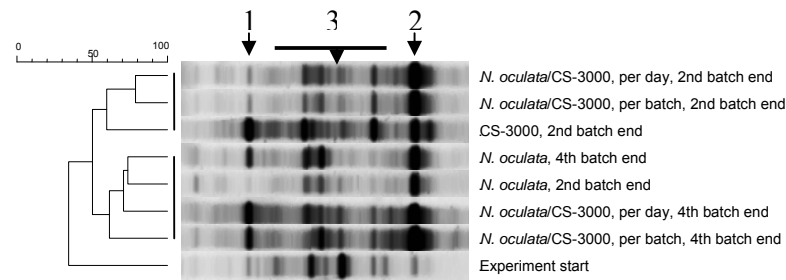
Table 5.2. Growth rate (day^{-1}) of each batch over 3 days (mean \pm SD, n = 3) of rotifer batch culture.

Feeding regime	Batch			
	1	2	3	4
CS-3000 + MC	0.39 ± 0.06^b	0.36 ± 0.09^b	0.36 ± 0.10^b	0.47 ± 0.05^b
CS-3000 + MC + prob	0.38 ± 0.05^b	0.46 ± 0.12^a	0.36 ± 0.07^b	0.45 ± 0.04^b
Nanno + MC	0.55 ± 0.01^a	0.51 ± 0.10^a	0.54 ± 0.12^a	0.60 ± 0.03^a
Nanno + MC + prob	0.50 ± 0.01^a	0.50 ± 0.03^a	0.53 ± 0.04^a	0.58 ± 0.07^a

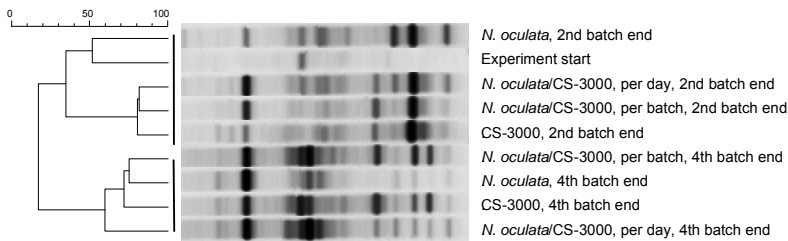
Treatments with different superscripts in each batch are significantly different from each other (Tukey test, $p < 0.05$). (CS-3000 + MC = CS-3000 as diet with the addition of microbial communities (MC); prob = mixture of probiotics; Nanno = *N. oculata*).

3.3. Microbial community shifts

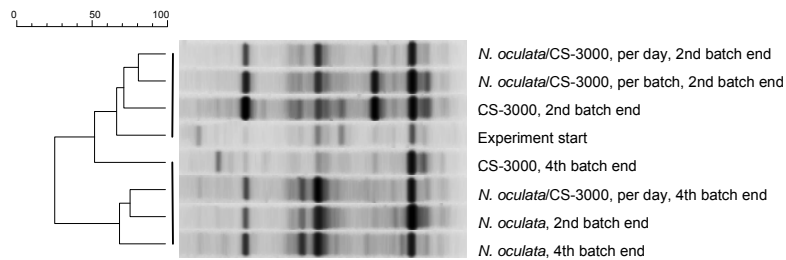
To analyze the microbial community associated with different feed types, samples were collected at the beginning of experiment 1 and at the end of batch culture 2 and 4. The DGGE patterns of different 16S rRNA gene V3 fragments amplified from the DNA extracted from the culture water of the rotifer batch cultures are presented in Fig. 5.2. Large shifts (60% changes of similarity) in DGGE banding patterns were found between the start and the end of batch 2 and 4 (Fig. 5.2a). This shift appeared also in the second (Fig. 5.2b) and to a lesser extent in the third (Fig. 5.2c) experimental runs. The two types of feed (CS-3000 and *N. oculata*) did not generate highly distinguishable banding patterns as their associated MC often clustered together in all 3 experimental runs. In the cultures in which the feed was changed per day or per batch no difference in terms of DGGE banding patterns appeared. However, considering the 3 experimental runs together (Fig. 5.2a, b and c), it was found that some band positions (arrow 1 and 2) appeared consistently no matter what type of feed or feeding regimes were given, while other band positions (region 3) appeared to be highly variable in response to different feeds or feeding regimes. Across the 3 experimental runs and independent from the feed type, MCs of the second batch and fourth batch tended to cluster together (with some exceptions). This indicates that the microbial communities in these experiments were still in strong evolution and suggests that, at the end of batch 4, MC might not have stabilized.



(a)



(b)



(c)

Fig. 5.2. DGGG profiles of the microbial community associated with different feed types and feeding regimes. (CS-3000/*N. oculata*: switching 2 feed types; panels (a), (b) and (c) were started at different times as indicated by “Experiment start”). Clustering of the patterns (using the Ward method based on Dice coefficients) is shown on the left (Scale 0–100 corresponds to similarity levels ranging from 0% to 100%). (1 and 2 are the band positions existing consistently; 3 is the band region which is highly variable). Bars indicate clusters that are dominated by MCs for batch 2 or batch 4 with some exceptions.

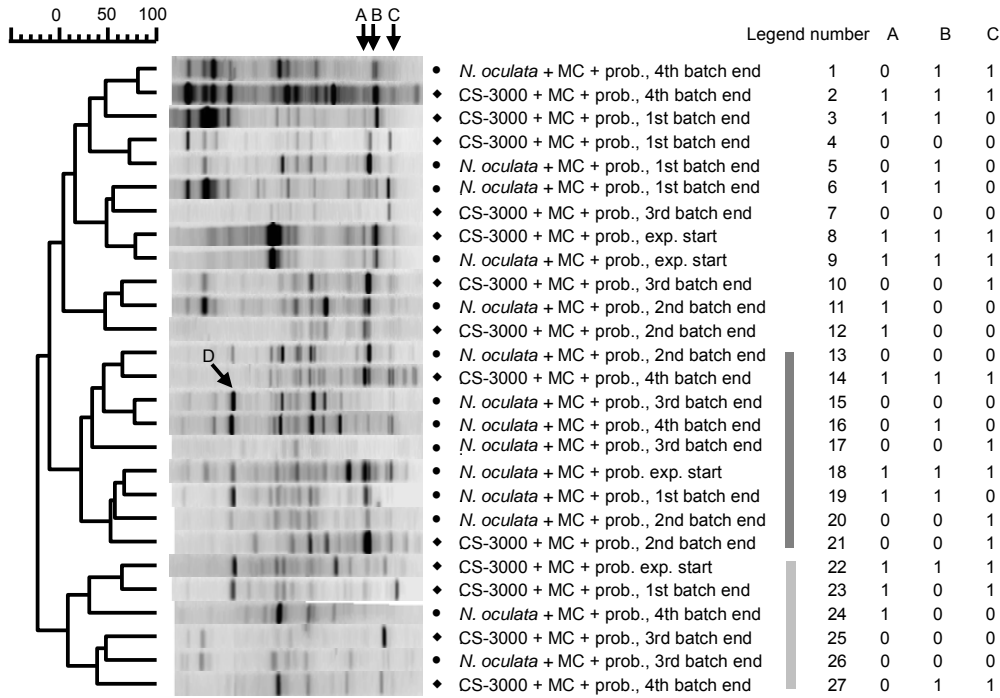


Fig. 5.3. DGGE profiles of the microbial community associated with the inoculations of Microbial community (MC) and mixed probionts (prob.) (CS-3000 + MC + prob.: rotifer cultures received CS-3000 with the inoculation of MC and prob.; Arrows: (A) probiont *Gluconobacter* sp., (B) probiont *Phenylobacterium* sp., (C) probiont *Paracoccus denitrificans* and (D) band number 24 in Fig. 5.4; '1': band detected; '0': no band). Clustering of the patterns (using the Ward method based on Dice coefficients) is shown on the left (Scale 0–100 corresponds to similarity levels ranging from 0% to 100%). Bars indicate clusters that are dominated by MC grown in batch culture fed *N. oculata* or CS-3000 with some exceptions.

Figure 5.3 shows the DGGE gel pattern profiles of the bacterial communities upon the addition of mixtures of probionts in rotifer batch cultures. The band intensity of the probiont *Gluconobacter* sp. became weaker after one batch culture and tended to disappear by the end of batch 4 (Fig 5.3. number 15-17 and 25-27). On the contrary, the probiotic *Phenylobacterium* sp. was still present at the end of batch 4 (Fig 5.3: numbers 2, 14, and 27). The probiont *Paracoccus denitrificans* followed the same

trend as *Phenylobacterium* sp. in spite of the fact that the band patterns generally became weaker towards the last batch culture (Fig. 5.3: numbers 1, 2 and 27). Probiotics took the dominant positions in several cases (e.g. numbers 12 and 19), but this was not constant during the subsequent batch cultures (numbers 10, 20 and 26). Changes of DGGE band patterns were observed in consecutive batch cultures (numbers 22, 23, 21, 25 and 27). *N. oculata* or CS-3000 based major clusters were formed although there were some exceptions (numbers 13-21 and 22-27).

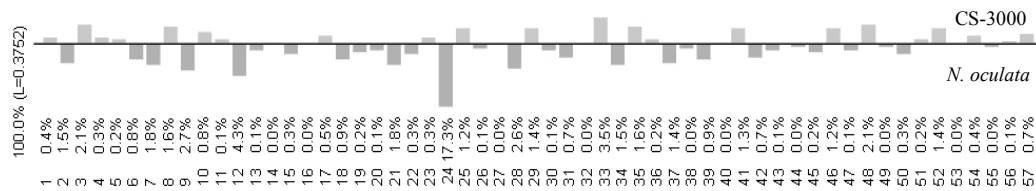


Fig. 5.4. Multivariate analysis of variance (MANOVA) and discriminant analysis of DGGE banding patterns. Numbers 1 to 57 are different band positions which served as the different characters. Each character was evaluated for its contribution to defined group separation; Characters which contributed positively to the CS-3000-fed group are indicated by grey bars above the line; Characters which contributed more to the *N. oculata*-fed group are indicated by grey bars below the line; The relative contributions of each band position to group separation are shown in percentage below each bar (and also correspond to the height of the bars). Total contributions accumulate to 100%. L: Value of Wilk's Lambda likelihood ratio test. Band number 42 corresponds to *Gluconobacter* sp., 46 to *Phenylobacterium* sp. and 51 to *Paracoccus denitrificans*. Band number 24 corresponds to arrow D in Fig.5.3.

Multivariate analysis of variance (MANOVA) and discriminant analysis of DGGE banding patterns are shown in Fig. 5.4. Both *Phenylobacterium* sp. (band number 46) and *Paracoccus denitrificans* (band number 42) appeared to be more dominant in the group fed CS-3000, while *Gluconobacter* sp. (band number 51) was more dominant in the group fed *N. oculata*. Apparently, the 3 added bacteria did not take exclusively the

dominant positions in DGGE banding patterns, since their contributions to the total percentage of separation were rather low. However, one band (number 24, indicated as arrow D in Fig. 5.3) was found typically in the group fed *N. oculata* and contributed 17.3% of the total percentage of separation.

Table 5.3 shows the total BSE equality measures of the rotifer batch culture samples with different feeding regimes of experiment 1. Relatively low Gini coefficient values were found when *N. oculata* was present in the feeding regimes. From the end of batch 2 to the end of batch 4, the community became more equal (Gini coefficient values from 0.54 to 0.68, Table 5.3) in the group in which feed types were switched per batch, while the BSEs of others batches appeared stable (Fig. 5.5).

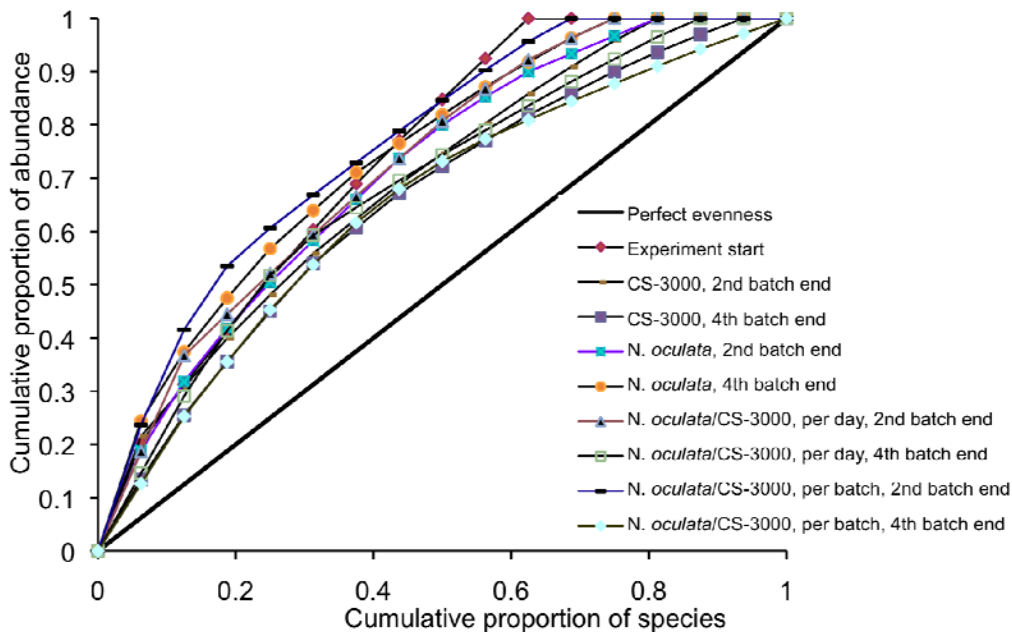


Fig. 5.5. Lorenz curves of the rotifer batch culture samples with different feeding regimes (CS-3000/*N. oculata*: switching 2 feed types; DGGE banding pattern data of Fig. 5.2 (b) were used).

Table 5.3. Gini coefficient, as total bacterial species distribution equality measure, of the rotifer batch culture samples with different feeding regimes, on the basis of 16 S rRNA gene fragment DGGE fingerprinting data.

Samples	Gini coefficient of batch 2 and 4 (mean \pm SD, n =2*, 3)	Gini coefficient of each feeding regime (mean \pm SD, n = 5*, 6)
Experiment start	0.62 \pm 0.09	
CS-3000	0.66 \pm 0.04	
2nd batch end		0.66 \pm 0.03
CS-3000	0.67 \pm 0.03	
4th batch end		
<i>N. oculata</i>	0.64 \pm 0.09	
2nd batch end		0.63 \pm 0.08
<i>N. oculata</i>	0.62 \pm 0.09	
4th batch end		
<i>N. oculata</i> /CS-3000 per day	0.61 \pm 0.11	
2nd batch end		0.63 \pm 0.08
<i>N. oculata</i> /CS-3000 per day	0.66 \pm 0.05	
4th batch end		
<i>N. oculata</i> /CS-3000 per batch	0.54 \pm 0.08	
2nd batch end		0.60 \pm 0.09
<i>N. oculata</i> /CS-3000 per batch	0.68 \pm 0.07	
4th batch end		

*: Two data, one for CS-3000 at 4th batch end and one for *N. oculata*/CS-3000 per batch at 4th batch end were not presented due to the failed PCR reactions. Treatments are not significantly different from each other (Tukey test, $p > 0.05$).

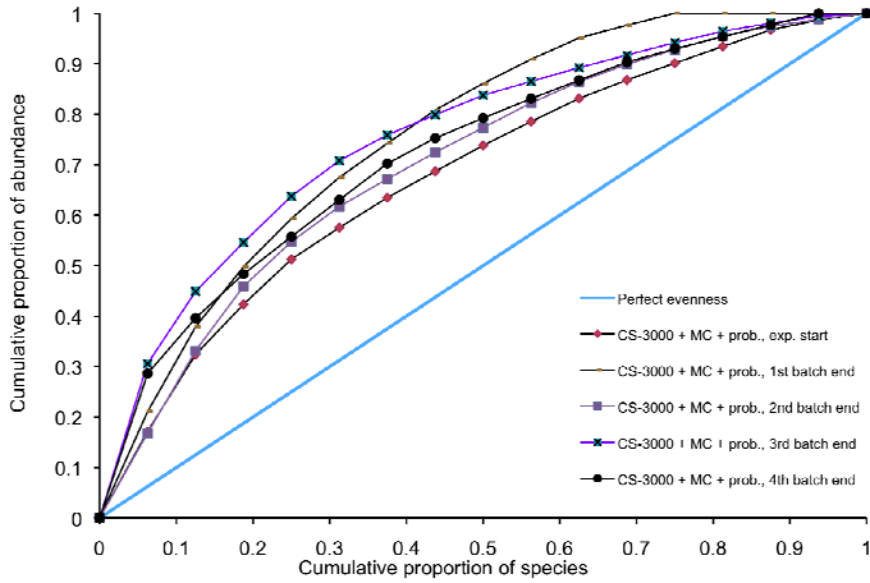
The mixture of 3 probionts in experiment 2 decreased the equality of BSE from batch culture 1 to 3 when CS-3000 was supplied as feed. An opposite trend was found when

N. oculata was fed. Across all batches, the BSEs were apparently independent from the feed type (Table 5.4). From batch 3 to 4, an increased equality in BSEs was found both for CS-3000 (Gini coefficient values 0.53 to 0.72) and *N. oculata* (Gini coefficient values 0.57 to 0.65).

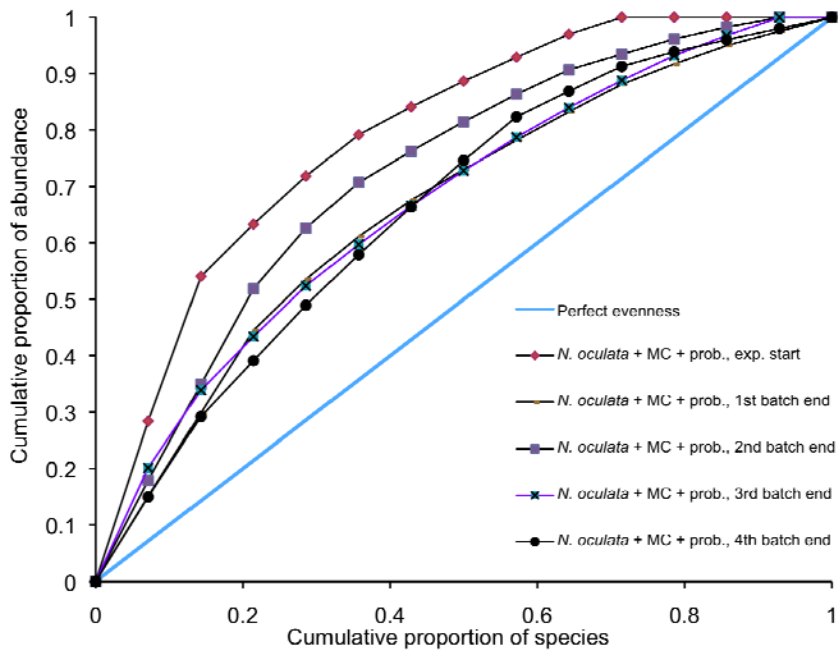
Table 5.4. Gini coefficient, as total bacterial species distribution equality measure, of the rotifer batch culture samples with the addition of mixed probiotics under different feeding regimes, on the basis of 16S rRNA gene fragment DGGE fingerprinting data.

Samples	Gini coefficient of per batch (mean \pm SD, n =2 or 3*)	Gini coefficient of each feeding regime (mean \pm SD, n = 11 or 12*)
CS-3000 + MC + prob., exp. start	0.63 \pm 0.02	
CS-3000 + MC + prob., 1st batch end	0.53 \pm 0.04	
CS-3000 + MC + prob., 2nd batch end	0.49 \pm 0.15	
CS-3000 + MC + prob., 3rd batch end	0.53 \pm 0.04	0.57 \pm 0.13
CS-3000 + MC + prob., 4th batch end	0.72 \pm 0.15	
<i>N. oculata</i> + MC + prob., exp. start	0.52 \pm 0.08	
<i>N. oculata</i> + MC + prob., 1st batch end	0.58 \pm 0.18	
<i>N. oculata</i> + MC + prob., 2nd batch end	0.55 \pm 0.05	
<i>N. oculata</i> + MC + prob., 3rd batch end	0.57 \pm 0.08	0.59 \pm 0.07
<i>N. oculata</i> + MC + prob., 4th batch end	0.65 \pm 0.06	

*: One sample, CS-3000 + MC + prob., 2nd batch end data, was not presented due to the failed PCR reactions. Treatments are not significantly different from each other (Tukey test, $p > 0.05$).



(a)



(b)

Fig. 5.6. Lorenz curves of the rotifer batch culture samples with the addition of mixed probiotics under two feed types (CS-3000 + MC + prob.: rotifer cultures received CS-3000 with the inoculation of MC and prob.; DGGE banding patterns data (legend numbers 2, 3, 8, 10, 12 for CS-3000 and 9, 5, 13, 1,16 for *N. oculata*) of Fig. 5.3 were used).

4. Discussion

The microbiota associated with rotifers is one of the major factors for the initial establishment of a MC in fish larvae during the first feeding stages (Dhert *et al.* 2001). Previously identified microbiota in rotifer mass cultures included: *Pseudomonas* spp., *Pseudoalteromonas* spp., *Vibrio* spp., *Moraxella* spp., *Flavobacterium* spp. and *Marinomonas* spp. (Skjermo and Vadstein 1993; Verdonck *et al.* 1994; Rombaut *et al.* 2001). The oral infection of larval fish through live feed has been proven (Grisez *et al.* 1996). Different control measures such as physical disinfection, antibiotics, as well as the addition of probiotics have been used (Hirata *et al.* 1998; Planas and Cunha 1999; Douillet 2000b; Verschuere *et al.* 2000). In a number of studies, probiotic strains such as *Lactobacillus* sp., terrestrial lactic acid bacteria, *Alteromonas* sp. or a probiotic mixture of 40 species have proven effective against pathogens and/or considerably enhanced the growth of rotifers and fish larvae (Gatesoupe 1991; Gatesoupe 1997; Douillet 2000b; Planas *et al.* 2004). Douillet (2000a) demonstrated a consistently enhanced rotifer growth rate with the addition of an *Alteromonas* strain and an unidentified Gram negative strain (B3) under all feeding regimes, in comparison with control cultures inoculated with microbial communities naturally present in seawater, or with cultures that were maintained bacteria-free. Tinh *et al.* (2006) developed a test system with gnotobiotically grown *Brachionus plicatilis* sensu strictu for the evaluation of the microbial functions and the nutritional value of

different feed types. The authors found that all of the tested MCs were able to increase the rotifer growth rate when yeasts were used as major feed source, whereas the MCs had no effect on rotifer performance when fed with *Chlorella*. In our study, a single addition of a mixture of 3 probionts (*Phenylobacterium* sp.; *Gluconobacter* sp. and *Paracoccus denitrificans*) had no significant effect on rotifer growth in batch culture irrespective of the feed type. On the other hand, rotifer growth rates were significantly higher when *N. oculata* was provided instead of a yeast based diet (CS-3000).

Despite considerable attention in recent years (Rombaut *et al.* 2001; Martínez-Díaz *et al.* 2003), the dynamics of bacterial communities over consecutive rotifer batch cultures are poorly understood. Many studies only mentioned effects on rotifer growth performance without MC information (Gatesoupe *et al.* 1991; Hagiwara *et al.* 1994). By conventional culture methods, Skjermo and Vadstein (1993) observed a shift in the bacterial composition from *Cytophaga/Flavobacterium* dominance to *Pseudomonas/Alcaligenes* dominance after enrichment of the rotifer cultures with squid meal. The microbiota of the rotifer cultures were presumably dominated by opportunistic species after enrichment. In natural aquatic systems, the composition of microbial communities and the abundance of the members of the MC are known to be influenced by resource availability and the food web, and bacterial populations differ in their response to shifts in resource availability (Fisher *et al.* 2000; Langenheder and Juergens 2001; Crump *et al.* 2003; Kent *et al.* 2006). Olsen *et al.* 2000 showed that the microalgae *Tetraselmis* sp. lowered the numbers of the associated microbiota in

Artemia, and a more diverse bacterial community developed. Our batch culture system constitutes a rather simple food web. The feed types CS-3000 and *N. oculata* could be considered as organic matter with different quality. In addition, the practice of rotifer batch cultivation characterized by high rotifer densities, rotifer population changes, debris, high loads of organic matter, water changes and rinsing may not be suitable for the establishment of stable microbial communities. In this system, microbial communities were associated to a certain extent with either *N. oculata* or CS-3000 (Fig. 5.3) when the rotifer growth rate was above 0.36 (Fig. 5.1). However, when the rotifer growth rate was lower such as in experiment 1 (batch 2), feed did not determine the type of 16S rRNA fragment in the DGGE pattern. Rather, the bacterial communities appeared in strong evolution, as MC's from the same batches tended to cluster together irrespective of the feed types. As a mixture of 3 different cryptic *B. plicatilis* species was used in our study, the question whether different cryptic *B. plicatilis* species have different associated microbial communities may not be neglected. In another study, we also found that the evolving microbial communities were different with different cryptic *Brachionus plicatilis* species cultures (data not shown).

In highly dynamic systems, questions related to the capability of the added probiont to persist in the MC (eventually depending on the feed type), are not yet answered completely. Also their effect on MC composition needs to be addressed. In our study, the 3 added probiont strains, which were isolated from a well-performing rotifer

culture, were found inconsistently in the MC fingerprint, although they could be detected up to batch 4 of some cultures. There was no clear association between either the feed type or the batch number and the presence of the 3 added probiotics. However, by group discriminant analysis, it was found that *Phenylobacterium* sp. and *Paracoccus* sp. contributed positively to the CS-3000 fed group, while *Gluconobacter* sp. contributed positively to the *N. oculata* fed group. Also the presence of one of the 3 probiotics (or a combination) in a sample did not determine the position of that sample in a cluster, indicating that the added probiotics were not able to steer the MC composition in a certain direction.

It is known that MCs consisting of the same bacterial species member can have large population size differences among the different species. Using Lorenz curve and Gini coefficient analysis, species distribution can be relatively quantified serving as indicator for the MC internal structure. In our study, we found no significant BSE differences between the yeast-based and microalgae-based fed rotifer cultures. Apparently, there were no clear correlations between rotifer growth performance and BSEs of MC in experiment 1. However in experiment 2, from batch 1 to batch 3, a relatively constant evenness (Gini coefficient value 0.49-0.53 for CS-3000; 0.55-0.58 for *N. oculata*) coincided with stable rotifer growth rates (Fig. 5.1). Although the added probiotics had apparently no effect on rotifer growth rates and they were not appearing in dominant positions in the MC, it is worth to identify their contributions to the total BSE in further studies.

Summarizing, the evolution of the MC composition as well as BSEs in rotifer batch culture, seems to be very complex. No clear-cut and strong correlation between MC composition and environmental variables could be detected, although feed type might be one of the strong drivers for creating diversity between rotifer microbial communities. The BSEs appeared independent of feed types, which suggest other factors may influence it.

Acknowledgements

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Chapter

6

Evaluation of pre/probiotics effects in turbot (*Psetta maxima* L.) larviculture by DGGE

Abstract

The effects of a commercial probiotic *Bacillus* mixture and a yeast cell wall mutant mnn9 were evaluated in flatfish larviculture. DGGE was used to monitor the bacterial community composition during larviculture, starting from fertilized eggs and through the initial larval stages of turbot (*Psetta maxima* L.). The results showed positive effects on larval survival and the diverse shifts of bacterial communities when probiotics and prebiotics were given. The agar-plating results also showed a considerable decrease of *Vibrio* loading. The analysis by Lorenz curve and Gini coefficient showed an increased bacterial species evenness when *Bacillus* mixture or wild type yeast was applied; On the contrary, a decreased evenness was observed when mutant yeast mnn9 was given.

Key words: probiotics, prebiotics, DGGE, turbot, bacterial species evenness

1. Introduction

Global flatfish production increased from 35,938 metric tons in 2002 to 135,782 tons in 2005 and China accounted for over 60% of this production (FAO 2007). However, the flatfish industry of China suffered greatly from an antibiotic residue crisis in 2006. It was estimated that at least 25,000 tons of turbot could not be sold and the economic losses due to this crisis are estimated to be several hundred million Euro (Lei 2007). The use of probiotics or beneficial bacteria, which control pathogens through a variety of mechanisms, is increasingly viewed as an alternative to antibiotic treatment (Balcázar *et al.* 2006a). Probiotics will probably help reducing the use of chemicals and drugs in aquaculture, and will thereby not only contribute to reducing the negative environmental impacts of aquaculture, but will also make aquaculture products more acceptable to consumers (Subasinghe 1997).

After more than 20 years of application, the use of probiotics in aquaculture is becoming well established as a biocontrol measure. The modes of action are being unravelled and *in vitro* screening protocols have been developed (Verschuere *et al.* 2000). Vine *et al.* (2006) summarized over 40 studies on intestinal probiotics used in fish and shellfish larviculture. In most of the studies, improved larval survival rates and enhanced growth have been described and for 30% of the probiotics that were considered, there was evidence of antagonistic effects on pathogens or opportunistic pathogenic bacteria. *Bacillus* spp, one of the earliest probiotics used in aquaculture,

display various interesting features of which antagonistic effects appeared to be most important (Gatesoupe 1991; Sugita *et al.* 1998; Rengpipat *et al.* 2000, 2003; Gullian *et al.* 2004; Vaseeharan and Ramasamy 2003; Kumar *et al.* 2006). *Bacillus* is a group of rod-shaped, Gram-positive bacteria, commonly found in diverse natural habitats and hosts, including fish and crustaceans (Gatesoupe 1999; Hong *et al.* 2005). Despite their wide application in agriculture (Nicholson 2002; Hong *et al.* 2005; Guo *et al.* 2006), a large scale screening of *Bacillus* spp for their use in aquaculture has rarely been conducted. Based on in vitro antagonistic effects and full-scale evaluations, several *Bacillus* strains were selected and commercial mixtures were developed for the use in shrimp and fish cultures (Balcázar *et al.* 2007).

With respect to the application of probiotics in aquatic environments, it was soon realized that a single addition of probiotics was not sufficient to obtain beneficial effects during prolonged periods. The proliferation in and colonization of the gastrointestinal tract of the animals by the probiotics seems to play an important role in displaying their beneficial effects. Thus, prebiotics, which are defined as non-digestible feed ingredients that benefit the host by stimulating growth and activity of health-promoting bacteria, has recently attracted more attention (Burr *et al.* 2005).

The ideal and most effective prebiotic would also be able to reduce or suppress the numbers and/or activities of known pathogens (Steer *et al.* 2000). Oligosaccharides that have been proposed as prebiotics for human gut microbiota include lactulose,

galacto-oligosaccharides, fructo-oligosaccharides, malto-oligosaccharides, xylo-oligosaccharides and soyabean oligosaccharides. β -glucans are a class of naturally occurring polysaccharides that have received particular interest as prebiotic substrates. An enhanced resistance to infections caused by the pathogens *Vibrio anguillarum*, *V. salmonicida*, *V. vulnificus*, *Yersinia. ruckeri* and *Edwardsiella. ictaluri* has been reported in shrimp, salmon and catfish when β -glucans from cell walls of *Saccharomyces cerevisiae* were used as prebiotic (Chen and Ainsworth 1992; Sung *et al.* 1994; Raa *et al.* 1996; Robertsen *et al.* 1999). Recently, the immunostimulatory nature of β -glucans and baker's yeast have been confirmed in gnotobiotic *Artemia* (Marques *et al.* 2006a; 2006b). The latter suggested that the increase in β -glucans and chitin in the cell wall, rather than an overall better nutritional value, of the yeast mnn9 strain is responsible for the protection against a virulent *Vibrio campbellii* strain and an opportunistic *Vibrio proteolyticus* strain.

The present study aimed to evaluate the potential probiotic effect of a *Bacillus* spp. mixture in the turbot yolk-sac larvae under starvation. In addition, DGGE was used to monitor the bacterial community composition associated with the cultures. The yeast cell wall mutant mnn9 was also tested as a prebiotic and its effect on the bacterial communities in the cultures was also evaluated.

2. Materials and methods

2.1. Activation of the *Bacillus* mixture

The *Bacillus* mixture (which was obtained under the form of spores) from INVE Technologies, Belgium, was activated by suspending the product in sterilized seawater with filtered aeration for 1 h at room temperature. The germinated *Bacillus* were counted microscopically and the appropriate amount was immediately applied to the cultures to have a final concentration of 5×10^6 cells mL⁻¹.

2.2. Preparation of the yeast strains

Two strains of axenic baker's yeast (*Saccharomyces cerevisiae*) were used for the experiment: the wild type (WT) strain (BY4741 [genotype, Mata his3Δ1 leu2Δ0 met15Δ0 ura3Δ0]) and its mnn9 isogenic mutant (BY4741 [genotype, Mata his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 YPL050c::kanMX4]), which has a null mutation resulting in a lower concentration of mannose linked to mannoproteins and higher concentrations of chitin and glucans in the cell wall (Aguilar-Uscanga and François 2003; Marques *et al.* 2004). Both strains were provided by the European *Saccharomyces cerevisiae* Archive for Functional Analysis, University of Frankfurt, Frankfurt, Germany. Yeast cultures were grown in sterile Erlenmeyer flasks with a cotton cap placed on a shaker in the dark (30 °C, 150 RPM). Both strains were cultured in a complete yeast extract-peptone-dextrose medium with the following composition: 1% yeast extract, 1%

bacteriological-grade peptone, 2% D-glucose (Sigma), 1% bacteriological-grade peptone (Sigma), and 2% D-glucose (Sigma). This medium was prepared in natural seawater (salinity 32 g L⁻¹) previously filtered (0.22- μ m filter) and sterilized by autoclaving at 120 °C for 20 min. The growth curve of each yeast strain was established by regularly measuring the absorbance at 600 nm with a spectrophotometer. Both strains were harvested by centrifugation (800 g for 10 min) in the stationary growth phase (after 3 days of culture, starting from a single colony). Cells were resuspended twice in sterile Falcon tubes with 20 mL of filtered and autoclaved seawater (FASW) (0.22 μ m). All handlings were performed in a laminar flow hood to maintain sterility. The harvested cells were applied to the cultures to have a final concentration of 5×10^5 cells mL⁻¹.

2.3. Lab scale experimental set-ups

The effect of the *Bacillus* mixture and yeast cell wall mutant *mnn9* on unfed turbot larvae was evaluated using a protocol similar to the one described by Bergh *et al.* (1992). Fertilized turbot (*Psetta maxima* L.) eggs were obtained from a commercial hatchery (Mingbo hatchery, Laizhou, China). The eggs were rinsed 3 times with filtered seawater and transferred to 6-well dishes. The *Bacillus* or the yeast cells were added once to the wells. Five eggs were transferred to each well containing 5 mL sterile seawater. One dish was used as control in which only sterilized seawater was added. The dishes were kept in the dark; dead eggs or larvae were removed from the dishes daily, and mortality was recorded. The survival of the turbot larvae was marked

daily.

2.4. Microbiological analyses

Samples were taken from the culture water at the start and at the end of the experiments to determine the number of colony-forming units (CFU). Tenfold dilutions in sterilized seawater were plated on marine agar and TCBS agar (Difco, Detroit, MI, USA).

One mL of pooled culture water with rotifers was collected in sterile eppendorf 1.5-mL tubes at the experiment start and at the end of each batch culture. Samples were centrifuged at 5000 g for 5 min and stored at -20 °C. DNA extraction, PCR-DGGE and DGGE banding pattern analysis using indexes for bacterial species evenness on the basis of Lorenz curve and Gini coefficient were performed using the same methods as described in previous chapters.

3. Results

During the first experiment in which turbot larvae were starved, the hatching percentage was high in all treatments (96%), probably reflecting the high quality of the eggs. The addition of bacteria and yeast in the wells had no influence on the hatching rate. Figure 6.1 shows the survival of the turbot larvae on each day. High mortalities occurred from day 3 onwards after mouth opening. Larvae treated with the yeast strain mnn9 or the *Bacillus* mixture showed a higher survival than that in the

control treatment in the treatment with wild type yeast. However, the differences were not significant ($P > 0.05$) due to a high variability.

Figure 6.2 shows the plate counts on marine agar and TCBS agar at the end of the experiment. The lowest number of bacteria on TCBS agar (10^3 CFU mL⁻¹) was found in the presence of the *Bacillus* mixture which was significant lower ($p < 0.05$) than in the other treatments. In addition, the addition of wild type yeast significantly reduced the TCBS count. However, there was no significant difference between the two types of yeasts ($p > 0.05$).

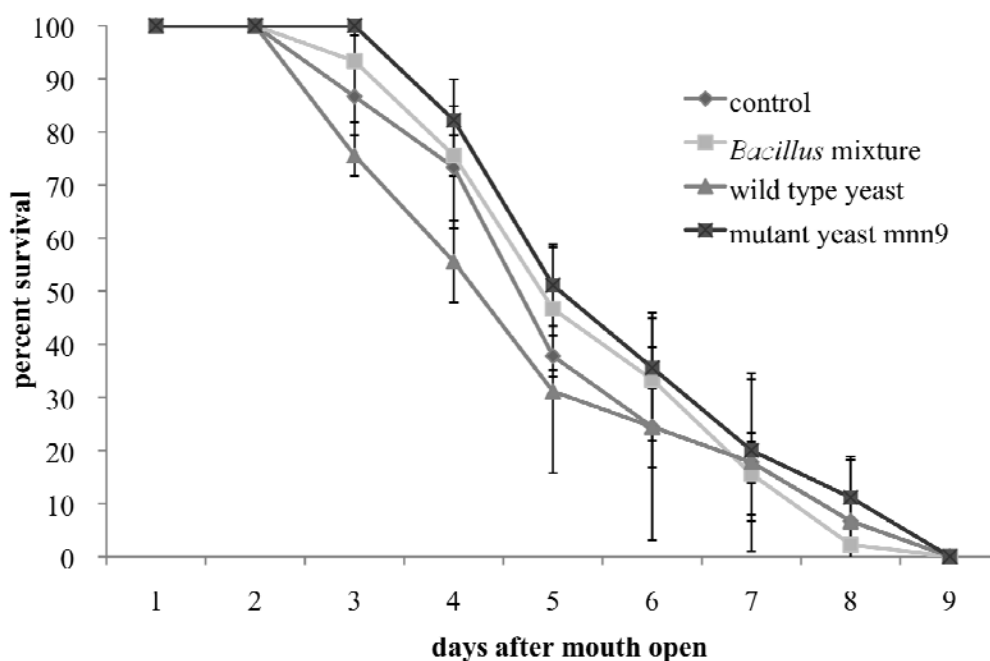


Fig. 6.1. Survival of starving turbot larvae in the presence of *Bacillus* and two types of yeast ($n = 6$ for control; $n = 3$ for other treatments; error bars = SD).

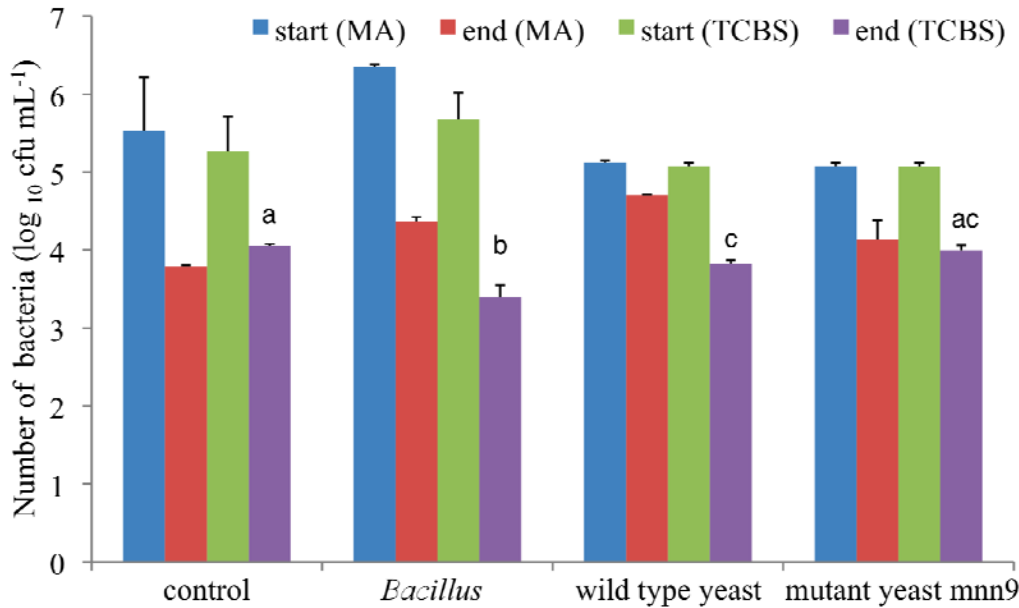


Fig. 6.2. Agar plating results of larviculture water containing starving turbot at the start and the end of the experiment (MA: marine agar, TCBS: Thiosulfate Citrate Bile Sucrose agar; a, b and c are significant different from each other at $P < 0.05$; error bars = standard deviation, $n=6$ for control; $n = 3$ for other treatments).

To analyze the bacterial community of the culture water in the presence of the *Bacillus* mixture or the yeasts, pooled samples collected at the beginning and end of the experiment were subjected to PCR-DGGE analysis. The analysis yielded highly distinguishable DGGE profiles for the different treatments (Fig. 6.3). Cluster analysis showed two clusters with a similarity of 23%. A clear separation of the control from the other treatment groups and low similarity of DGGE banding patterns between the start and the end of experiments of treatment groups were found. DGGE profiles showed that there was a similarity of 62% of the bacterial community between the two controls. There was a 56% similarity between profiles of the cultures treated with the two yeast types at the end of the experiment.

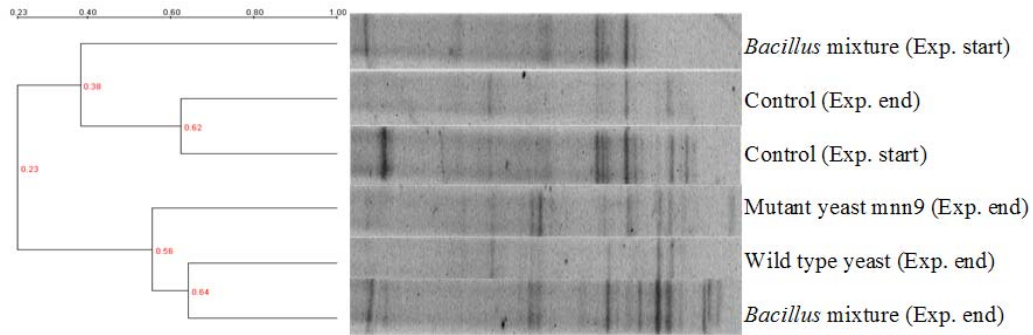


Fig. 6.3. DGGE profiles and clustering analysis of the bacterial community associated with the starving turbot cultures in the presence of a *Bacillus* mixture and two types of yeast (Exp. = experiment; Experiment starts of wild type yeast and mnn9 yeast were considered the same as control start). Clustering of the patterns (using the Ward method based on Dice coefficients) is shown on the left (Scale The scale 0.23 – 1.00 shows similarity levels ranging from 23% to 100%).

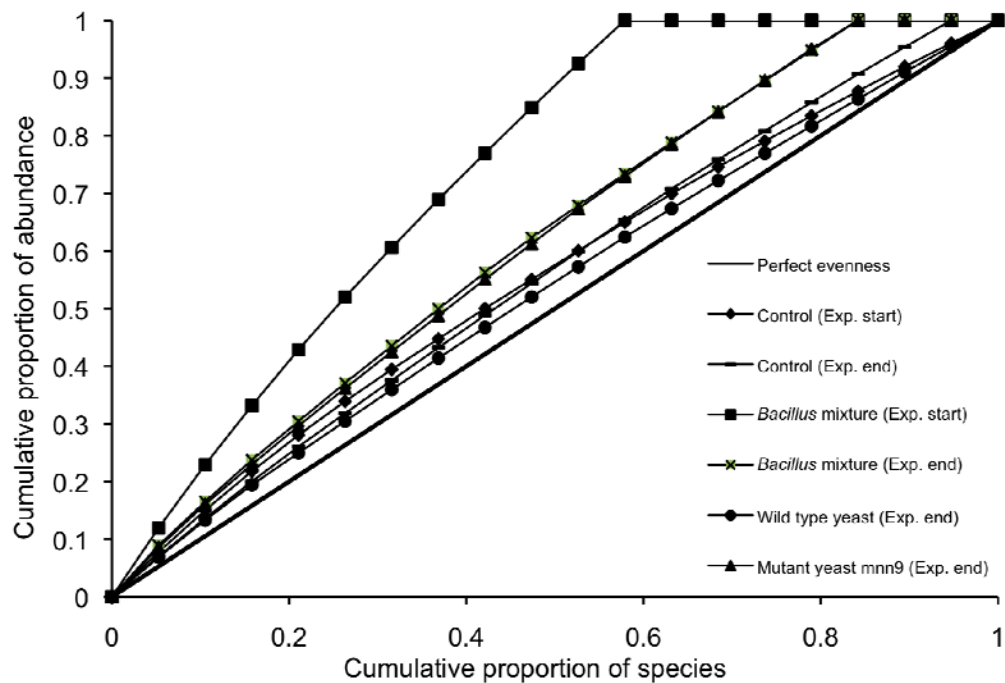


Fig. 6.4. Lorenz curves of the samples from the start and end of the experiment based on DGGE banding pattern.

Lorenz curves of the samples from the control group showed that there is very little difference present between the start and end of the experiment (Fig. 6.4: Control). An increasing evenness at the end of experiment was found for the treatments *Bacillus* mixture and wild type yeast.

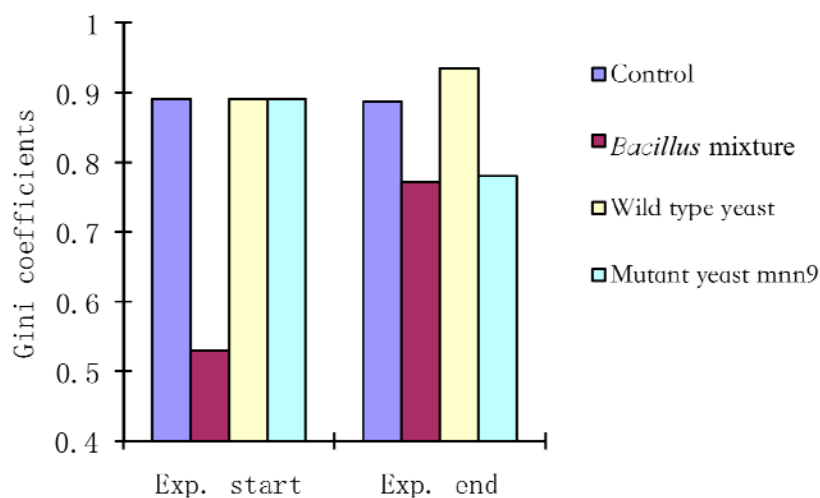


Fig. 6.5. Gini coefficient of the samples taken at the start of the experiment (Exp. start) and end of the experiment (Exp. end), based on the calculation from DGGE banding pattern.

Fig. 6.5. showed clear differences of GC between the sampling points (Exp. start and Exp. end). A large increase of GC value (from 0.53 to 0.77) was found in the *Bacillus* mixture treatment. While a decrease of GC value from 0.89 to 0.78 was observed when mutant yeast mnn9 was given. For wild type yeast, the GC value increased from 0.89 to 0.93. Nearly no difference was found for control group (GC 0.89 to 0.88).

4. Discussion

The *Bacillus* mixture used in this study has been evaluated before as a candidate probiotic mixture, mainly for shrimp but also in fish larviculture. In those experiments, increased growth rates of shrimp and fish larvae and reduced *Vibrio* levels were obtained (Balcázar *et al.* 2006b; 2007). *Bacillus subtilis* and *B. licheniformis* were found to increase resistance to pathogenic *Yersinia ruckeri* in rainbow trout (Raida *et al.* 2003). In gilthead seabream, an improved cellular innate immune response was found when *B. subtilis* and *Lactobacillus delbrueckii* were given (Salinas *et al.* 2005). Recently, newly isolated strains of *Bacillus subtilis*, *Bacillus cereus* and *Bacillus licheniformis* were selected and evaluated as potential biological agents for the enhancement of the water quality in cultures of ornamental fish, and it was found that the selected isolates reduced together synergistically the level of pathogens and the concentrations of waste ions *in vitro* and *in vivo* and therefore, the strains were considered to be safe for use in ornamental aquaculture (Laloo *et al.* 2007). Our *in vivo* evaluations have proven that the *Bacillus* mixture has no deleterious effects on turbot. Furthermore, our experiments also confirmed the positive effects of the *Bacillus* mixture in terms of survival rate and reduction of *Vibrio* numbers, although the differences were not always significant.

Yeasts, which are traditionally used as feed additives, have been promoted and used as probiotics and prebiotics in aquaculture (Tovar *et al.* 2002; Li and Gatlin III 2005).

Currently, more specific yeast products that are derived from the cell wall of a specific yeast strain (*Saccharomyces cerevisiae*1026), are developed and intended for aquacultural on-farm use, either in farm-made feeds or as a top-coat on commercial feeds (<http://www.alltech.com/About/aquaproducts.cfm>). The yeast mutant mnn9 used in this study has been shown to have a positive effect (improved growth and protection against pathogen vibrios) on both rotifers and *Artemia*, under both gnotobiotic and open culture conditions (Tinh *et al.* 2006; Marques 2006a, b; Soltanian *et al.* 2007a, b). However, it was not known so far whether they have a beneficial or deleterious effect on fish larvae. Our study on mnn9 showed a slight positive effect on the survival of turbot larvae, however the reduction on vibrios was not significant.

With the aid of molecular tools, studies on intestinal microbiota of fish revealed a great diversity and variation between animals of different ages and differences between different individuals of the same age (Ringo *et al.* 2003; Romero *et al.* 2006; Fjellheim *et al.* 2007; Hovda *et al.* 2007). Although the factors influencing the microbial diversity and variations are not fully described, several studies showed that these variations could be related to the specific fish diets, different feeding regimes or exogenous live feed (Ringø *et al.* 2006; Martin-Antonio *et al.* 2007; Brunvold *et al.* 2007). These variations indicate that there might be some space for probiotics aiming at establishing a more stable and mature intestinal microbiota. Our study showed strong evolution in the bacterial community of turbot yolk-sac larvae in sterile

seawater in the absence of any external input (Fig. 6.3, control).

By adding a *Bacillus* mixture, larger changes in the bacterial communities between the initial and final experiment were observed. This implied that, by the end of the experiment, the *Bacillus* mixture probably was not able to maintain their initial composition. The subtle difference of cell wall composition of the two types of yeast strain, resulted in a nearly 40% difference in bacterial community similarity at the end of the experiment. The Lorenz curve and Gini coefficient analyses revealed that the different treatments resulted in the different internal species structures of the MCs. The two types of yeast strains induced divergent change in the distribution of bacterial species. These results indicate changes in the composition and the evenness in a MC can be directed by the inputs of probiotics or different yeast strains. These changes might have an impact on the development of the intestinal microbiota of the fish larvae.

With respect to the application of probiotics in aquaculture, farmers often consider the probiotic as a replacement of chemicals and antibiotics, and as a result misused them as drugs and expected to see instant effects on the fish (Moriarty 2003). Although they possess several different of modes of action against pathogenic bacteria, probiotics should not be used as therapeutic agents. In conclusion, a *Bacillus* mixture and yeast strain mnn9 were evaluated as putative pro- and prebiotics and showed some effects on starving turbot yolk-sac larvae. Moreover, bacterial communities can be influenced

in terms of species evenness (as analysed by Gini coefficient) and diversity (Dice coefficient) through the addition of *Bacillus* of yeast strains. Finally, the agar plating confirmed the possibility of a *Bacillus* mixture to reduce the numbers of *Vibrio* in turbot.

Chapter

7

Discussion, conclusion and future perspectives

Chapter 7

Discussion, conclusion and future perspectives

In this chapter, the importance of characterizing microbial communities in larviculture, the probionts as shaping forces of microbial community composition as well as the possible relationships between microbial community composition and health of larvae are discussed in the light of the obtained results. Also, the statistical strategy of analysing microbial community fingerprinting data is evaluated. Finally, further research perspectives dealing with probiotics in complex microbial community in larviculture are discussed.

1. Monitoring of microbes in live feed and larviculture

Microorganisms play an important role in larviculture as they can either negatively interact causing disease or positively interact with the host. The goal of present study is to understand the role and dynamics of probiotics in the microbial community composition in the larviculture. Before adding exogenous microbial agents, the characteristics of the microbial community at different culture stages (such as the introduction of live feed, the changes of diets and the eggs incubation) need to be understood. In the present study, we analyzed the microbial communities in consecutive rotifer batch cultures and we found a correlation between the rotifer growth rate and the microbial community composition (chapter 4, Fig. 4.1 and 4.4). In

addition, we characterized the bacterial community associated with the early life stages of turbot. A “plant-specific” microbial community appeared to be present in turbot larval culture (chapter 3, Fig. 3.1).

1.1. Limiting or diverting microbial communities in larviculture?

It is known that the specific bacterial species cohabiting with microalgae and rotifers are not always beneficial to fish larvae which feed on algae and rotifers (Maeda *et al.* 1997). Similarly, *Artemia*-fed halibut larvae and juveniles were found to be dominated by feed-associated *Vibrio alginolyticus* and *V. splendidus*, switching to a different microbial community after weaning (Verner-Jeffreys *et al.* 2003). In a study of Verner-Jeffreys *et al.* (2006), egg-associated microbiota of Pacific threadfin, *Polydactylus sexfilis* and amberjack, *Seriola rivoliana*, were characterized. The results showed that egg-associated transfer of bacterial pathogens could be significant in Pacific threadfin culture, illustrating the importance of treating eggs. At early stages of intensively reared cod, feeding contributed to the introduction of bacterial communities (Brunvold *et al.* 2007). This study uncovered significant food and environmentally induced changes in the microbial community compositions in larviculture. Hence some authors focused on limiting microbial inputs, for example, a 90% reduction of total heterotrophic bacteria and *Vibrionaceae* were obtained in rotifers exposed for 15 min to 40 mg L⁻¹ of a hydrogen peroxide-based product. In the case of *Artemia* nauplii, 95% reduction of heterotrophic bacteria were obtained after 5 min exposure to 8000 mg L⁻¹ with an associated 83% reduction of *Vibrionaceae*

(Giménez *et al.* 2006). It is now believed that sterilities cannot eliminate all microbes. Nutrients and dissolved organics create a favorable condition for the growth of diverse bacteria, fungi, protozoa and micrometazoa (Sharrer and Summerfelt 2007). The bacterial concentrations in cod incubators can increase a thousand-fold during hatching (Hansen and Olafsen 1999). Therefore the establishment of a microbial community with known un-harmful properties would be desired. Martínez-Díaz *et al.* (2003) reported that it was possible to replace the microbial community in rotifer cultures, started from disinfected amictic eggs, with selected bacterial strains. However, in larviculture, due to the complex nature of microbial community, reports of a consistent persistence of added bacterial strains are still rare. In our study of rotifer batch culture, we inoculated the mixed microbial culture at the start of batch culture. The introduced MCs were examined by a molecular tool DGGE; The results showed that the inoculum was still dominant in the ‘Nevada’ strain culture after the first batch (chapter 4, Fig. 4.2).

1.2. Using molecular tools to monitor microbial communities in larviculture

Molecular tools have allowed to characterize of the microbial community of the larviculture without the need to first culture the organisms of interest. PCR based analysis of the 16S ribosomal RNA genes can address some aspects such as population abundances and species discrimination (Crosby 2005). The “fingerprint” method of PCR-DGGE is known to amplify and display the predominant bacteria in samples, and allows for the detection the predominant bacterial components present in

a sample (Hovda 2007). Brunvold *et al.* (2007) concluded that DGGE is a suitable method for characterizing bacterial communities in hatcheries. The changes in the number of bands and banding positions indicate that a new bacterial community was established between the samples taken on days 5 and 13 in a hatchery. In our study (chapter 3), the DGGE profiles revealed bacterial community changes in the turbot larviculture water before and after first feeding and it was found that the microbial communities present in samples from systems with diseased animals were more complex and more evenly distributed than samples coming from healthy systems (chapter 3, Fig. 3.1). PCR-DGGE can also be used for the discrimination of virus, fungi and protozoa or analysis a specific function by targeting different genes. DGGE can generate results rapidly (in 2 days), acquiring the microbial profiles of a complex unknown samples. Using fluorescence *in situ* hybridisation (FISH), the sample can be analysed directly for a specific group or species in few hours without PCR procedure, however no total community diversity information is generated in this way (Dahllof 2002). By continuously monitoring the microbial community, the linkage between MC changes and larviculture performance can be defined, and the interesting microbes can be selected for further study. For example, in analysing the MCs associated with different cultures of rotifer strains, we found dominant DGGE bands (correspond to bacterial species) that are specific for the communities associated with *B. plicatilis* Nevada and *sensu strictu*, but they were not present in the patterns of the inoculum (chapter 4, Fig. 4.2).

2. Parameters governing microbial community composition in larviculture

2.1. The influence of nutritional input to microbial community composition

It is known that physical (salinity, temperature, pH etc.) and biological parameters (competition, mutualistic interaction, predation etc.) govern the composition of aquatic microbial communities (Nold and Zwart 1998). In a rotifer batch culture, the net energy inputs through feeding may not only encourage rapid growth of rotifer but also of the microorganisms. Among many biotic and abiotic factors, organic matter in an ecosystem is frequently the limiting factor for growth of heterotrophic microorganisms. Environmental transitions (such as regular nutrient pulses) can cause changes in bacterial community structure and these changing conditions will govern the fate of the microbial community behavior and processes (Van Hannen *et al.* 1999; Carrero-Colón *et al.* 2006). Evidences from laboratory and field studies show also that nutrient addition can influence bacterial diversity, and the relationship between energy inputs and diversity differed for different taxonomic groups of bacteria (Horner-Devine *et al.* 2003). It is also known that specific organic substrates often favor the growth of particular populations with specific catabolic activities (Atlas and Bartha 1987). Specific carbon, nitrogen, phosphorus, some amino acids sources and their respective ratios can result in strong alternating competition between different groups of microorganisms (Hargreaves 2006; Veuger *et al.* 2007). Larviculture systems are

typically subjected to periodic defined nutrient addition; therefore, the further study of the effects of periodic exogenous feeding on the physiological functionality of individual microorganisms and the dynamics of microbial community composition are required.

2.2. Predation as a selective force to shape the microbial community composition

Predation has to be considered a major selective force strongly influences microbial community composition (Jürgens and Matz 2002). In a study on three eutrophic *Daphnia*-dominated ponds using bacterial group-specific oligonucleotide probes, the authors demonstrated that predation is a major structuring force for planktonic bacterial communities (Langenheder and Jurgens 2001). The changes in predation regime probably had a much stronger impact on the structure of the bacterial community than on bacterial abundance and biomass. In another study, bacterial community composition was monitored in four shallow eutrophic lakes during one year using DGGE of PCR-amplified prokaryotic rRNA genes. The authors found no evidence for top-down (grazers) regulation of bacterial community composition in the turbid lakes, while grazing by ciliates and daphnids (*Daphnia* and *Ceriodaphnia*) was significantly related to changes in the bacterial community in the clear water lakes (Muylaert *et al.* 2002). Although field experiments have indicated that rotifer grazing should generally play a minor role in bacteria consumption compared to feeding by coexisting protozoans, laboratory experiments have revealed that rotifers may

structure the microbial web and filter-feeding rotifers brachionids seem to be significant feeders on the bacteria, flagellates and small ciliates (Arndt 1993). *Brachionus* can directly ingest high quantity of bacteria, and they may ingest large particles of detritus, on which bacteria may be present as aggregates. Consistent positive correlation between the density of *B. calyciflorus* and the bacteria was found in a tropical reservoir (Bonecker and Aoyagui 2005). In our study (Chapter 4), large changes in rotifer growth rate were found to be associated with large changes in the microbial community composition, suggesting that there might be a causal link. As a mixture of three different rotifer *B. plicatilis* biotype strains was used in some experiments, different rotifer species competed each other, resulting in rotifers composition changes under specific feed types. For example, rotifer strain *B. plicatilis sensu strictu* dominated the rotifer populations by the end of batch culture cycle 4 when CS-3000 alone used as feed (unpublished results, EU research project: ROTIGEN, Q5RS-2002-01302). As our studies found a correlation between different rotifer *B. plicatilis* strains and associated MC compositions, it would be worthwhile to evaluate to what degree rotifer can divert MC compositions under specific feed regime.

In recent decade, viruses are recognized as abundant and dynamic members of marine microbial communities (Wommack and Colwell 2000). They may selectively kill the most abundant members of the prokaryotic community and virus-mediated genetic exchange may involve in shaping prokaryotic community structure (Winter *et al.*

2004). However, in the field of aquaculture, the viral community is rarely monitored, resulting in a lack of a direct demonstrations of the relationship between viral and microbial community.

3. Effect of exogenous bacteria for the shaping of microbial community

It can be assumed that a large input of allochthonous bacteria may change the composition of microbial communities in targeted site. The influence of inlet bacteria on the assemblage composition of bacterioplankton was investigated in two Swedish forest lakes of different hydraulic retention time. In the lake with short retention time, the lake bacterioplankton assemblage largely resembled the riverine assemblages (Lindstroem and Bergstroem 2004). However, a number of biotic and abiotic factors possible limited the survival of allochthonous bacteria in aquatic environments (Barcina *et al.* 1997). In a study of bacterial community in a great scallop (*Pecten maximus*) hatchery, DGGE banding pattern indicated a change in the bacterial composition even as the water has passed the pipes (Sandaa *et al.* 2003). Community hybridization of amplified 16S ribosomal RNA demonstrated that the bacterial biota of the chicken gastrointestinal tract changed significantly in response to the probiotic treatments and shifted differently when genetically modified and unmodified probiotics were introduced (Netherwood *et al.* 1999). In our study (chapter 5), the addition of a mixture of 3 probionts (*Phenylobacterium* sp.; *Gluconobacter* sp. and *Paracoccus denitrificans*), which were isolated from a well-performing rotifer culture,

were found inconsistently in the MC fingerprint. Also the presence of one of the 3 probionts (or a combination) in a sample did not determine the position of that sample in a DGGE banding pattern cluster, indicating that the added probionts were not able to steer the MC composition in a certain direction (chapter 5, Fig. 5.3). In aquaculture, microbial probiotics have been reported to have many beneficial effects. However, it is unclear whether these beneficial effects are directly or indirectly due to the interaction with host microbial community. At present, advanced molecular methods allow detecting specific bacterial species and groups, and studying the impact of probiotics on microbial community structure in the targeted sites.

4. Links between microbial community composition, function and health of larvae

The existence of stable indigenous microbiota in fish is accepted, but the bacterial composition may change with age, nutritional status and environmental conditions (Ringø *et al.* 2003). To the species Atlantic halibut *Hippoglossus hippoglossus* L., the bacterial community of yolk sac larvae from different production units could be highly variable (Verner-Jeffreys *et al.* 2003) or adapted to be relatively similar with a distinct and specific community (Jensen *et al.* 2004; Bjornsdottir *et al.* 2008). In our study (chapter 6, Fig.6.3), the results showed the strong evolution in the bacterial community of unfed turbot yolk-sac larvae in sterile seawater, and this evolution could be largely diverted by addition of the *Bacillus* mixture. The study of

Bjornsdottir (*et al.* 2008) found that *Marinomonas* spp. were only present in the successful first feeding larvae and concluded that individual bacterial groups may affect the overall success of halibut larvae in intensive farming. The intestinal microbiota in Senegalese sole (*Solea senegalensis*) showed also the significant differences between intestinal bacterial composition in soles fed commercial diets and natural preys (Martin-Antonio *et al.* 2007). The cold temperatures brought about a change in the percentages of *Vibrio* species and a higher representation of α -*proteobacteria* in both extensive and semi-extensive systems. An effective management strategy in the production of marine fish larvae may rely on promoting of the MC stability of the feed chain.

There is much evidence that microbial community have important and specific metabolic, trophic, and protective functions to the hosts (Guarner and Malagelada 2003). Bacteria from surrounding water can enter digestive tract at yolk sac stage of larvae through drinking (Olafsen 2001). With the development of fish larval digestive system, niche heterogeneity may determine the structure and spatial organization of the microbial community in the larval gut. Therefore bacterial community structure could be different between the larvae and culture water, and vary from fish to fish (Sandaa *et al.* 2003; Huber *et al.* 2004). Maintenance of a diverse microbial community that includes innocuous and beneficial bacteria may be the key to a successful culture environment in an aquatic hatchery (Schulze *et al.* 2006). Using microbial matured water, dominated by non-opportunistic bacteria, it is possible to

prevent the fast colonization by opportunistic bacteria (Skjermo and Vadstein 1999). Potential pathogens like some vibrios, which adapt to a wide range of aquatic environments, may flourish quickly in a hatchery system, leaving little space for competitors to act. Our results in chapter 6 show a group of *Bacillus* mixture is able to inhibit vibrios in fish larviculture. It is also worthwhile to further investigate whether subtle changes (different yeast cell wall compositions) in the substrate can favour probiotics, allowing them to compete with a pathogen such as certain vibrios. Using prebiotics will be a good approach to support probiotics and modulate microbial community in larviculture.

5. Approaches to assess microbial communities

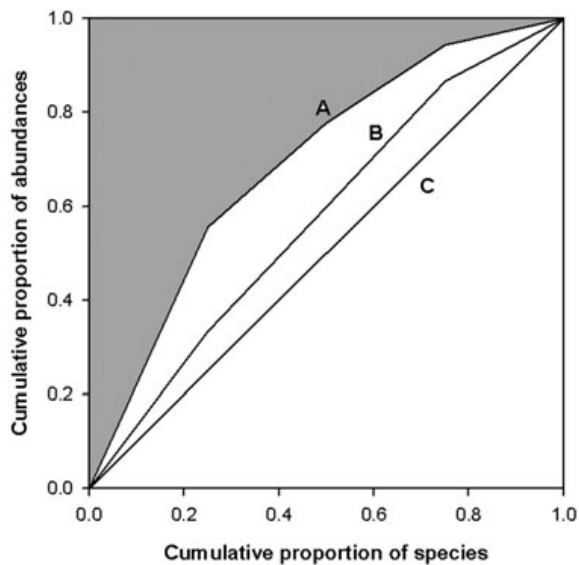
Larviculture is a complex and dynamic environment that varies temporally, spatially, and different aquacultural practices are also likely to influence the microbial community. Most molecular analysis of microbial communities involves the retrieval of 16S rRNA genes either by cloning and sequencing or by fingerprinting the PCR products (McCaig *et al.* 2001). PCR-DGGE has been successfully used in many fields of microbial ecology to assess the diversity of microbial communities and to determine the community dynamic in response to environmental variations. The presence and relative intensities of individual bands in DGGE gels can be compared and various diversity indices and cluster analysis can be calculated.

$$H = - \sum_{i=1}^N p_i * \ln p_i; E_N = H/H_{\max} = H/\ln S$$

The Shannon diversity index (H) is the index that is most commonly used to characterize species diversity in a community. The proportion of individuals found in the i^{th} species relative to the total number of individuals (p_i) is calculated, and then multiplied by the natural logarithm of this proportion ($\ln p_i$). The resulting product is summed across species, and multiplied by -1. To measure the species evenness, Shannon's equitability (E) is often used. Shannon's equitability can be calculated by dividing H by H_{\max} ($H_{\max} = \ln S$, S = total number of species in a sample). Equitability assumes a value between 0 and 1 with 1 being complete evenness. Shannon's index combined with Shannon's equitability can account for both abundance and evenness of the species present.

Frequently, a more complete picture of the distribution of species can be formed by plotting proportional abundance (usually on a logarithmic scale) against rank abundance (Stiling 2001). New alternative diversity analysis, based on the Lorenz curve (1905), was applied to PCR-DGGE banding patterns analysis (Mertens *et al.* 2005). In the Lorenz curve, species are often ranked by size, and the cumulative proportion of species (x-axis) is plotted against the corresponding cumulative proportion of their total abundances on the y-axis. For Lorenz curve applied to DGGE data the abscissas are the cumulative proportions of bands and the ordinates the cumulative proportions of the peak heights of the densitometric curves. If all species

are the same size, the Lorenz curve is a straight diagonal line, called the line of equality (Fig. 7.1 curve C). If there is any inequality in size, then the Lorenz curve is above the line of equality. The total amount of inequality can be summarized by the Gini coefficient, which is the ratio between the area enclosed by the line of equality and the Lorenz curve, and the total triangular area above the line of equality.



species	abundance		
	curve A	curve B	curve C
1	10	10	2
2	4	8	2
3	3	8	2
4	1	4	2

Fig. 7.1. Lorenz curves of different populations A, B and C. The number of species and abundances of the populations is indicated in the table. For curve A the Gini coefficient is twice the shaded area (from Mertens *et al.* 2005).

Diversity analyses based on Lorenz curve and Gini coefficient was applied to assess a number of microbial communities. This approach was applied to the analysis of substrate utilization data obtained from using BIOLOG microtiter plates (Harch *et al.* 1997; Pankhurst *et al.* 2001; Preston-Mafham *et al.* 2002; Pankhurst *et al.* 2005).

When PCR-DGGE fingerprinting data were used, diversity analysis based on Lorenz curve showed that the type I methanotrophic community was less evenly distributed in historically HCH-polluted soils compared with less polluted reference soils (Mertens *et al.* 2005). The authors also pointed out the drawbacks of the commonly used Shannon and Simpson diversity indices such as un-standardized and intuitive way of combining species richness and evenness. In another study, the Gini coefficient was adapted, represented by twice the area above the curve, was used as a diversity measure to describe methanotrophic community (Halet *et al.* 2006). It was seen that the diversity of the methanotrophic community was low during the thermophilic phase and increased during the final maturation phase. In a recent study (Wittebolle *et al.* 2008), the Lorenz curves were also evaluated based on the Pareto principle (a 20% of a country's population possesses 80% of its wealth) (Pareto 1897). According to this principle, the cumulative y axis value (the proportion of intensities) corresponding with the 20% level on the x axis (the cumulative proportion of species) is evaluated. During the quantifying community dynamics of nitrifiers in functionally stable reactors, based on the Pareto-Lorenz curves, it was observed that only a small group of ammonia-oxidizing bacterial species played a numerically dominant role in the nitrification of both reactors.

For PCR-DGGE fingerprinting analysis of MC in our studies, the number of bands in the DGGE gel indicated species richness. Due to the detection limit of PCR-DGGE method (i.e. only dominant species are detectable in an environmental sample),

species richness was determined by the samples that had the maximum number of bands. For other samples which had the less number of bands, more species were added until the equal species richness was reached. The added species abundance was assigned a value as '0'. In this way, the relative rare species are been included and it has a significant impact on MC interspecies structure analysis (table 7.1). Using the DGGE data in chapter 5, it shows that the addition of rare species dramatically changes the Gini coefficient values (from 0.915 to 0.530 in group *Bacillus* mixture Exp. start), but to a less extends on the values of Shannon evenness (from 0.915 to 0.811 in group *Bacillus* mixture Exp. start). It is known that Shannon evenness and Gini coefficient index are invariant with respect to sample size (proportion does not change), but indeed rare species add to them. Although small amounts of data contamination in the upper tail of the data distribution (dominant species) can reverse unambiguous conclusions in Lorenz curve analysis (Cowell and Victoria-Feser 2007), it is remain questionable whether Gini coefficient index is over estimating the differences between samples when more rare species are added. Nevertheless, Gini coefficient index appears more sensitive than Shannon evenness index no matter whether the rare species are included (on the basis of the equalized number of bands) or excluded (on the basis of the actual bands present) Table 7.1 shows the Gini coefficient ranged from 0.891 to 0.935 when the actual number of bands present was used, while the Shannon Evenness indices shows little difference among each other (0.994-0.998). When the equalized number was used, the difference between the Gini

coefficients is even larger ($0.935 - 0.530 = 0.405$) than the difference between the Shannon Evenness ($0.998 - 0.811 = 0.187$).

Table 7.1. Shannon evenness, Gini coefficient and Lorenz asymmetry coefficient of the microbial community in starving turbot larviculture system on the basis of PCR-DGGE data.

Indices	Control (Exp. start)	Control (Exp. end)	<i>Bacillus</i> mixture (Exp. start)	<i>Bacillus</i> mixture (Exp. end)	Wild type yeast (Exp. end)	Mutant yeast mnn9 (Exp. end)
Shannon Evenness (bands present*)	0.994	0.998	0.995	0.996	0.998	0.997
Shannon Evenness (equalized number*)	0.994	0.979	0.811	0.938	0.998	0.939
Gini coefficient (bands present)	0.891	0.937	0.915	0.916	0.935	0.927
Gini coefficient (equalized number)	0.891	0.888	0.530	0.772	0.935	0.781
Lorenz asymmetry coefficient (bands present)	1.172	0.977	1.193	1.035	1.106	1.049
Lorenz asymmetry coefficient (equalized number)	1.172	0.725	0.421	0.384	1.106	0.327

*: ‘Bands present’ represents that the calculation of species richness was on the basis of actual number of bands; ‘Equalized number’ represents that the calculation of species richness was on the basis of maximum number of bands in one DGGE lane.

The Gini coefficient has been showed to be an effective measure of inequality in our studies. However, Gini coefficient does not contain all the information in the Lorenz

curve and it has been pointed out that different Lorenz curves can have the same Gini coefficient (Shumway and Koede 1995). Damgaard and Weiner (2000) proposed a second and complementary statistic, the Lorenz asymmetry coefficient (LAC), which characterized an important aspect of the shape of a Lorenz curve. Statistics tells us which size classes contribute most to the population's total inequality. A simplified version of this can be demonstrated by example populations consisting of only three sizes. Populations a, b, and c in Fig. 7.2 have the same mean (10), the same Gini coefficient (0.6), but different Lorenz curves (Fig. 7.3). A Lorenz curve is called symmetric if the curve is parallel with the line of equality at the axis of symmetry with LAC equals to 1. If $LAC > 1$, then the point where the Lorenz curve is parallel with the line of equality is below the axis of symmetry. Correspondingly, if $LAC < 1$, then the point where the Lorenz curve is parallel with the line of equality is above the axis of symmetry (Fig. 7.3). Considering the original data distributions, in case of Fig. 7.2, most of the inequality within the population is due to the very few largest individuals, which contain a very large percentage of the population's biomass. In case b, the same overall degree of inequality is due primarily to the relatively large number of very small individuals, which are contributing very little to the population's total biomass. Interestingly, the Lorenz curve is always symmetric when the data are lognormal distributed (Damgaard and Weiner 2000). Obviously, adding rare species showed a large reduction of LAC (from 1.193 to 0.421 in group *Bacillus* mixture Exp. start), therefore LAC should be used with great caution when rare

species are counted. In most of our studies, LAC analysis would result that the less dominate species are the major contributors to the total species distributions if rare species were added.

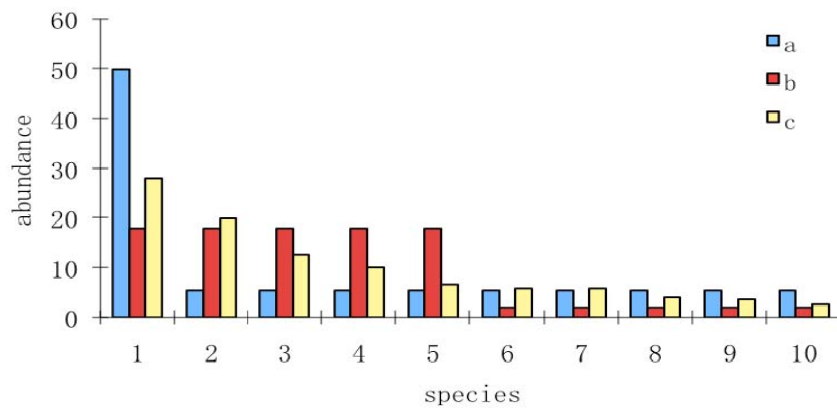


Fig. 7.2 Three simple populations with the same mean (10), Gini coefficient (0.6), and sample size (10), but with different Lorenz asymmetry coefficients: (a) 1.46; (b) 0.74; (c) 0.995 (data a and b from Damgaard and Weiner 2000).

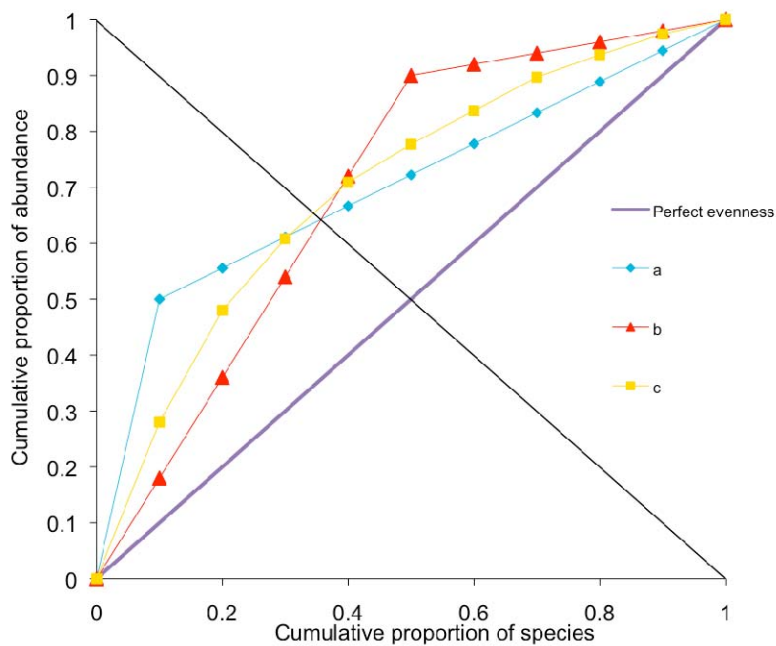


Fig. 7.3 Three Lorenz curves: a symmetric case (c), and two asymmetric cases (a and b) (black line is axis of symmetry).

Lorenz curve and Gini coefficient analyses are generally regarded as golden standard in the field economics. They incorporate comprehensive individual level data and allow direct comparison between units with different size populations (Coulter 1989). However, in microbial community studies, changes in microbial community internal structures may not necessarily lead to altered diversities and distribution, because changes of some taxonomic groups may be compensated by changes of others. Furthermore, Lorenz curve and Gini coefficient analyses treated all bacterial individual equally without considering the activity differences within or inter populations. For a soil bacterial communities study, it showed standard anonymous diversity indices such as Shannon, Chao1 (estimates richness by adding a correction factor to the observed number of species), and rarefaction (estimates the decrease in apparent species richness of a community with decreasing subsample size) analysis did not allow detection of management-dependent influences on the soil bacterial community, while the gene library approach allowed identification of potential management-specific indicator taxa (Hartmann and Widmer 2006). Nevertheless, Lorenz curve analysis of DGGE fingerprinting data in MC is still valued for its sensitive, rapid and high-throughput. As a large number of functional forms can be chosen for the best fits the data when estimating a Lorenz curve (Chotikapanich and Griffiths 2005), together with power law probability distribution such as Pareto distribution, Lorenz curve analysis may offer as a meaningful way of assessing MC.

6. Strategy of using probiotics in larviculture and approaches of evaluation efficiency

Probiotics are generally administered either as a live microbial feed supplements or as an additive to the culture medium (Moriarty 2003). The viable probiotics are expected to be at effective concentration and to be persisted for a period of time. For example, the ratios of 10 to the sixth power colony forming units' probionts per gram of fish weight (CFU g⁻¹) or per mL of pond water (CFU mL⁻¹) are often indicated. However, the ratio of probionts to total bacterial biomass in culture system should not be neglected. In realistic, it appears to be impossible to apply probionts daily or even weekly at dominant amount, especially in earthen pond. It is confirmed by Ziaei-Nejad *et al.* (2006) that the colonization rates of post-larval shrimp digestive tracts by probionts *Bacillus* bacteria were very low in all treatments in earthen ponds (0.07% - 0.30% of total bacteria). But *Bacillus* bacteria became artificially dominant (87.2–93.0% of total bacteria for one treatment) in both the water and the digestive tracts of zoea stage 3 larvae in the 10 L tanks. It is also indicated that shrimp fed probiotic-enriched *Artemia* had significantly ($P < 0.05$) higher *Bacillus* counts than did shrimp administered probiotic in the water. It is now feasible to add probionts via live feed, to be present in dominant, particularly in the early larval stages.

As reviewed in chapter 1 table 1.1, a number of different approaches are available for characterizing probiotics in complex community. One of the best descriptions of the

probiotics *in vivo* today is the exploitation the taxonomic information of the 16S rRNA gene. Techniques for fingerprinting microbial communities, including DGGE and T-RFLP analyses, can discern different dominant bacterial populations. However, these methods generally allow the detection of a phylogenetic group or a bacteria species only if it represents 1% of the total microbiota (Seksik *et al.* 2003), although the ratio of 0.1% was achieved in our study *in vitro* (chapter 2, Fig. 2.2). Studies of human intestine microbiota show many of the species that establish smaller populations in the intestine, including *E. coli* and *Staphylococcus aureus*, known to play significant role in health (Walker and Buckley 2005). In order to detect probiotics by fingerprinting methods, the ratio of probiotics in a total microbiota must be guaranteed and specific probes and primers must be used to specifically target probiotics if they are present in minority groups.

The advent of various high-throughput techniques, such as the combination of phylogenetic and functional gene arrays (PhyloChip and GeoChip), represents a high-throughput approach to elucidating mechanism responsible for beneficial effects of probiotics at targeted sites. Metagenomic approaches, with the help of 454 FLX pyrosequencing, can help to characterize the entire microbiota of an individual and/or a culture system, and show microbial biodiversity at very high resolution. This would also help to verify species-distribution model and non-parametric estimation coefficient such as Gini coefficient, and the questions about the functional

relationship between probiotics and microbial community *in vivo* could be addressed more effectively.

7. China case: promote probiotics and prepare prebiotics.

As in many Asian countries, there is a strong research and commercial interest in animal probiotics in China (Crittenden *et al.* 2005). As listed in chapter 2, *Bifidobacterium*, *Lactobacillus*, *Bacillus*, photosynthetic bacteria and *Bdellovibrio bacteriovorus* have been used as probiotics in different animal species including fish. Although a number of research groups are screening novel probiotics for aquaculture, most emphasis has been on the introduction of probiotics from terrestrial animal feed additives. This approach might be proved useful to inland aquaculture, but remains to be evaluated in marine culture. Our studies proved PCR-DGGE analysis is a good method to evaluate rotifer autochthonous probiotics, a commercial *Bacillus* mixture and MCs in larviculture system. As modern molecular techniques are available, both researchers and manufacturers should apply these techniques to ensure the species of bacteria are correctly identified, for quality assurance as well as safety (Wang *et al.* 2008).

Despite China is also a major producer of prebiotic oligosaccharides, the use of prebiotics with marine fishes has been poorly reported to date in China. It is worth noting that prebiotics select for the enrichment of beneficial bacteria, such as *Lactobacillus* spp. and *Bifidobacterium* spp. (Burr and Gatlin III 2005). Our study

also shows two types of yeast strains with different cell wall compositions induced divergent change in the distribution of bacterial species. Locally in China, a number of Chinese medicinal herbs have been found to promote the growth of intestinal commensal bacteria, and several studies show the inhibition of Vibrios in sea bream *Lutjanus sanguineus*, large yellow croaker *Pseudosciaena crocea* and sea bass *Dicentrarchus labrax* (Jin *et al.* 1999; Tong *et al.* 2007; Lu *et al.* 2008). As rich natural resources, such as macro algae, are widely available for production of prebiotics, the application of prebiotics in aquaculture has shown great potential.

8. Conclusion and future perspectives

In summary, the following conclusion can be draw from this study:

- PCR-DGGE analysis of 16S rRNA gene fragments is a good method to study of MCs in larviculture system and ensure probiotics quality and efficacy.
- The MCC changes and shifts revealed by DGGE band patterns link to the growth rates of rotifer.
- Different diets and different rotifer strains but not probionts shape MCC in rotifer batch culture.
- Lorenz curves analysis of PCR-DGGE fingerprinting data is an effective and sensitive way of interpreting internal structure of MC in comparison with

other diversity indices.

With advanced molecular tools and well-developed gnotobiotic animal system (*Artemia*, zebrafish, soon to be extended with sea bass), priority could be given to the host-microbe interaction research. As the host cells responses can provide direct evidences of beneficial/ detrimental effects of implanted microbes. To the field study of MC larviculture, it is still unclear what the effects of dominant substrate composition (diets) and food web structure (including virus) for the shaping of MC in larviculture. What are the effects of perturbations (diets, antibiotics, probiotics) to the larvae microbiota? It might provide an opportunity to introduce beneficial changes if the effects are significant and reproducible. As the data from advanced molecular tools are accumulating, more effective analysis such as species-distribution model could help us to better understand the species-habitat relationship and the effects of introducing probiotics.

Summary

In this PhD study, we investigated the dynamic of microbial community compositions (MCCs) in rotifer batch culture and fish larviculture by PCR-DGGE approach. The shifts of MCCs link to specific cultures (different diets, rotifer *Brachionus plicatilis* cryptic strains and probiotics) and culture performance were evaluated in rotifer batch cultures and fish larvicultures.

Chapter 1 provides an overview of current constraints for larviculture, the role of probiotics in larviculture and the methods used for the *in vivo* evaluation. Following the food chain of larviculture, the interaction of bacteria with microalgae, rotifer and *Artemia* are being discussed. The influence of probiotics to the microbial community composition in the larvicultures is summarised.

Chapter 2 presents the status of probiotics in aquaculture in China. Various types of probiotic products and relevant research are described. Improper labelling, unidentified strains and the lack of shelf-life and *in vivo* monitoring are discussed. Finally, it outlines the types of effective evaluation methods that can be used to ensure the quality control of probiotic products.

Chapter 3 describes a 16S rRNA gene based PCR-DGGE approach to monitor microbial communities associated with turbot (*Psetta maxima* L.) larviculture in a commercial hatchery. In addition, the colour changes of the green water as well as the

larval health status were monitored. The DGGE profiles revealed bacterial community changes in the culture water before and after first feeding. Lorenz curve and Gini coefficients analyses suggested that the microbial communities present in samples from systems with diseased animals were more complex and more evenly distributed than samples coming from healthy systems.

In Chapter 4, the evolution of the composition of bacterial communities associated with 3 different strains belonging to different cryptic species of the rotifer *Brachionus plicatilis* was monitored during four subsequent cycles of batch cultures using DGGE, cluster analysis, multidimensional scaling and principal component analysis. The data suggest that the evolving bacterial communities are *Brachionus plicatilis* strain specific. Moreover, large changes in rotifer growth rate were found to be associated with large changes in the bacterial community composition, suggesting that there might be a causal link. Finally, Lorenz curves and Gini coefficient analysis revealed the different bacterial species distributions among different *B. plicatilis* strain cultures, and those species distributions were evolving differently during four subsequent cycles.

In chapter 5, different feed types, different feeding regimes and mixtures of 3 probionts were evaluated for their effects on rotifer growth performance and for the composition of the microbial community associated with the rotifers. DGGE analysis showed that the initial microbial community had a major impact on the evolution of

the microbial community in the cultures. It was shown that the dominant bacterial species in the cultures receiving either *N. oculata* or CS-3000 were different. However, in cultures receiving both feeds (either switching between feeds on a daily basis or on a batch basis), a high similarity in microbial community fingerprint was found. The presence of probionts was detected by the end of 4 batch culture cycles in spite of strong shifts of the bacterial community. By group discriminant analysis, it was found that *Phenylobacterium* sp. and *Paracoccus* sp. contributed positively to the CS-3000 fed group, while *Gluconobactera* sp. contributed positively to the *N. oculata* fed group although they were not considered as a very dominant species.

In chapter 6, the effects of a probiotic *Bacillus* mixture and yeast cell wall mutant mnn9 were evaluated in turbot (*Psetta maxima* L.) larviculture. DGGE was used to monitor the bacterial community composition during larviculture, starting from the fertilized eggs and through the initial larval stages of turbot. The results revealed the positive effects for larval survival rates and the diverse shifts of bacterial communities when probiont and prebiotics were given. The agar-plating results also showed a considerable decrease of *Vibrio* loading. The further assessment by Lorenz curve and Gini coefficient revealed an increased bacterial species distribution when *Bacillus* mixture or wild type yeast was served; On the contrary, a decreasing of bacterial species distribution was observed when mutant yeast mnn9 was given.

In chapter 7, the importance of characterizing microbial communities in larviculture,

parameters that governing microbial community composition in larviculture, the probionts as shaping forces of microbial community composition, as well as the possible relationships between microbial community composition and health of larvae are discussed in the light of the obtained results. The advantages and drawbacks of fingerprinting methods for the study of MC are discussed. Also, the statistical strategy of analyzing microbial community fingerprinting data is evaluated. Finally, further research perspectives, such as gene arrays and metagenomic approaches which can deal with probiotics in complex microbial community in laviculture, are prospected.

In conclusion, PCR-DGGE analysis of 16S rRNA gene fragments is so far still a good method to study of MCs in larviculture system and ensure probiotics quality and efficacy. PCR-DGGE revealed the dynamic changes of MCCs linked to a various bio-factors in rotifer cultures and larvicultures. Different diets and different rotifer strains but not probionts shape MCC in rotifer batch culture. Lorenz curves analysis of PCR-DGGE fingerprinting data is an effective and sensitive way of interpreting internal structure of MC in comparison with other diversity indices.

Samenvatting

In dit PhD werk werd de dynamiek van de samenstelling van de microbiële gemeenschappen (“microbial community composition: MCC) onderzocht in rotiferen batch culturen en vislarvenculturen met een PCR-DGGE benadering. De verschuivingen in de MCC werd bestudeerd in functie van de volgende parameters: verschillende diëten, cryptische rotiferen stammen en probiotica.

Hoofdstuk 1 geeft een overzicht van de huidige situatie van de larvicultuur, de rol van de probiotica in de larvicultuur en de gebruikte methoden voor de *in vivo* evaluatie van hun activiteit. De interactie van bacteriën met microalgen, rotiferen en *Artemia* wordt samengevat.

Hoofdstuk 2 vat de situatie samen rond het gebruik van probiotica in China. Verschillende types van probiotische producten en relevant onderzoek worden beschreven. Onjuiste labelling, niet-geïdentificeerde stammen, het gebrek aan voldoende “shelf-life” en *in vivo* monitoring worden besproken als mogelijke pijnpunten. De beschikbare methoden om een effectieve evaluatie uit te voeren van de kwaliteit van probiotica worden beschreven.

Hoofdstuk 3 beschrijft de 16S rRNA gen-gebaseerde PCR-DGGE methode om de microbiële gemeenschappen geassocieerd met de larvicultuur van tarbot (*Psetta maxima* L.) op te volgen in een commerciële broedhuis. Bovendien werd de

kleurverandering van het “groen water” en gezondheidstoestand van de larven opgevolgd. De DGGE profielen tonen aan dat de bacteriële gemeenschappen in het cultuurwater veranderen voor en na de eerste voeding. De Lorenz en Gini coëfficiënten analyse laat veronderstellen dat de microbiële gemeenschap aanwezig in de monsters van systemen die zieke dieren huisvesten meer complex en meer gelijk verdeeld zijn dan in de monsters genomen uit systemen die gezonde dieren huisvesten.

In hoofdstuk 4, wordt de evolutie van de MCC geassocieerd met 3 verschillende cryptische rotiferen (*Brachionus plicatilis*) stammen opgevolgd gedurende 3 opeenvolgende cycli van batch culturen. Hierbij werd DGGE, cluster analyse en multidimensionele scaling en principiële component analyse gebruikt. De gegevens suggereren dat de bacteriële gemeenschappen geassocieerd met *Brachionus* stam-specifiek zijn. Bovendien blijken grote veranderingen in de groeisnelheid van de rotiferen geassocieerd te zijn met grote veranderingen in MCC, wat een causaal verband laat veronderstellen. De Lorenz curven en de Gini coëfficiënten analyse lieten uitschijnen dat er verschillende bacteriële soorten distributies bestaan bij de verschillende rotiferen stammen en dat de soorten distributie anders evolueerde gedurende de vier cycli.

In hoofdstuk 5 werden verschillende voedertypes, verschillende voederregimes en mengsels van 3 probiotica geëvalueerd voor hun effect op de performance van

rotiferen en de MCC geassocieerd met de rotiferen. De DGGE analyse toonde aan dat de initiële microbiële gemeenschap een belangrijke impact had op de evolutie van de microbiële gemeenschappen in de culturen. Er werd aangetoond dat de dominante bacteriële species in de culturen die *N. oculata* of CS3000 gevoederd kregen, verschillend waren. In culturen die beide types van voeders kregen werd een hoge gelijkensis in de microbiële gemeenschap fingerprint vastgesteld. De aanwezigheid van toegevoegde probionten werd vastgesteld op het einde van de 4^{de} batch cultuur niettegenstaande de sterke verschuivingen in de microbiële gemeenschappen. Met behulp van discriminant analyse, kon aangetoond worden dat *Phenylobacterium* sp. en *Paracoccus* sp. op een positieve wijze bijdroegen tot de CS300 gevoede groep, terwijl *Gluconobacteria* sp. op een positieve wijze bijdroeg tot de groep gevoed met *N. oculata*, alhoewel ze niet konden beschouwd worden als dominante soorten.

In hoofdstuk 6 werden de effecten van een probiotisch mengsel van *Bacillus* en van de gistcelwand mutanten mnn9 geëvalueerd in een tarbot (*Psetta maxima* L.) larvicultuur. DGGE analyse werd gebruikt om de microbiële gemeenschap op te volgen van het ei-stadium tot the initiële larvale fase. De resultaten toonden positieve effecten van beide addities op de larvale overleving en veroorzaakten uitlopende veranderingen in de MCC. Agar uitplantingen toonden een reductie aan van de *Vibrio* belasting. Via Lorenz curve en Gini coëfficiënten analyse kan aangetoond worden dat het *Bacillus* mengsel en wildtype gist zorgden voor een verhoogde bacteriële

soortenspreiding, terwijl de *mnn9* gistmutant zorgde voor een verlaagde bacteriële soortenspreiding.

In hoofdstuk 7 wordt het belang van de karakterisering van de microbiële gemeenschappen in larvicultuur, de parameters die de MCC bepalen, de probionten met hun MCC beïnvloedend karakter en de mogelijke relatie tussen MCC en de gezondheidstoestand van larven besproken in het licht van de bekomen resultaten. De voor- en nadelen van de gebruikte fingerprinting methode worden belicht. De statistische strategieën voor de analyse van microbiële gemeenschap fingerprints worden geëvalueerd. Onderzoeksperspectieven, zoals gen array en metagenomische benaderingen, die kunnen gebruikt worden voor de analyse van de effecten van probionten in complexe microbiële gemeenschappen worden besproken.

Ter conclusie kan gesteld worden dat PCR-DGGE analyse op basis van 16S rRNA genfragmenten een goede methode is om de microbiële gemeenschap in larvicultuur op te volgen. Met DGGE analyse konden de dynamische veranderingen in MCC in verband gebracht worden met veranderende factoren in de rotiferenculturen en larviculturen. De verschillende diëten en de verschillende rotiferenstammen, maar niet de probionten, konden de MCC in rotiferen culturen bepalen. Lorenz curven analyse van de PCR-DGGE fingerprint data is een doeltreffende en gevoelige manier om de structuur van een microbiële gemeenschap te karakteriseren in vergelijking met andere methoden.

References

- Aguilar-Uscanga B, Francois JM (2003) A study of the yeast cell wall composition and structure in response to growth conditions and mode of cultivation. *Lett Appl Microbiol* 37: 268-274.
- Alonso-Saez L, Aristegui J, Pinhassi J, Gomez-Consarnau L, Gonzalez JM, Vaque D, Agusti S, Gasol JM (2007) Bacterial assemblage structure and carbon metabolism along a productivity gradient in the NE Atlantic Ocean. *Aquat Microb Ecol* 46: 43-53.
- Alves D, Specker JL, Bengtson DA (1999) Investigations into the causes of early larval mortality in cultured summer flounder (*Paralichthys dentatus* L.). *Aquaculture* 176: 155-172.
- Amann RI, Ludwig W, Schleifer KH (1995) Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. *Microbiol Rev* 59:143-169.
- Amann RI, Binder BJ, Olson RJ, Chisholm SW, Devereux R, Stahl DA (1990) Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. *Appl Environ Microbiol* 56: 1919-1925.
- Ampe F, Miambi E (2000) Cluster analysis, richness and biodiversity indexes derived from denaturing gradient gel electrophoresis fingerprints of bacterial communities demonstrate that traditional maize fermentations are driven by the transformation process. *Intern J Food Microbiol* 60: 91-97.
- Arndt H (1993) Rotifers as predators on components of the microbial web (bacteria, heterotrophic flagellates, ciliates)-a review. *Hydrobiologia* 255: 231-246.

- Asfie M, Yoshijima T, Sugita H (2003) Characterization of the goldfish fecal microflora by the fluorescent *in situ* hybridization method. *Fish Sci* 69: 21-26.
- Atlas RM, Bartha R (1987) *Microbial ecology: fundamentals and applications*. Menlo Park, Calif.: Benjamin/Cummings Pub. Co.
- Austin B (2006) The bacterial microflora of fish, revised. *TSWJ* 6: 931-945.
- Avendano RE, Riquelme CE (1999) Establishment of mixed-culture probiotics and microalgae as food for bivalve larvae. *Aquacult Res* 30: 893-900.
- Azad SA, Chong VC, Vikineswary S (2002) Phototrophic bacteria as feed supplement for rearing *Penaeus monodon* larvae. *J World Aquacult Soc* 33: 158-168.
- Balcázar JL, Rojas-Luna T, Cunningham DP (2007) Effect of the addition of four potential probiotic strains on the survival of pacific white shrimp (*Litopenaeus vannamei*) following immersion challenge with *Vibrio parahaemolyticus*. *J Invertebr Pathol* 96: 147-150.
- Balcázar JL, Blas Id, Ruiz-Zarzuela I, Cunningham D, Vendrell D, Muzquiz JL (2006a) The role of probiotics in aquaculture. *Vet Microbiol* 114: 173-186.
- Balcázar JL, Decamp O, Vendrell D, De Blas I, Ruiz-Zarzuela I (2006b) Health and nutritional properties of probiotics in fish and shellfish. *Microb Ecol Health Dis* 18: 65-70.
- Barcina I, Lebaron P, Vives-Rego J (1997) Survival of allochthonous bacteria in aquatic systems: A biological approach. *FEMS Microbiol Ecol* 23: 1-9.
- Barlaan EA, Sugimori M, Furukawa S, Takeuchi K (2005) Profiling and monitoring of microbial populations by denaturing high-performance liquid chromatography. *J Microbiol Methods* 61: 399-412.

- Barlass a PJ, Houston CW, Clements b MO, Moir A (2002) Germination of *Bacillus cereus* spores in response to L-alanine and to inosine: the roles of gerL and gerQ. *Microbiology* 148: 2089-2095.
- Bergh O, Hansen GH, Taxt RE (1992) Experimental infection of eggs and yolk sac larvae of halibut, *Hippoglossus hippoglossus* L. *J Fish Dis* 15: 379-391.
- Bjornsdottir R, Johannsdottir J, Coe J, Smaradottir H, Agustsson T, Sigurgisladottir S, Gudmundsdottir BK (2008) Survival and quality of halibut larvae (*Hippoglossus hippoglossus* L.) in intensive farming: Possible impact of the intestinal bacterial community. *Aquaculture* doi:10.1016/j.aquaculture.2008.09.003
- Blanch AR, Alsina M, Simon M, Jofre J (1997) Determination of bacteria associated with reared turbot (*Scophthalmus maximus*) larvae. *J Appl Microbiol* 82: 729-734.
- Bomba A, Nemcova R, Gancarcikova S, Herich R, Guba P, Mudronova D (2002) Improvement of the probiotic effect of micro-organisms by their combination with maltodextrins, fructo-oligosaccharides and polyunsaturated fatty acids. *Br J Nutr* 88: 95-99.
- Bondad-Reantaso MG, Subasinghe RP, Arthur JR, Ogawa K, Chinabut S, Adlard R, Tan Z, Shariff M (2005) Disease and health management in Asian aquaculture. *Vet Parasitol* 132: 249-272.
- Bonecker CC, Aoyagui ASM (2005) Relationships between rotifers, phytoplankton and bacterioplankton in the corumbá reservoir, Goiás State, Brazil. *Hydrobiologia* 546: 415-421.
- Boon N, De Windt W, Verstraete W, Top EM (2002) Evaluation of nested PCR-DGGE (denaturing gradient gel electrophoresis) with group-specific 16 S rRNA

- primers for the analysis of bacterial communities from different wastewater treatment plants. *FEMS Microbiol Ecol* 39: 101-112.
- Boon N, Marlé C, Top EM, Verstraete W (2000) Comparison of the spatial homogeneity of physico-chemical parameters and bacterial 16S rRNA genes in sediment samples from a dumping site for dredging sludge. *Appl Microbiol Biotechnol* 53: 742-747.
- Boström KH, Simu K, Hagstrom, Riemann L (2004) Optimization of DNA extraction for quantitative marine bacterioplankton community analysis. *Limnol Oceanogr: Methods* 2: 365-373.
- Bottari B, Ercolini D, Gatti M, Neviani E (2006) Application of FISH technology for microbiological analysis: current state and prospects. *Appl Microbiol Biotechnol* 73: 485-494.
- Bourne DG, Young N, Webster N, Payne M, Salmon M, Demel S, Hall M (2004) Microbial community dynamics in a larval aquaculture system of the tropical rock lobster, *Panulirus ornatus*. *Aquaculture* 242: 31-51.
- Brunvold L, Sandaa R-A, Mikkelsen H, Welde E, Bleie H, Bergh O (2007) Characterisation of bacterial communities associated with early stages of intensively reared cod (*Gadus morhua*) using Denaturing Gradient Gel Electrophoresis (DGGE). *Aquaculture* 272: 319-327.
- Bryant DA, Frigaard NU (2006) Prokaryotic photosynthesis and phototrophy illuminated. *Trends Microbiol* 14: 488-496.
- Buchmann K, Dalsgaard I, Nielsen ME, Pedersen K, Uldal A, Garcia JA, Larsen JL (1997) Vaccination improves survival of Baltic salmon (*Salmo solar*) smolts in delayed release sea ranching (net-pen period). *Aquaculture* 156: 335-348.

- Buller NB (2004) Bacteria from fish and other aquatic animals: a practical identification manual. CABI Publishing, Wallingford, UK.
- Burr G, Gatlin D, Ricke S (2005) Microbial ecology of the gastrointestinal tract of fish and the potential application of prebiotics and probiotics in finfish Aquaculture. J World Aquacult Soc 36: 425-436.
- Cahu C, Zambonino Infante J (2001) Substitution of live food by formulated diets in marine fish larvae. Aquaculture 200: 161-180.
- Cai JP, Zhao J (2006) The recent research progress on *Bdellovibrio bacteriourus*. Acta Microbiol Sinica 6: 1028-1032 (In Chinese, Abstract in English).
- Callanan M (2005) Mining the Probiotic Genome: advanced strategies, enhanced benefits, perceived obstacles. Curr Pharm Des 11: 25-36.
- Carmen Collado M, Hernández M (2007) Identification and differentiation of *Lactobacillus*, *Streptococcus* and *Bifidobacterium* species in fermented milk products with *bifidobacteria*. Microbiol Res 162: 86-92.
- Carrero-Colón M, Nakatsu CH, Konopka A (2006) Microbial community dynamics in nutrient-pulsed chemostats. FEMS Microbiol Ecol 57: 1-8.
- Castell J, Blair T, Neil S, Howes K, Mercer S, Reid J, Young-Lai W, Gullison B, Dhert P, Sorgeloos P (2003) The effect of different HUFA enrichment emulsions on the nutritional value of rotifers (*Brachionus plicatilis*) fed to larval haddock (*Melanogrammus aeglefinus*). Aquacult Int 11: 109-117.
- Cerda-Cuellar M, Blanch AR (2004) Determination of *Vibrio scophthalmi* and its phenotypic diversity in turbot larvae. Environ Microbiol 6: 209-217.
- Chen D, Ainsworth AJ (1992) Glucan administration potentiates immune defence mechanisms of channel catfish, *Ictalurus punctatus* Rafinesque. J Fish Dis 15:

295-304.

Chen JC, Lin ZH, Ma XX (2003) Evidence of the production of silver nanoparticles via pretreatment of *Phoma* sp. 3. 2883 with silver nitrate. *Lett Appl Microbiol* 37: 105-108.

Chotikapanich D, Griffiths WE (2005) Averaging Lorenz curves. *J Econ Inequal* 3: 1-19.

Collado MC, Meriluoto J, Salminen S (2007a) Development of new probiotics by strain combinations: is it possible to improve the adhesion to intestinal mucus? *J Dairy Sci* 90: 2710-2716.

Collado MC, Meriluoto J, Salminen S (2007b) *In vitro* analysis of probiotic strain combinations to inhibit pathogen adhesion to human intestinal mucus. *Food Res Int* 40: 629-636.

Collins MD, Gibson GR (1999) Probiotics, prebiotics, and synbiotics: approaches for modulating the microbial ecology of the gut. *Am J Clin Nutr* 69: 1052-1057.

Corcoran BM, Ross RP, Fitzgerald GF, Stanton C (2004) Comparative survival of probiotic *Lactobacilli* spray-dried in the presence of prebiotic substances. *J Appl Microbiol* 96: 1024-1039.

Coulter PB (1989) *Measuring inequality: a methodological handbook*. Westview Press Boulder, CO.

Cowell FA, Victoria-Feser MP (2007) Robust stochastic dominance: A semi-parametric approach. *J Econ Inequal* 5: 21-37.

Crespi V (2005) *FAO Fact Sheets on Aquaculture*, *FAO Aquacult Newslett* p 32, 44.

- Crittenden R, Bird AR, Gopal P, Henriksson A, Lee YK, Playne MJ (2005). Probiotic research in Australia, New Zealand and the Asia-Pacific region. *Curr Pharmaceutical Design* 11: 37-53.
- Croft MT, Lawrence AD, Raux-Deery E, Warren MJ, Smith AG (2005) Algae acquire vitamin B12 through a symbiotic relationship with bacteria. *Nature* 438: 90-94.
- Crosby LD (2005) Molecular microbial community analysis: Understanding and overcoming the limitations. Thesis, Stanford University.
- Crump BC, Kling GW, Bahr M, Hobbie JE (2003) Bacterioplankton community shifts in an arctic lake correlate with seasonal changes in organic matter source. *Appl Environ Microbiol* 69: 2253-2268.
- Dahllof I (2002) Molecular community analysis of microbial diversity. *Curr Opin Biotechnol* 13: 213-217.
- Damgaard C, Weiner J (2000) Describing Inequality in Plant Size or Fecundity. *Ecology* 81: 1139-1142.
- Danovaro R, Luna GM, Dell'Anno A, Pietrangeli B (2006) Comparison of two fingerprinting techniques, terminal restriction fragment length polymorphism and automated ribosomal intergenic spacer analysis, for determination of bacterial diversity in aquatic environments. *Appl Environ Microbiol* 72: 5982-5989.
- De Liphay JR, Johnsen K, Albrechtsen HJ, Rosenberg P, Aamand J (2004) Bacterial diversity and community structure of a sub-surface aquifer exposed to realistic low herbicide concentrations. *FEMS Microbiol Ecol* 49: 59-69.

- Defoirdt, T. (2007) Quorum sensing disruption and the use of short-chain fatty acids and polyhydroxyalkanoates to control luminescent vibriosis. PhD thesis, Ghent University, Belgium.
- Defoirdt T, Boon N, Sorgeloos P, Verstraete W, Bossier P (2007) Alternatives to antibiotics to control bacterial infections: luminescent vibriosis in aquaculture as an example. *Trends Biotechnol* 25: 472-479.
- Defoirdt T, Crab R, Wood TK, Sorgeloos P, Verstraete W, Bossier P (2006) Quorum sensing-disrupting brominated furanones protect the gnotobiotic brine shrimp *Artemia franciscana* from pathogenic *Vibrio harveyi*, *Vibrio campbellii*, and *Vibrio parahaemolyticus* isolates. *Appl Environ Microbiol* 72: 6419-6423.
- Defoirdt T, Boon N, Bossier P, Verstraete W (2004) Disruption of bacterial quorum sensing: an unexplored strategy to fight infections in aquaculture. *Aquaculture* 240: 69-88.
- Dehasque, M., Devresse, B., Sorgeloos, P (1993) Effective suppression of bacteria bloom during hatching and enrichment of *Artemia* and its applicability in fish/shrimp hatcheries. In: World Aquaculture'93 Proceedings. Book of Abstracts. European Aquaculture Society, Special Publication No. 22, Ostend, 1993.
- Dejonghe W, Boon N, Seghers D, Top EM, Verstraete W (2001) Bioaugmentation of soils by increasing microbial richness: missing links. *Environ Microbiol* 3: 649-657.
- Dhert P, Rombaut G, Suantika G, Sorgeloos P (2001) Advancement of rotifer culture and manipulation techniques in Europe. *Aquaculture* 200: 129-146.

- Dhert P (1996) Rotifers. In: Lavens P, Sorgeloos P (Eds.), Manual on the production and use of live food for aquaculture FAO Fisheries Technical Paper 361, pp 61-100.
- Dhert P, Sorgeloos P (1993) Contributions towards a specific DHA enrichment in the live food *Brachionus plicatilis* and *Artemia* sp. Fish Farming Technology: In: Proceedings of the First International Conference on Fish Farming Technology, Trondheim, Norway, 9-12 August 1993.
- Dong X, Li Y, Zhang XH, Liu J, Hu Z, Chen J (2007) Identification and inhibitory activity to pathogenic *Vibrio* species of a marine bacterium *Phaeobacter* DL2. J Fish Sci China 14: 996-1003. (in Chinese, Abstract in English).
- Dong YH, Zhang LH (2005) Quorum sensing and quorum-quenching enzymes. J Microbiol 43: 101-109.
- Dooms S, Papakostas S, Hoffman S, Delbare D, Dierckens K, Triantafyllidis A, De Wolf T, Vadstein O, Abatzopoulos TJ, Sorgeloos P (2007) Denaturing gradient gel electrophoresis (DGGE) as a tool for the characterisation of *Brachionus* sp. strains. Aquaculture 262: 29-40.
- Douglas SE (2006) Microarray studies of gene expression in fish. OMICS J Integr Biol 10: 474-489.
- Douillet PA (2000a) Bacterial additives that consistently enhance rotifer growth under synxenic culture conditions 1. Evaluation of commercial products and pure isolates. Aquaculture 182: 249-260.
- Douillet PA (2000b) Bacterial additives that consistently enhance rotifer growth under synxenic culture conditions 2. Use of single and multiple bacterial probiotics. Aquaculture 182: 241-248.

- Dubois JW, Hill S, England LS, Edge T, Masson L, Trevors JT, Brousseau R (2004) The development of a DNA microarray-based assay for the characterization of commercially formulated microbial products. *J Microbiol Methods* 58: 251-262.
- Eddy SD, Jones SH (2002) Microbiology of summer flounder *Paralichthys dentatus* fingerling production at a marine fish hatchery. *Aquaculture* 211: 9-28.
- FAO (2007) Aquaculture production: quantities 1950-2005. FISHSTAT Plus - Universal software for fishery statistical time series [online or CD-ROM]. Available at: <http://www.fao.org/fi/statist/FISOFT/FISHPLUS.asp>. FAO, UN.
- FAO (2006) State of world aquaculture 2006. FAO Fisheries Department. FAO Fisheries Technical Paper No. 500. Rome, Italy.
- FAO (2003) The role of aquaculture in improving food security and nutrition. FAO Committee on world food security 29 sessions, Rome, Italy.
- Farzanfar A (2006) The use of probiotics in shrimp aquaculture. *FEMS Immunol Med Mic* 48: 149-158.
- Fasoli S, Marzotto M, Rizzotti L, Rossi F, Dellaglio F, Torriani S (2003) Bacterial composition of commercial probiotic products as evaluated by PCR-DGGE analysis. *Int J Food Microbiol* 82: 59-70.
- Ferguson RL, Buckley EN, Palumbo AV (1984) Response of marine bacterioplankton to differential filtration and confinement. *Appl Environ Microbiol* 47: 49-55.
- Fisher MM, Klug JL, Lauster G, Newton M, Triplett EW (2000) Effects of resources and trophic interactions on freshwater bacterioplankton diversity. *Microb Ecol* 40: 125-138.
- Fjellheim AJ, Playfoot KJ, Skjermo J, Vadstein O (2007) *Vibrionaceae* dominates the microflora antagonistic towards *Listonella anguillarum* in the intestine of

- cultured Atlantic cod (*Gadus morhua* L.) larvae. *Aquaculture* 269: 98-106.
- Fromin N, Hamelin J, Tarnawski S, Roesti D, Jourdain-Miserez K, Forestier N, Teyssier-Cuvelle S, Gillet F, Aragno M, Rossi P (2002) Statistical analysis of denaturing gel electrophoresis(DGE) fingerprinting patterns. *Environ Microbiol* 4: 634-643.
- Fukami K, Nishijima T, Ishida Y (1997) Stimulative and inhibitory effects of bacteria on the growth of microalgae. *Hydrobiologia* 358: 185-191.
- Gafan GP, Lucas VS, Roberts GJ, Petrie A, Wilson M, Spratt DA (2005) Statistical analyses of complex denaturing gradient gel electrophoresis profiles. *J Clin Microbiol* 43: 3971-3978.
- Gatesoupe FJ (1999) The use of probiotics in aquaculture. *Aquaculture* 180: 147-165.
- Gatesoupe FJ (1997) Siderophore production and probiotic effect of *Vibrio* sp. associated with turbot larvae, *Schthalmus maximus*. *Aquat Living Resour* 10: 239-246.
- Gatesoupe FJ (1991) The effect of three strains of lactic bacteria on the production rate of rotifers, *Brachionus plicatilis*, and their dietary value for larval turbot, *Scophthalmus maximus*. *Aquaculture* 96: 335-342.
- Giménez G, Padrós F, Roque A, Estévez A, Furones D (2006) Bacterial load reduction of live prey for fish larval feeding using Ox-Aquaculture[®]. *Aquacult Res* 37: 1130-1139.
- Goldenberg O, Herrmann S, Marjoram G, Noyer-Weidner M, Hong G, Bereswill S, Gobel UB (2007) Molecular monitoring of the intestinal flora by denaturing high performance liquid chromatography. *J Microbiol Methods* 68: 94-105.

- Gomez-Gil B, Thompson FL, Thompson CC, Swings J (2003) *Vibrio rotiferianus* sp. nov., isolated from cultures of the rotifer *Brachionus plicatilis*. Int J Syst Evol Microbiol 53: 239-243.
- Gomez-Gil B, Roque A, Turnbull JF (2000) The use and selection of probiotic bacteria for use in the culture of larval aquatic organisms. Aquaculture 191: 259-270.
- Gomez-Gil B, Herrera-Vega MA, Abreu-Grobois FA, Roque A (1998) Bioencapsulation of two different *Vibrio* species in nauplii of the brine shrimp (*Artemia franciscana*). Appl Environ Microbiol 64: 2318-2322.
- Grisez L Chair M., Sorgeloos P, Ollevier F (1996) Mode of infection and spread of *Vibrio anguillarum* in turbot *Scophthalmus maximus* larvae after oral challenge through live feed. Dis Aquat Organ 26: 181-187.
- Grossart HP, Levold F, Allgaier M, Simon M, Brinkhoff T (2005) Marine diatom species harbour distinct bacterial communities. Environ Microbiol 7: 860-873.
- Guarner F, Malagelada JR (2003) Gut flora in health and disease. The Lancet 361: 512-519.
- Gullian M, Thompson F, Rodriguez J (2004) Selection of probiotic bacteria and study of their immunostimulatory effect in *Penaeus vannamei*. Aquaculture 233: 1-14.
- Guo X, Li D, Lu W, Piao X, Chen X (2006) Screening of *Bacillus* strains as potential probiotics and subsequent confirmation of the in vivo effectiveness of *Bacillus subtilis* MA139 in pigs. Antonie Van Leeuwenhoek 90: 139-146.
- Hagiwara A, Hamada K, Hori S, Hirayama K (1994) Increased sexual reproduction in *Brachionus plicatilis* (Rotifera) with the addition of bacteria and rotifer extracts. J Exp Mar Biol Ecol 181: 1-8.

- Halet D, Defoirdt T, Van Damme P, Vervaeren H, Forrez I, Van de Wiele T, Boon N, Sorgeloos P, Bossier P, Verstraete W (2007) Poly--hydroxybutyrate-accumulating bacteria protect gnotobiotic *Artemia franciscana* from pathogenic *Vibrio campbellii*. FEMS Microbiol Ecol 60: 363-369.
- Halet D, Boon N, Verstraete W (2006) Community dynamics of methanotrophic bacteria during composting of organic matter. J Biosci Bioeng 101: 297-302.
- Hansen GH, Olafsen JA (1999) Bacterial interactions in early life stages of marine cold water fish. Microb Ecol 38: 1-26.
- Harch BD, Correll RL, Meech W, Kirkby CA, Pankhurst CE (1997) Using the Gini coefficient with BIOLOG substrate utilisation data to provide an alternative quantitative measure for comparing bacterial soil communities. J Microbiol Methods 30: 91-101.
- Hargreaves JA (2006) Photosynthetic suspended-growth systems in aquaculture. Aquacult Eng 34: 344-363.
- Hartmann M, Widmer F (2006) Community structure analyses are more sensitive to differences in soil bacterial communities than anonymous diversity Indices Appl Environ Microbiol 72: 7804-7812.
- Hernández Serrano P (2005) Responsible use of antibiotics in aquaculture. FAO Fisheries Technical Paper No. 469. Rome, Italy p 97.
- Hino A (1993) Present culture systems of the rotifer (*Brachionus plicatilis*) and the function of micro-organisms. In: Lee CS, Su MS, Liao IL (Eds.), Proceedings of Finfish Hatchery in Asia 91, 17–19 December 1991, Tungking, Taiwan. Tungking Marine Laboratory Conference Proceedings 3, pp 51–59.

- Hirata H, Murata O, Yamada S, Ishitani H, Wachi M (1998) Probiotic culture of the rotifer *Brachionus plicatilis*. *Hydrobiologia* 387: 495-498.
- Hjelm M, Bergh O, Riaza A, Nielsen J, Melchiorsen J, Jensen S, Duncan H, Ahrens P, Birkbeck H, Gram L (2004) Selection and identification of autochthonous potential probiotic bacteria from turbot larvae (*Scophthalmus maximus*) rearing units. *Syst Appl Microbiol* 27: 360-371.
- Holmstrøm K, Gram L (2003) Elucidation of the *Vibrio anguillarum* Genetic response to the potential fish probiont *Pseudomonas fluorescens* AH 2, Using RNA-Arbitrarily Primed PCR. *J Bacteriol* 185: 831-842.
- Holyoak M, Loreau M (2006) Reconciling empirical ecology with neutral community models. *Ecology* 87: 1370-1377.
- Hong HA, Duc LH, Cutting SM (2005) The use of bacterial spore formers as probiotics. *FEMS Microbiol Rev* 29: 813-835.
- Hori T, Haruta S, Ueno Y, Ishii M, Igarashi Y (2006) Direct comparison of single-strand conformation polymorphism (SSCP) and denaturing gradient gel electrophoresis (DGGE) to characterize a microbial community on the basis of 16S rRNA gene fragments. *J Microbiol Methods* 66: 165-169.
- Horner-Devine MC, Leibold MA, Smith VH, Bohannan BJ (2003) Bacterial diversity patterns along a gradient of primary productivity. *Ecol Lett* 6: 613-622.
- Hovda MB (2007) Application of PCR and DGGE to characterise the microflora of farmed fish. Thesis, University of Bergen.
- Hovda MB, Lunestad BT, Fontanillas R, Rosnes JT (2007) Molecular characterisation of the intestinal microbiota of farmed Atlantic salmon (*Salmo salar* L.). *Aquaculture* 272: 581-588.

- Hu K, Yang XL (2006) Current progress of microbial ecological agents in aquaculture in China. *Fish Mod* 6: 36-38. (in Chinese)
- Huang H, Yao Z, Zheng J (1990) The study of the applications of photosynthetic bacteria to aquaculture (3)-The value of using photosynthetic bacteria as the initial feed for fish larvae. *J of Zhanjiang Fish Coll* 10: 58-61. (in Chinese, Abstract in English).
- Huang YC, Wang SL, Yang ZC, Li CN (1999) Effect of effective microorganisms (EM) on the bacteria in intestina tract and proteas activity in the intestine and hepatopancreas of Jian carp. *Fujian Fish* 1: 24-29. (in Chinese)
- Hubbell SP (2005) Neutral theory and the evolution of ecological equivalence. *Ecology* 87: 1387-1398.
- Hubbell SP (2001) *The unified neutral theory of biodiversity and biogeography*. Princeton University Press, Princeton.
- Huber I, Spanggaard B, Appel K, Rossen L, Nielsen T, Gram L (2004) Phylogenetic analysis and *in situ* identification of the intestinal microbial community of rainbow trout (*Oncorhynchus mykiss*, Walbaum). *J Appl Microbiol* 96: 117-132.
- Huervana FH, de la Cruz JJY, Caipang CMA (2006) Inhibition of luminous *Vibrio harveyi* by “green water” obtained from tank culture of tilapia, *Oreochromis mossambicus*. *Acta Ichthyol Piscat* 36 (1): 17-23.
- Hurt RA, Qiu X, Wu L, Roh Y, Palumbo AV, Tiedje JM, Zhou J (2001) Simultaneous recovery of RNA and DNA from soils and sediments. *Appl Environ Microbiol* 67: 4495-4503.
- Hurtle W, Shoemaker D, Henschel E, Norwood D (2002) Denaturing HPLC for identifying bacteria. *Biotechniques* 33: 386-388.

- Huys G, Vancanneyt M, D'Haene K, Vankerckhoven V, Goossens H, Swings J (2006) Accuracy of species identity of commercial bacterial cultures intended for probiotic or nutritional use. *Res Microbiol* 157: 803-810.
- Huys L, Dhert P, Robles R, Ollevier F, Sorgeloos P, Swings J (2001) Search for beneficial bacterial strains for turbot (*Scophthalmus maximus* L.) larviculture. *Aquaculture* 193: 25-37.
- Irianto A, Austin B (2002) Probiotics in aquaculture. *J Fish Dis* 25: 633-642.
- Ishibashi N, Yamazaki S (2001) Probiotics and safety. *Am J Clin Nutr* 73: 465-470.
- Jansen GJ, Wildeboer-Veloo ACM, Tonk RHJ, Franks AH, Welling GW (1999) Development and validation of an automated, microscopy-based method for enumeration of groups of intestinal bacteria. *J Microbiol Methods* 37: 215-221.
- Jasti S, Sieracki ME, Poulton NJ, Giewat MW, Rooney-Varga JN (2005) Phylogenetic diversity and specificity of bacteria closely associated with *Alexandrium spp.* and other phytoplankton. *Appl Environ Microbiol* 71: 3483-3494.
- Jin S, Wang GL, Zhao QS, Lu TX (1999) The inhibition of Chinese herbal medicines to pathogenic bacteria of marine cage-cultured sea bass. *Mar Sci* 5: 8-10. (in Chinese).
- Joint I, Tait K, Callow ME, Callow JA, Milton D, Williams P, Camara M (2002) Cell-to-cell communication across the prokaryote-eukaryote Boundary. *Science* 298: 1207-1207.
- Jorquera MA, Lody M, Leyton Y, Riquelme C (2004) Bacteria of subclass γ -*Proteobacteria* associated with commercial *Argopecten purpuratus* (Lamarck, 1819) hatcheries in Chile. *Aquaculture* 236: 37-51.

- Jürgens K, Matz C (2002) Predation as a shaping force for the phenotypic and genotypic composition of planktonic bacteria. *Antonie Van Leeuwenhoek* 81: 413-434.
- Jurkevitch E (2007) Predatory behaviors in bacteria-Diversity and transitions. *Microbe, Am Soci Microbiol* 2: 67-73.
- Kanazawa A (2003) Nutrition of Marine Fish Larvae. In: Jana BB, Webster CD (eds) *Sustainable Aquaculture: Global Perspectives*. Binghamton, New York.
- Kasai Y, Kishira H, Sasaki T, Syutsubo K, Watanabe K, Harayama S (2002) Predominant growth of *Alcanivorax* strains in oil-contaminated and nutrient-supplemented sea water. *Environ Microbiol* 4: 141-147.
- Keller M, Zengler K (2004) Tapping into microbial diversity. *Nat Rev Microbiol* 2: 141-150.
- Kent AD, Jones SE, Lauster GH, Graham JM, Newton RJ, McMahon KD (2006) Experimental manipulations of microbial food web interactions in a humic lake: shifting biological drivers of bacterial community structure. *Environ Microbiol* 8: 1448-1459.
- Kesarcodi-Watson A, Kaspar H, Lategan MJ, Gibson L (2008) Probiotics in aquaculture: The need, principles and mechanisms of action and screening processes. *Aquaculture* 274: 1-14.
- Kim DH, Austin B (2006) Innate immune responses in rainbow trout (*Oncorhynchus mykiss*, Walbaum) induced by probiotics. *Fish and Shellfish Immunology* 21: 513-524.
- Kobayashi M, Kurata S (1978) The mass culture and cell utilization of photosynthetic bacteria. *Process Biochem* 13: 27-30.

- Kumar R, Mukherjee SC, Prasad KP, Pal AK (2006) Evaluation of *Bacillus subtilis* as a probiotic to Indian major carp *Labeo rohita* (Ham.). *Aquacult Res* 37: 1215-1221.
- Kushal R, Anand SK, Chander H (2006) *In vivo* demonstration of enhanced probiotic effect of co-immobilized *Lactobacillus acidophilus* and *Bifidobacterium bifidum*. *Int J Dairy Technol* 59: 265-271.
- Kysela DT, Palacios C, Sogin ML (2005) Serial analysis of V6 ribosomal sequence tags (SARST-V6): a method for efficient, high-throughput analysis of microbial community composition. *Environ Microbiol* 7: 356-364.
- Laloo R, Ramchuran S, Ramduth D, Gorgens J, Gardiner N (2007) Isolation and selection of *Bacillus* spp. as potential biological agents for enhancement of water quality in culture of ornamental fish. *J Appl Microbiol* 103: 1471-1479.
- Lam P, Cowen JP (2004) Processing deep-sea particle-rich water samples for Fluorescence *in situ* Hybridization: consideration of storage effects, preservation, and sonication. *Appl Environ Microbiol* 70: 25-33.
- Lambert C, Morehouse KA, Chang CY, Sockett RE (2006) *Bdellovibrio*: growth and development during the predatory cycle. *Curr Opin Microbiol* 9: 639-644.
- Lan J, Zhang XH, Wang Y, Chen J, Han Y (2008) Isolation of an unusual strain of *Edwardsiella tarda* from turbot and establish a PCR detection technique with the *gyrB* gene. *J Appl Microbiol* 105: 644-651.
- Langenheder S, Juergens K (2001) Regulation of bacterial biomass and community structure by metazoan and protozoan predation. *Limnol Oceanogr* 46: 121-134.
- Langenheder S, Jurgens K (2001) Regulation of bacterial biomass and community structure by metazoan and protozoan predation. *Limnol Oceanogr* 46: 121-134.

- Lavens P, Sorgeloos P (1996) Manual on the production and use of live food for aquaculture. FAO, Rome, Italy.
- Lee CS, Ostrowski AC (2001) Current status of marine finfish larviculture in the United States. *Aquaculture* 200: 89-109.
- Lee SH, Fuhrman JA (1991) Species composition shift of confined bacterioplankton studies at the level of community DNA. *Mar Ecol Prog Ser* 79: 195-201.
- Lei QL (2007) Promotion for the healthy development of industrial mariculture - appearing of the famous brand turbot with electronic labels on the market. *Oceano Limnol Sinica* 38: 186. (in Chinese)
- Lei QL (2003) Turbot culture technology. Shanghai Science and Technology Publishing House.
- Leonel Ochoa-Solano J, Olmos-Soto J (2006) The functional property of *Bacillus* for shrimp feeds. *Food Microbiol* 23: 519-525.
- Lerner KL, Lerner BW (2003) World of microbiology and immunology. Gale, Detroit.
- Li H, Yu Y, Li Y, Chen G, Ji W, Xu HS (2001) Selection and identification of marine probiotics. *High Technol Lett* 11: 18-22. (in Chinese, Abstract in English).
- Li P, Gatlin III DM (2005) Evaluation of the prebiotic GroBiotic[®]-A and brewers yeast as dietary supplements for sub-adult hybrid striped bass (*Morone chrysops* × *M. saxatilis*) challenged *in situ* with *Mycobacterium marinum*. *Aquaculture* 248: 197-205.
- Li P, Zhu Y, Jiang HY (2002) Application of EM in the cultures of *Macrobrachium rosenbergii*. *J Aquacult* 4: 23-24. (in Chinese).

- Li RW, Waldbieser GC (2006) Production and utilization of a high-density oligonucleotide microarray in channel catfish, *Ictalurus punctatus*. BMC Genomics 7: 134-140.
- Li WJ, Ni YZ (1995) Effective microorganisms research and its application. Chinese J Ecol 14(5): 58-62.
- Liao S, Zheng G, Wang A, Huang H, Sun R (2006) Isolation and characterization of a novel aerobic denitrifier from shrimp pond. ACTA Ecol Sinica 26: 3018-3724. (in Chinese, Abstract in English).
- Lindstroem ES, Bergstroem AK (2004) Influence of inlet bacteria on bacterioplankton assemblage composition in lakes of different hydraulic retention time. Limnol Oceanogr 49: 125-136.
- Liu SX, Xie QS, Yang ZC (2006) Status and prospect of application EM in aquaculture. Hebei Fish 12: 5-7. (in Chinese)
- Liu XM, Nie JH, Wang QR (2002) Research progress in the probiotics of compound microorganisms. Chinese J Eco-Agr 10: 80-83. (in Chinese)
- Lu ZK, Jian JC, Wu ZH, Lu YS, Tang JF (2008) The application of Chinese herbal feed additives in Net-cage culture of *Lutjanus sanguineus*. J Guangdong Ocean U 28: 59-64. (in Chinese, abstract in English).
- Lubzens E, Zmora O, Barr Y (2001) Biotechnology and aquaculture of rotifers. Hydrobiologia 446: 337-353.
- Lubzens E, Rothbard S, Blumenthal A, Kolodny G, Perry B, Olund B, Wax Y, Farbstein H (1987) Possible use of *Brachionus plicatilis* (O.F. Muller) as food for freshwater Cyprinid larvae. Aquaculture 60: 143-155.

- Macey BM, Coyne VE (2005) Improved growth rate and disease resistance in farmed *Halotis midae* through probiotic treatment. *Aquaculture* 245: 249-261.
- Maeda M, Nogami K, Kanematsu M, Hirayama K (1997) The concept of biological control methods in aquaculture. *Hydrobiologia* 358: 285-290.
- Makridis P, Costa RA, Dinis MT (2006) Microbial conditions and antimicrobial activity in cultures of two microalgae species, *Tetraselmis chuii* and *Chlorella minutissima*, and effect on bacterial load of enriched *Artemia metanauplii*. *Aquaculture* 255: 76-81.
- Makridis P, Fjellheim JA, Skjermo J, Vadstein O (2000a) Colonization of the gut in first feeding turbot by bacterial strains added to the water or bioencapsulated in rotifers. *Aquacult Int* 8: 367-380.
- Makridis P, Fjellheim AJ, Skjermo J, Vadstein O (2000b) Control of the bacterial flora of *Brachionus plicatilis* and *Artemia franciscana* by incubation in bacterial suspensions. *Aquaculture* 185: 207-218.
- Mandal D, Bolander ME, Mukhopadhyay D, Sarkar G, Mukherjee P (2006) The use of microorganisms for the formation of metal nanoparticles and their application. *Appl Microbiol Biotechnol* 69: 485-492.
- Marco ML, Pavan S, Kleerebezem M (2006) Towards understanding molecular modes of probiotic action. *Curr Opin Biotechnol* 17: 204-210.
- Marques A, Dhont J, Sorgeloos P, Bossier P (2006a) Immunostimulatory nature of β -glucans and baker's yeast in gnotobiotic *Artemia* challenge tests. *Fish Shellfish Immuno* 20: 682-692.

- Marques A, Huynh Thanh T, Verstraete W, Dhont J, Sorgeloos P, Bossier P (2006b) Use of selected bacteria and yeast to protect gnotobiotic *Artemia* against different pathogens. *J Exp Mar Biol Ecol* 334: 20-30.
- Marques A, Dinh T, Ioakeimidis C, Huys G, Swings J, Verstraete W, Dhont J, Sorgeloos P, Bossier P (2005) Effects of Bacteria on *Artemia franciscana* Cultured in Different Gnotobiotic Environments. *Appl Environ Microbiol* 71: 4307-4317.
- Marques A, Dhont J, Sorgeloos P, Bossier P (2004) Evaluation of different yeast cell wall mutants and microalgae strains as feed for gnotobiotically grown brine shrimp *Artemia franciscana*. *J Exp Mar Biol Ecol* 312: 115-136.
- Martin-Antonio B, Manchado M, Infante C, Zerolo R, Labella A, Alonso C, Borrego JJ (2007) Intestinal microbiota variation in Senegalese sole (*Solea senegalensis*) under different feeding regimes. *Aquacult Res* 38: 1213-1222.
- Martínez-Díaz SF, Álvarez-González CA, Moreno Legorreta M, Vázquez-Juárez R, Barrios-González J (2003) Elimination of the associated microbial community and bioencapsulation of bacteria in the rotifer *Brachionus plicatilis*. *Aquacult Int* 11: 95-108.
- Marzorati M, Wittebolle L, Boon N, Daffonchio D, Verstraete W (2008) How to get more out of molecular fingerprints: practical tools for microbial ecology. *Environ Microbiol* 10: 1571-1581.
- McCaig AE, Glover LA, Prosser JI (2001) Numerical analysis of grassland bacterial community structure under different land management regimens by using 16S ribosomal DNA sequence data and denaturing gradient gel electrophoresis banding patterns. *Appl Environ Microbiol* 67: 4554.

- McCartney AL (2002) Application of molecular biological methods for studying probiotics and the gut flora. *Br J Nutr* 88: 29-37.
- McFall-Ngai MJ (2002) Unseen Forces: The influence of bacteria on animal development. *Dev Biol* 242: 1-14.
- Mertens B, Boon N, Verstraete W (2005) Stereospecific effect of hexachlorocyclohexane on activity and structure of soil methanotrophic communities. *Environ Microbiol* 7: 660-669.
- Míguez B, Combarro MP (2003) Bacteria associated with sardine (*Sardina pilchardus*) eggs in a natural environment (Ria de Vigo, Galicia, northwestern Spain). *FEMS Microbiol Ecol* 44: 329-334.
- Mo Z, Yu Y, Li H, Li Y, Ji, W, Xu HS (2001) Selection of vibrios-antagonism bacteria. *Period Ocean U Qingdao* 31: 225-231.
- Moretti A, Pedini Fernandez-Criado M, Cittolin G, Guidastrì R. (1999) Manual on hatchery production of seabass and gilthead seabream. FAO, Rome, Italy.
- Moriarty DJW (2003) Probiotics in aquaculture. *Microbiol Australia* 24: 15-17.
- Muroga K (2001) Viral and bacterial diseases of marine fish and shellfish in Japanese hatcheries. *Aquaculture* 202: 23-44.
- Muylaert K, Van der Gucht K, Vloemans N, De Meester L, Gillis M, Vyverman W (2002) Relationship between bacterial community composition and bottom-up versus top-down Variables in four eutrophic shallow lakes. *Appl Environ Microbiol* 68: 4740-4750.
- Muyzer G, Smalla K (1998) Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. *Antonie Van Leeuwenhoek* 73: 127-141.

- Muyzer G, de Waal EC, Uitterlinden AG (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl Environ Microbiol* 59: 695-700.
- Nakase G, Eguchi M (2007) Analysis of bacterial communities in *Nannochloropsis* sp. cultures used for larval fish production. *Fish Sci* 73: 543-549.
- Netherwood T, Gilbert HJ, Parker DS, O'Donnell AG (1999) Probiotics shown to change bacterial community structure in the avian gastrointestinal tract. *Appl Environ Microbiol* 65: 5134-5138.
- Neufeld JD, Mohn WW (2005) Unexpectedly high bacterial diversity in arctic tundra relative to boreal forest soils, revealed by serial analysis of ribosomal sequence tags. *Appl Environ Microbiol* 71: 5710-5718.
- Nicholson WL (2002) Roles of *Bacillus* endospores in the environment. *Cell Mol Life Sci* 59: 410-416.
- Nicolas JL, Corre S, Cochard JC (2004) Bacterial population association with phytoplankton cultured in a bivalve hatchery. *Microb Ecol* 48: 400-413.
- Nicolas JL, Robic E, Ansquer D (1989) Bacterial flora associated with a trophic chain consisting of microalgae, rotifers and turbot larvae: influence of bacteria on larval survival. *Aquaculture* 83: 237-248.
- Nikoskelainen S, Ouwehand AC, Bylund G, Salminen S, Lilius EM (2003) Immune enhancement in rainbow trout (*Oncorhynchus mykiss*) by potential probiotic bacteria (*Lactobacillus rhamnosus*). *Fish Shellfish Immunol* 15: 443-452.

- Nocker A, Camper AK (2006a) Selective removal of DNA from dead cells of mixed bacterial communities by use of ethidium monoazide. *Appl Environ Microbiol* 72: 1997-2004.
- Nocker A, Cheung CY, Camper AK (2006b) Comparison of propidium monoazide with ethidium monoazide for differentiation of live vs. dead bacteria by selective removal of DNA from dead cells. *J Microbiol. Methods* 67: 310-320.
- Nold SC, Zwart G (1998) Patterns and governing forces in aquatic microbial communities. *Aquat Ecol* 32: 17-35.
- O'Sullivan DJ (2000) Methods for analysis of the intestinal microflora. *Curr Issues Intest Microbiol* 1: 39-50.
- Olafsen JA (2001) Interactions between fish larvae and bacteria in marine aquaculture. *Aquaculture* 200: 223-247.
- Olsen AI, Olsen Y, Attramadal Y, Christie K, Birkbeck TH, Skjermo J, Vadstein O (2000) Effects of short term feeding of microalgae on the bacterial flora associated with juvenile *Artemia franciscana*. *Aquaculture* 190: 11-25.
- Olsen Y (1997) Larval-rearing technology of marine species in Norway. *Hydrobiologia* 358: 27-36.
- Olsson JC, Joeborn A, Westerdahl A, Blomberg L, Kjelleberg S, Conway PL (1996) Is the turbot, *Scophthalmus maximus* (L.), intestine a portal of entry for the fish pathogen *Vibrio anguillarum*? *J Fish Dis* 19: 225-234.
- Ortells R, Gomez A, Serra M (2003) Coexistence of cryptic rotifer species: ecological and genetic characterisation of *Brachionus plicatilis*. *Freshw Biol* 48: 2194-2202.

- Ortuno J, Esteban MA, Meseguer J (2002) Effects of four anaesthetics on the innate immune response of gilthead seabream (*Sparus aurata* L.). *Fish Shellfish Immunol* 12: 49-59.
- Pankhurst CE, Stirling GR, Magarey RC, Blair BL, Holt JA, Bell MJ, Garside AL (2005) Quantification of the effects of rotation breaks on soil biological properties and their impact on yield decline in sugarcane. *Soil Biol Biochem* 37: 1121-1130.
- Pankhurst CE, Yu S, Hawke BG, Harch BD (2001) Capacity of fatty acid profiles and substrate utilization patterns to describe differences in soil microbial communities associated with increased salinity or alkalinity at three locations in South Australia. *Biol Fertil Soils* 33: 204-217.
- Papakostas S, Dooms S, Christodoulou M, Triantafyllidis A, Kappas I, Dierckens K, Bossier P, Sorgeloos P, Abatzopoulos TJ (2006) Identification of cultured *Brachionus* rotifers based on RFLP and SSCP Screening. *Mar Biotechnol* 8: 547-559.
- Papakostas S, Triantafyllidis A, Kappas I, Abatzopoulos TJ (2005) The utility of the 16S gene in investigating cryptic speciation within the *Brachionus plicatilis* species complex. *Mar Biol* 147: 1129-1139.
- Papandroulakis N, Divanach P, Anastasiadis P, Kentouri M (2002) The pseudo-green water technique for intensive rearing of sea bream (*Sparus aurata*) larvae. *Aquacult Int* 9: 205-216.
- Pareto V (1897). *Le cours d'économie politique*. Macmillan, London, United Kingdom.
- Parvez S, Malik KA, Ah, Kang S, Kim HY (2006) Probiotics and their fermented food products are beneficial for health. *J Appl Microbiol* 100: 1171-1185.

- Patra SK, Mohamed KS (2003) Enrichment of *Artemia* nauplii with the probiotic yeast *Saccharomyces boulardii* and its resistance against a pathogenic *Vibrio*. *Aquacult Int* 11: 505-514.
- Petersen DG, Dahlloef I (2005) Improvements for comparative analysis of changes in diversity of microbial communities using internal standards in PCR-DGGE. *FEMS Microbiol Ecol* 53: 339-348.
- Pineiro M, Stanton C (2007) Probiotic Bacteria: Legislative framework-requirements to evidence basis. *J Nutr* 137: 850-853.
- Planas M, Vazquez JA, Marques J, Perez-Lomba R, Gonzalez MP, Murado M (2004) Enhancement of rotifer (*Brachionus plicatilis*) growth by using terrestrial lactic acid bacteria. *Aquaculture* 240: 313-319.
- Planas M, Cunha I (1999) Larviculture of marine fish: problems and perspectives. *Aquaculture* 177: 171-190.
- Plante S, Pernet F, Hache R, Ritchie R, Ji B, McIntosh D (2007) Ontogenetic variations in lipid class and fatty acid composition of haddock larvae *Melanogrammus aeglefinus* in relation to changes in diet and microbial environment. *Aquaculture* 263: 107-121.
- Pond MJ, Stone DM, Alderman DJ (2006) Comparison of conventional and molecular techniques to investigate the intestinal microflora of rainbow trout (*Oncorhynchus mykiss*). *Aquaculture* 261: 194-203.
- Preston-Mafham J, Boddy L, Randerson PF (2002) Analysis of microbial community functional diversity using sole-carbon-source utilisation profiles- a critique. *FEMS Microbiol Ecol* 42: 1-14.

- Prosser JI, Bohannon BJM, Curtis TP, Ellis RJ, Firestone MK, Freckleton RP, Green JL, Green LE, Killham K, Lennon JJ (2007) Essay: The role of ecological theory in microbial ecology. *Nat Rev Microbiol* 5: 384-392.
- Raa J (1996) The use of immunostimulatory substances in fish and shellfish farming. *Rev Fish Sci* 4: 229-288.
- Raida MK, Larsen JL, Nielsen ME, Buchmann K (2003) Enhanced resistance of rainbow trout, *Oncorhynchus mykiss* (Walbaum), against *Yersinia ruckeri* challenge following oral administration of *Bacillus subtilis* and *B. licheniformis* (BioPlus2B). *J Fish Dis* 26: 495-498.
- Ramette A (2007) Multivariate analyses in microbial ecology. *FEMS Microbiol Ecol* 62: 142-160.
- Rana K, Immink A (2001) *Trends in Global Aquaculture Production: 1984-1996*. Rome, Italy.
- Reitan KI, Rainuzzo JR, ie G, Olsen Y (1997) A review of the nutritional effects of algae in marine fish larvae. *Aquaculture* 155: 207-221.
- Rengpipat S, Tunyanun A, Fast AW, Piyatiratitivorakul S, Menasveta P (2003) Enhanced growth and resistance to *Vibrio* challenge in pond-reared black tiger shrimp *Penaeus monodon* fed a *Bacillus* probiotic. *Dis Aquat Organ* 55: 169-173.
- Rengpipat S, Rukpratanporn S, Piyatiratitivorakul S, Menasaveta P (2000) Immunity enhancement in black tiger shrimp (*Penaeus monodon*) by a probiont bacterium (*Bacillus* S11). *Aquaculture* 191: 271-288.
- Ringo E, Sperstad S, Myklebust R, Refstie S, Krogdahl A (2006) Characterisation of the microbiota associated with intestine of Atlantic cod (*Gadus morhua* L.): The

- effect of fish meal, standard soybean meal and a bioprocessed soybean meal. *Aquaculture* 261: 829-841.
- Ringø E, Olsen RE, Mayhew TM, Myklebust R (2003) Electron microscopy of the intestinal microflora of fish. *Aquaculture* 227: 395-415.
- Ringø E, Birkbeck TH (1999) Intestinal microflora of fish larvae and fry. *Aquacult Res* 30: 73-93.
- Ritar AJ, Dunstan GA, Nelson MM, Brown MR, Nichols PD, Thomas CW, Smith EG, Crear BJ, Kolkovski S (2004) Nutritional and bacterial profiles of juvenile *Artemia* fed different enrichments and during starvation. *Aquaculture* 239: 351-373.
- Rittmann BE, Hausner M, Löffler F, Love NG, Muyzer G, Okabe S, Oerther DB, Peccia J, Raskin L, Wagner M (2006) A vista for microbial ecology and environmental biotechnology. *Environ Sci Technol* 40: 1096-1103.
- Robertsen B (1999) Modulation of the non-specific defence of fish by structurally conserved microbial polymers. *Fish Shellfish Immunol* 9: 269-290.
- Rogosky AM, Moak PL, Emmert EAB (2006) Differential predation by *Bdellovibrio bacteriovorus* 109. *J Curr Microbiol* 52: 81-85.
- Rombaut G, Grommen R, Zizhong Q, Vanhoof V, Suantika G, Dhert P, Sorgeloos P, Verstraete W (2003) Improved performance of an intensive rotifer culture system by using a nitrifying inoculum (ABIL). *Aquacult Res* 34: 165-174.
- Rombaut G (2001) Control of the microbial community in rotifer cultures (*Brachionus plicatilis*). Dissertation, Ghent University.
- Rombaut G, Suantika G, Boon N, Maertens S, Dhert P, Top E, Sorgeloos P, Verstraete W (2001) Monitoring of the evolving diversity of the microbial community

- present in rotifer cultures. *Aquaculture* 198: 237-252.
- Rombaut G, Dhert P, Vandenberghe J, Verschuere L, Sorgeloos P, Verstraete W (1999) Selection of bacteria enhancing the growth rate of axenically hatched rotifers (*Brachionus plicatilis*). *Aquaculture* 176: 195-207.
- Romero J, Navarrete P (2006) 16S rDNA-based analysis of dominant bacterial populations associated with early life stages of coho salmon (*Oncorhynchus kisutch*). *Microb Ecol* 51: 422-430.
- Ronen A, Perelberg A, Abramowitz J, Hutoran M, Tinman S, Bejerano I, Steinitz M, Kotler M (2003) Efficient vaccine against the virus causing a lethal disease in cultured *Cyprinus carpio*. *Vaccine* 21: 4677-4684.
- Roux KH (2002) Single-step PCR optimization using touchdown and stepdown PCR programming. Humana Press, New Brunswick.
- Ruby EG (1991) The genus *Bdellovibrio*. In: The Prokaryotes, 2nd Edn. (Balows A, Truper, HG, Dworkin, M., Harder, W. and Schleifer, KH, Eds.), pp 3400–3415.
- Salinas I, Cuesta A, Esteban M, Meseguer J (2005) Dietary administration of *Lactobacillus delbrückii* and *Bacillus subtilis*, single or combined, on gilthead seabream cellular innate immune responses. *Fish Shellfish Immunol* 19: 67-77.
- Sandaa RA, Magnesen T, Torkildsen L, Bergh (2003) Characterisation of the Bacterial Community Associated with Early Stages of Great Scallop (*Pecten maximus*), Using Denaturing Gradient Gel Electrophoresis (DGGE). *Syst Appl Microbiol* 26: 302-311.
- Santosa S, Farnworth E, Jones PJH (2006) Probiotics and their potential health claims. *Nutr Rev* 64: 265-274.

- Sapp M, Schwaderer A, Wiltshire K, Hoppe H-G, Gerds G, Wichels A (2007a) Species-specific bacterial communities in the phycosphere of microalgae. *Microb Ecol* 53: 683-699.
- Sapp M, Wichels A, Gerds G (2007b) Impacts of cultivation of marine diatoms on the associated bacterial community. *Appl Environ Microbiol* 73: 3117-3120.
- Sastry M, Ahmad A, Khan MI, Kumar R (2003) Biosynthesis of metal nanoparticles using fungi and actinomycete. *Curr Sci* 85: 162-170.
- Schramm A, Larsen LH, Revsbech NP, Ramsing NB, Amann R, Schleifer KH (1996) Structure and function of a nitrifying biofilm as determined by *in situ* hybridization and the use of microelectrodes. *Appl Environ Microbiol* 62: 4641-4647.
- Schulze AD, Alabi AO, Tattersall-Sheldrake AR, Miller KM (2006) Bacterial diversity in a marine hatchery: balance between pathogenic and potentially probiotic bacterial strains. *Aquaculture* 256: 50-73.
- Schwartz A, Le Blay G, Blaut M (2000) Quantification of different *Eubacterium* spp. in human fecal samples with species-specific 16S rRNA-targeted oligonucleotide probes. *Appl Environ Microbiol* 66: 375-382.
- Seksik P, Rigottier-Gois L, Gramet G, Sutren M, Pochart P, marteau P, Jian , Dore J (2003) Alterations of the dominant faecal bacterial groups in patients with Crohn's disease of the colon. *Gut* 52: 237-242.
- Serra M, Gomez A, Carmona MJ (1998) Ecological genetics of *Brachionus* sympatric sibling species. *Hydrobiologia* 387: 373-384.

- Shabarinath S, Sanath Kumar H, Khushiramani R, Karunasagar I, Karunasagar I (2007) Detection and characterization of *Salmonella* associated with tropical seafood. *Int J Food Microbiol* 114: 227-233.
- Sharrer MJ, Summerfelt ST (2007) Ozonation followed by ultraviolet irradiation provides effective bacteria inactivation in a freshwater recirculating system. *Aquacult Eng* 37: 180-191.
- Sheffield VC, Cox DR, Lerman LS, Myers RM (1989) Attachment of a 40-base-pair G+ C-rich sequence (GC-clamp) to genomic DNA fragments by the polymerase chain reaction results in improved detection of single-base changes. *Proc Natl Acad Sci USA* 86: 232-236.
- Shields RJ (2001) Larviculture of marine finfish in Europe. *Aquaculture* 200: 55-88.
- Shumway DL, Koide RT (1995) Size and reproductive inequality in mycorrhizal and nonmycorrhizal populations of *Abutilon Theophrasti*. *J Ecol* 83: 613-620.
- Skjermo J, Salvesen I, Oie G, Olsen Y, Vadstein O (1997) Microbially matured water: a technique for selection of a non-opportunistic bacterial flora in water that may improve performance of marine larvae. *Aquacult Int* 5: 13-28.
- Skjermo J, Vadstein O (1999) Techniques for microbial control in the intensive rearing of marine larvae. *Aquaculture* 177: 333-343.
- Skjermo J, Vadstein O (1993) Characterization of the bacterial flora of mass cultivated *Brachionus plicatilis*. *Hydrobiologia* 255: 185-191.
- Soltanian S, Dhont J, Sorgeloos P, Bossier P (2007a) Influence of different yeast cell-wall mutants on performance and protection against pathogenic bacteria (*Vibrio campbellii*) in gnotobiotically-grown *Artemia*. *Fish Shellfish Immunol* 23: 141-153.

- Soltanian S, Francois JM, Dhont J, Arnouts S, Sorgeloos P, Bossier P (2007b) Enhanced disease resistance in *Artemia* by application of commercial beta-glucans sources and chitin in a gnotobiotic *Artemia* challenge test. Fish Shellfish Immunol 1304-1314.
- Sorgeloos P, Dhert P, Candreva P (2001) Use of the brine shrimp, *Artemia spp.*, in marine fish larviculture. Aquaculture 200: 147-159.
- Sorgeloos P, Dehasque M, Dhert P, Lavens P (1994) Larviculture of marine finfish: the current status. Infofish Int 4: 49-54.
- Spanggaard B, Huber I, Nielsen J, Sick EB, Pipper CB, Martinussen T, Slierendrecht WJ, Gram L (2001) The probiotic potential against vibriosis of the indigenous microflora of rainbow trout. Environ Microbiol 3: 755-765.
- Spiegelman D, Whissell G, Greer CW (2005) A survey of the methods for the characterization of microbial consortia and communities. Can J of Microbiol 51: 355-386.
- Steer T, Carpenter H, Tuohy K, Gibson GR (2000) Perspectives on the role of the human gut microbiota and its modulation by pro-and prebiotics. Nutr Res Rev 13: 229-254.
- Stiling P (2001) Ecology: theories and applications. New Jersey. Prentice-Hall.
- Suantika G, Dhert P, Nurhudah M, Sorgeloos P (2000) High-density production of the rotifer *Brachionus plicatilis* in a recirculation system: consideration of water quality, zootechnical and nutritional aspects. Aquacult Eng 21: 201-213.
- Subasinghe RP (2005) Epidemiological approach to aquatic animal health management: opportunities and challenges for developing countries to increase aquatic production through aquaculture. Prev Vet Med 67: 117-124.

- Subasinghe RP, Curry D, McGladdery SE, Bartley D (2003) Recent technological innovations in aquaculture. In: Review of the state of world aquaculture, FAO Fisheries Circular pp 59-74.
- Subasinghe RP (1997) Fish health and quarantine. Review of the state of the world aquaculture. FAO Fisheries circular 45–49.
- Sugita H, Hirose Y, Matsuo N, Deguchi Y (1998) Production of the antibacterial substance by *Bacillus* sp. strain NM 12, an intestinal bacterium of Japanese coastal fish. *Aquaculture* 165: 269-280.
- Suminto, Hirayama K (1997) Application of a growth-promoting bacteria for stable mass culture of three marine microalgae. *Hydrobiologia* 358: 223-230.
- Sung HH, Kou GH, Song YL (1994) Vibriosis resistance induced by glucan treatment in tiger shrimp (*Penaeus monodon*). *Fish Pathol* 29: 11-17.
- Szymanski N, Patterson RA (2003) Effective Microorganisms (EM) and wastewater systems in future directions for on-site systems: Best management practice. In: Patterson, RA, Jones MJ (Eds). Proceedings of on-site '03 conference, Armidale, Australia, 2003.
- Tamaru CS, Murashige R, Lee CS (1994) The paradox of using background phytoplankton during the larval culture of striped mullet, *Mugil cephalus* L. *Aquaculture* 119: 167-174.
- Tanaka R, Ootsubo M, Sawabe T, Ezura Y, Tajima K (2004) Biodiversity and *in situ* abundance of gut microflora of abalone (*Haliotis discus hannai*) determined by culture-independent techniques. *Aquaculture* 241: 453-463.

- Temmerman R, Pot B, Huys G, Swings J (2003) Identification and antibiotic susceptibility of bacterial isolates from probiotic products. *Int J Food Microbiol* 81: 1-10.
- Tinh NTN, Dierckens K, Sorgeloos P, Bossier P (2008) A Review of the Functionality of Probiotics in the Larviculture Food Chain. *Mar Biotechnol* 10: 1-12.
- Tinh NTN, Linh ND, Wood TK, Dierckens K, Sorgeloos P, Bossier P (2007) Interference with the quorum sensing systems in a *Vibrio harveyi* strain alters the growth rate of gnotobiotically cultured rotifer *Brachionus plicatilis*. *J Appl Microbiol* 103: 194-203.
- Tinh NTN, Phuoc NN, Dierckens K, Sorgeloos P, Bossier P (2006) Gnotobiotically grown rotifer *Brachionus plicatilis* sensu strictu as a tool for evaluation of microbial functions and nutritional value of different food types. *Aquaculture* 253: 421-432.
- Tong GZ, Shi YS, Ma JY (2007) Antibacterial effect of Chinese herbal medicines on pathogenic bacteria in large yellow croaker *Pseudosciaena crocea* in marine cage culture *Fish Sci* 26: 436-439. (in Chinese, abstract in English).
- Tovar D, Zambonino J, Cahu C, Gatesoupe FJ, Vazquez-Juarez R, Lesel R (2002) Effect of live yeast incorporation in compound diet on digestive enzyme activity in sea bass (*Dicentrarchus labrax*) larvae. *Aquaculture* 204: 113-123.
- Tseng CK (1993) Notes on mariculture in China. *Aquaculture* 111: 21-30.
- Van der Lelie D, Lesaulnier C, McCorkle S, Geets J, Taghavi S, Dunn J (2006) Use of single-point genome signature tags as a universal tagging method for microbial genome surveys. *Appl Environ Microbiol* 72: 2092-2101.

- Van Hannen EJ, Zwart G, van Agterveld MP, Gons HJ, Ebert J, Laanbroek HJ (1999) Changes in bacterial and eukaryotic community structure after mass lysis of filamentous cyanobacteria associated with viruses. *Appl Environ Microbiol* 65: 795-801.
- Van Rijn J (1996) The potential for integrated biological treatment systems in recirculating fish culture-A review. *Aquaculture* 139: 181-201.
- Vanya Ewart K, Belanger JC, Williams J, Karakach T, Penny S, Tsoi SCM, Richards RC, Douglas SE (2005) Identification of genes differentially expressed in Atlantic salmon (*Salmo salar*) in response to infection by *Aeromonas salmonicida* using cDNA microarray technology. *Dev Comp Immunol* 29: 333-347.
- Vaseeharan B, Ramasamy P (2003) Control of pathogenic *Vibrio* spp. by *Bacillus subtilis* BT 23, a possible probiotic treatment for black tiger shrimp *Penaeus monodon*. *Lett Appl Microbiol* 36: 83-87.
- Vaughan EE, Mollet B, deVos WM (1999) Functionality of probiotics and intestinal *lactobacilli*: light in the intestinal tract tunnel. *Curr Opin Biotechnol* 10: 505-510.
- Verdonck L, Grisez L, Sweetman E, Minkhoff G, Sorgeloos P, Ollevier F, Swings J (1997) Vibrios associated with routine productions of *Brachionus plicatilis*. *Aquaculture* 149: 203-214.
- Verdonck L, Swings J, Kersters K, Dehasque M, Sorgeloos P, Léger P (1994) Variability of the Microbial Environment of Rotifer *Brachionus plicatilis* and *Artemia* Production Systems. *J World Aquacult Soc* 25: 55-59.

- Verner-Jeffreys DW, Nakamura I, Shields RJ (2006) Egg-associated microflora of Pacific threadfin, *Polydactylus sexfilis* and amberjack, *Seriola rivoliana*, eggs. characterisation and properties. *Aquaculture* 253: 184-196.
- Verner-Jeffreys DW, Shields RJ, Bricknell IR, Birkbeck TH (2003) Changes in the gut-associated microflora during the development of Atlantic halibut (*Hippoglossus hippoglossus* L.) larvae in three British hatcheries. *Aquaculture* 219: 21-42.
- Verschuere L, Rombaut G, Sorgeloos P, Verstraete W (2000) Probiotic bacteria as biological control agents in aquaculture. *Microbiol Mol Biol Rev* 64: 655-671.
- Verschuere L, Dhont J, Sorgeloos P, Verstraete W (1997) Monitoring biologic patterns and r/K-strategists in the intensive culture of *Artemia* juveniles. *J Appl Microbiol* 83: 603-612.
- Verstraete W (2007) Microbial ecology and environmental biotechnology. *ISME J* 1: 4-8.
- Verstraete W, Wittebolle L, Heylen K, Vanparys B, De Vos P, Van de Wiele T, Boon N (2007) Microbial Resource Management: The Road To Go for Environmental Biotechnology. *Eng Life Sci* 7: 117-126.
- Veuger B, Eyre BD, Maher D, Middelburg JJ (2007) Nitrogen incorporation and retention by bacteria, algae, and fauna in a subtropical intertidal sediment: An *in situ* ¹⁵N-labeling study. *Limnol Oceanogr* 52: 1930-1942.
- Vine NG, Leukes WD, Kaiser H (2006) Probiotics in marine larviculture. *FEMS Microbiol Rev* 30: 404-427.

- Von Wintzingerode F, Gobel UB, Stackebrandt E (1997) Determination of microbial diversity in environmental samples: pitfalls of PCR-based rRNA analysis. FEMS Microbiol Rev 21: 213-229.
- Walker R, Buckley M (2005) "Probiotic microbes: the scientific basis." Proceedings of a colloquium held by the American Academy of Microbiology (Baltimore). Washington, DC: American Society for Microbiology.
- Wang A, Zheng G, Liao S, Huang H, Sun R, (2007) Diversity analysis of bacteria capable of removing nitrate/nitrite in a shrimp pond. ACTA Ecol Sinica 27: 1937-1943. (in Chinese, Abstract in English).
- Wang QY, Cai SL, Li J (1997) The shrimp farming industry in China. World Aquacult 28: 23-29. (in Chinese).
- Wang X, Du Z, Chen G, Li Y, Ji W, Xu HS (2002) Application of probiotic A18 to larvae culture of bay scallop (*Argopecten irradians*). Chinese High Technol Lett 8: 86-90.
- Wang X, Sun Z, Liu X, Ma J, Wang B, Song X, Zhu Z, Shi J, Wang F (1994) Application of photosynthetic bacteria in scallop artificial seed breeding. J Fish China 18: 65-68. (in Chinese, Abstract in English).
- Wang B, Yu J, Li Y, Ji W, Xu HS (2002) Isolation and identification of pathogen (*Vibrio harveyi*) from sea perch, *Lateolabrax japonicus*. J Fish Sci of China 1: 52-55.
- Wang Y, Han Y, Li Y, Chen J, Zhang X-H (2007) Isolation of *Photobacterium damsela* subsp. *piscicida* from diseased tongue sole (*Cynoglossus semilaevis* Gunther) in China. ACTA Microbiol Sinica 47, 763-768. (in Chinese, Abstract in English).

- Wang YB, Li JB, Lin J (2008) Probiotics in aquaculture: Challenges and outlook. *Aquaculture* 281: 1-4.
- Ward and Moyer (1966) Ecologic relationships between bacteria and algae in mass culture. Paper presented at the conference on bioregenerative systems, Washington, D.C., 15-16 November, 1966.
- Watanabe T, Kiron V (1994) Prospects in larval fish dietetics. *Aquaculture* 124: 223-251.
- Watanabe T, Tamiya T, Oka A, Hirata M, Kitajima C, Fujita S (1983) Improvement of dietary value of live foods for fish larvae by feeding them on ω 3 highly unsaturated fatty acids and fat-soluble vitamins. *Bull Jpn Soc Sci Fish* 49: 471-479.
- Wei G, Pan L, Du H, Chen J, Zhao L (2004) ERIC-PCR fingerprinting-based community DNA hybridization to pinpoint genome-specific fragments as molecular markers to identify and track populations common to healthy human guts. *J Microbiol Methods* 59: 91-108.
- Wei Q, (2002) Social and economic impacts of aquatic animal health problems in aquaculture in China.. In: Arthur JR, Phillips MJ, Subasinghe RP, Reantaso MB, MacRae IH (Eds.). Primary aquatic animal health care in rural, small-scale, aquaculture development. FAO Fish. Tech. Pap. No. 406. pp55-61.
- Winter C, Smit A, Herndl GJ, Weinbauer MG (2004) Impact of virioplankton on archaeal and bacterial community richness as assessed in seawater batch cultures. *Appl Environ Microbiol* 70: 804-813.
- Wittebolle L, Vervaeren H, Verstraete W, Boon N (2008) Quantifying community dynamics of nitrifiers in functionally stable reactors. *Appl Environ Microbiol* 74: 286-293.

- Wittebolle L, Boon N, Vanparys B, Heylen K, De Vos P, Verstraete W (2005) Failure of the ammonia oxidation process in two pharmaceutical wastewater treatment plants is linked to shifts in the bacterial communities. *J Appl Microbiol* 99: 997-1006.
- Wommack KE, Colwell RR (2000) Virioplankton: Viruses in aquatic ecosystems. *Microbiol Mol Biol Rev* 64: 69-114.
- Wu L, Wang YM, Xu Q, Wei FJ (2004) The influence of EM to the hematological parameters of Southern catfish (*Silurus meriaionalis* Chen). *Feed Res* 5: 7-10. (in Chinese).
- Wu XY, Walker M, Vanselow B, Chao RL, Chin J (2007) Characterization of mesophilic *bacilli* in faeces of feedlot cattle. *J Appl Microbiol* 102: 872-879.
- Xiao H, Li Y, Wang XH, Ji WS, Xu HS (1999) Studies on pathogens of rotted gill and rotted caudal fins of sea perch (*Lateolabrax japonicus*) fry. *J Ocean U Qingdao* 29; 87-93. (in Chinese, Abstract in English).
- Xu B, Zhou X, Xu H, Shi J (1994) Promotion of plant extract on growth of photosynthetic bacteria. *J Ocean U Qingdao* 24: 47-51. (in Chinese, Abstract in English).
- Xu B, Ding M, Mao J, Xu HS (1992) The food value of *Phodopseudomonas spheroides* for *Brachionus plicatilis*. *T Oceanol Limnol* 2: 17-22. (in Chinese, Abstract in English).
- Yair S, Yaacov D, Susan K, Jurkevitch E (2003) Small eats big: ecology and diversity of *Bdellovibrio* and like organisms, and their dynamics in predator-prey interactions. *Agronomie* 23: 433-439.
- Ye MB, Gu HX, Chen HL, Lin RJ (2004). Application of EM in the cultures of

- juvenile green sea turtle (*Chelonia mydas*) China Fish 6: 51-52. (in Chinese)
- Ye Y (1999) Historical consumption and future demand for fish and fishery products: exploratory calculations for the years 2015/2030. *FAO Fisheries Circular* No. 946. Rome, Italy, p 31.
- Yeung PSM, Sanders ME, Kitts CL, Cano R, Tong PS (2002). Species-specific identification of commercial probiotic strains. *J Dairy Sci* 85: 1039-1051.
- Yoshimura K, Tanaka K, Yoshimatsu T (2003) A novel culture system for the ultra-high-density production of the rotifer, *Brachionus rotundiformis* - a preliminary report. *Aquaculture* 227: 165-172.
- Yoshimura K, Hagiwara A, Yoshimatsu T, Kitajima C (1996) Culture technology of marine rotifers and the implications for intensive culture of marine fish in Japan. *Mar Freshwat Res* 47: 217-222.
- Yúfera M (2001) Studies on *Brachionus* (Rotifera): an example of interaction between fundamental and applied research. *Hydrobiologia* 446: 383-392.
- Zambonino Infante JL, Cahu CL (2007) Dietary modulation of some digestive enzymes and Metabolic processes in developing marine fish: Applications to diet formulation. *Aquaculture* 268: 98-105.
- Zhang D, Sun Q, Naihong C, Qiao Z (1988) Isolation and cultivation of photosynthetic bacteria of *rhodospirillaceae* and its application as additive in feeds of fish and prawn. *J Fish China* 12, 367-369. (in Chinese).
- Zhao MS (2002) Roles of *Bdellovibrio bacteriovorus* on the treatment, prevention of shrimp and crab diseases and the application method. *Mod Fish Info*. 17: 14-16. (in Chinese).
- Zhao Y, Li W, Zhou Z, Wang L, Pan Y, Zhao L (2005) Dynamics of microbial

community structure and cellulolytic activity in agricultural soil amended with two biofertilizers. *European Journal of Soil Biology* 41: 21-29.

Zhong Y, Power G (1997) Fisheries in China: Progress, problems, and prospects. *Can J Fish Aquat Sci* 54: 224-238.

Zhou J (2003) Microarrays for bacterial detection and microbial community analysis. *Curr Opin Microbiol* 6: 288-294.

Ziaei-Nejad S, Rezaei MH, Takami GA, Lovett DL, Mirvaghefi AR, Shakouri M (2006) The effect of *Bacillus* spp. bacteria used as probiotics on digestive enzyme activity, survival and growth in the Indian white shrimp *Fenneropenaeus indicus*. *Aquaculture* 252: 516-524.

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Ecology

Excellent bachelor

Thesis: "Studies on the effects of different feeds on the health of larvae of *Penaeus chinensis* and the bacterial analysis in rearing water" performed at the Laboratory of Marine Microbiology. Promoter: Professor Huai-Shu Xu, Grade: A.

1999-2001

Master study in Ghent University, Faculty of Bioscience Engineering

Distinction

Thesis: "Using different formulations probiotics to improve the rotifer (*Brachionus plicatilis*) culture" performed at the Laboratory of Microbial Ecology and Technology. Promoter: Professor Willy Verstraete, Grade: 15/20.

Professional record

1995-1999

Research assistant at Laboratory of Marine Microbiology, Ocean University of Qingdao. Director: Prof. Huai-Shu Xu.

Participated in European Economic Community project: "Diagnosis and control of bacterial diseases in penaeid shrimp hatchery-relationships between microbial flora, nutrition, production techniques and health status of penaeid shrimp".

Participated in National Natural Science Foundation of China (NSFC) project: "Marine biofouling and microbial control".

Participated and assisted 4 times training courses of Marine biotechnology and aquaculture in Chinese Marine Biotechnology Center /BAC/UNESCO

Participated in the International Symposium on Progress and Prospect of Marine Biotechnology (ISPPMB'98).

Authored and co-authored 11 articles in local journals.

2002-2003

Research assistant at Laboratory of Marine Genetics and Breeding. Director: Prof. Zhenmin Bao.

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Doctoral research with Prof. dr. ir. P. Bossier (Laboratory of Aquaculture and Artemia Reference Center, Ghent University) and Prof. Zhenmin Bao (Laboratory of Marine Genetics and Breeding) as scientific promoters. Research was supported by a grant of VLIR scholarship.

Lecturing course "Live food production" for undergraduate students (2003).

Tutor of 5 undergraduate students in bioscience during their graduated thesis (2003-2006).

Collaborator in State Oceanic Administration project "Ocean resource survey-picoplankton and nanoplankton in North yellow sea and Qingdao coastal area".

Collaborator in the Key technology R&D program of Ministry and Science and Technology “Integrated algae and shellfish cultures in shallow seawater-Integration and Demonstration”

Collaborator in the High-tech program of Ministry and Science and Technology “Self-clean anti-fouling coat in deep sea environment”.

Collaborator in 4 NSFC projects “Hemolysin in marine vibrios”, “Functions of hemolysin in *V. harveyi*”, “CO₂ concentration gene clone and expression in *Arthrospira (Spirulina)*” and “Respiratory Metabolism to Sulfide Exposure in *Urechis unicinctus*”

Coordinator in NSFC project “The relationship of disease with the composition and dynamics of microbial communities on sea cucumber and abalone seeds settlement plates”

Co-inventor of Chinese patent n°2006100421190 “A low mucosity algin producing method”.

Publications in recent five years (including international peer-reviewed journals):

Rombaut, G., Grommen, R., Zizhong Qi., Vanhoof, V., Suantika, G., Dhert, P., Sorgeloos, P., Verstraete, W. (2003) Improved performance of an intensive rotifer culture system by using a nitrifying inoculum (ABIL). *Aquaculture Research* 34(2): 165-174. SCI IF: 1.051 (2006)

LI Yun , LV Yan , LI Jun , QI Zizhong , ZHANG Xiao-hua , WANG

Xianghong Xu Huaishu. (2004) Study of heterotrophic bacterial flora, dominant *Vibrios* composition of larvae and postlarvae of *Penaeus chinensis* and its relationship with disease. *Periodical of Ocean University of China*. 34(6):1003-1007 (in Chinese, Abstract in English).

MU Chunhua, BAO Zhenmin, CHEN Gang, HU Jingjie, HAO Lujiang, QI Zizhong, LI Guangxue, (2005) Bacterial diversity in the sediments collected from the Shikoku Basin. *Acta Oceanologica Sinica* 24(3) 114-121. SCI IF: 0.507 (2006)

MU Chun-hua, BAO Zhen-min, CHEN Gang, HAO Lu-jiang, QI Zi-zhong, LAO Jun, LI Guang-xue, HU Jing-jie. (2006) Bacterial diversity in the sediments collected from the Northwest Pacific. *Acta Oceanologica Sinica*. 2006(28) 4 121-128. SCI IF: 0.507 (2006).

Bai, B., Pang, L., Qi, Z., Chen J., Austin, B. and Zhang, X.-H. 2008. Distribution of five vibrio virulence-related genes among *Vibrio harveyi* isolates The Journal of General and Applied Microbiology (in press).

Qi, Z., Dierckens, K., Defoirdt, T., Sorgeloos, P., Boon, N., Bao, ZM., Bossier, P.. Effects of feeding regime and probionts on the diverting microbial communities in rotifer *Brachionus* culture. Aquaculture International (accepted)

Qi, Z., Defoirdt, T., Dierckens, K., Sorgeloos, P., Boon, N., Bao, ZM., Bossier, P. Analysis of the evolution of microbial communities associated with different cultures of rotifer strains belonging to different cryptic species of the *Brachionus plicatilis* species complex. (submitted).

Qi, Z., Zhang, X-H., Bossier, P. Current status and prospect of probiotics in aquaculture of China. (conditional accepted, in revising).

Papers in Conference Proceedings

ZZ. Qi, ZM. Bao, B. Sun, P. Sorgeloos, N. Boon, P. Bossier. 2005. Evaluating The Effect of potential synbiotics on in vitro Antagonism Against A Pathogen Using DGGE. Larvi 2005 fish & Shellfish larviculture Symposium C.I. Hendry, G. Van Stappen, M. Wille and P. Sorgeloos (Eds). European Aquaculture Society Special publication no 36, Oostende, Belgium.

Workshops and course

Workshop: Recirculation in marine fish/shellfish larviculture. 14 Nov. 2003 Ghent university, 't Pand Onderbergen 1 Gent, Belgium.

Workshop: Environment & Ecosystem preservation, 23-26, April 2005, Qingdao, PR China.

Mini-symposium: Biofilm. 13 Nov. 2007. Ghent university, 't Pand Onderbergen 1 Gent, Belgium.

Course: Molecular workshop: Fluorescent *in situ* hybridization (FISH) training course, 5-6, June, 2007, Ocean University of China, Qingdao, P.R. China.

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