Application of PCR and DGGE to characterise the microflora of farmed fish

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Stavanger, August 14, 2007

Introduction

The Norwegian fishing sector represents one of the largest national industries in terms of economic value, and was responsible for 5 % of the Norwegian export revenues in 2006 (Norwegian Seafood Export Council, 2007). The sector provides food of high nutritional quality for the consumers. Fish farming has, during the last decades, become an increasingly important part of the Norwegian fishing industry. In 2006 the total sales of farmed fish was 705 000 tons and accounted for 52 % of the export income (Directorate of Fisheries, 2007; Norwegian Seafood Export Council, 2007). From a producer's point of view, fish is a challenging product. The consumers request fresh, mild-processed products with a long shelf-life. However, fish is a perishable product and a suitable substrate for bacterial growth, in addition to its susceptibility to rapid chemical degradation.

Much research has been carried out on packaging and preservation techniques to prolong the shelf-life and product quality. To date, some of the better, and commercially utilised, methods include packaging in a modified atmosphere or under vacuum.

It is important to have robust methods for a rapid and trustworthy assessment of the total product quality, and to determine the product shelf-life. In addition, it is also necessary to study the effects of quality improving efforts. Traditional quality assessment analyses can be time consuming and not very discriminating for detection of specific bacteria found among the total flora present. Consequently, new complementary techniques have to be introduced. The work herein describes the application of molecular methods to detect and characterise the bacterial flora of farmed fish. Furthermore, the methods have been used to study the effects of shelf-life prolonging treatment and packaging.

Abstract

Farming of fish has become an increasingly important part of the Norwegian fishing industry. Furthermore, the Norwegian fishing sector represents one of the largest national industries in terms of economic value. Fish is a perishable product and a suitable substrate for chemical degradation and bacterial growth. Determinations of shelf-life of fish and fish products have traditionally been based on microbial, chemical and sensory evaluation. It is, however, important to have methods for a rapid and reliable assessment of the microflora, and to aid in the determination of the shelf-life of the fish products. This thesis discusses and describes the use of PCR and denaturing gradient gel electrophoresis (DGGE) to detect and characterise the microflora of farmed Atlantic halibut and cod. The aims were to introduce and apply molecular biological methods for characterisation of the microflora, and to use these methods to detect the changes in the microflora as a function of shelf-life extending treatments. An additional aim was to compare the results obtained from molecular-based and culture-based methods.

The DGGE and subsequent sequencing approach displayed the bacterial flora of the farmed fish, and identified the predominant microflora. When applying the sequencing approach, *Photobacterium* spp., *Pseudomonas* spp., *Brochothrix thermosphacta*, *Serratia* sp., *Yersinia* sp., *Micrococcus luteus* and *Shewanella* spp. were found to be the predominant bacteria in farmed Atlantic cod and halibut, stored under modified atmosphere (MA). The method detected a more diverse bacterial flora than previously obtained when culture-based methods were applied.

Bacterial DNA extracted directly from the sample, without prior cultivation, gave a more diverse bacterial community. Furthermore, the molecular methods have been used to study the effects of MA packaging and ozone treatment on the microflora composition. There was no observable effect of ozone treatment of farmed cod.

List of publications

Paper I Hovda, M. B., Sivertsvik, M., Lunestad, B. T., Lorentzen, G. & Rosnes, J. T. (2007): Characterisation of the dominant bacterial population in modified atmosphere packaged farmed halibut (*Hippoglossus hippoglossus*) based on 16S rDNA-DGGE. Food Microbiology, Vol. 24: 362-371.

Paper II Hovda, M. B., Lunestad, B. T., Sivertsvik, M. & Rosnes, J. T. (2007): Characterisation of the bacterial flora of modified atmosphere packaged farmed Atlantic cod (*Gadus morhua*) by PCR-DGGE of conserved 16S rRNA gene regions. International Journal of Food Microbiology, Vol. 117: 68-75.

Paper III Hovda, M. B., Sivertsvik, M., Lunestad, B. T. & Rosnes, J. T. (2007): Microflora assessments using PCR-DGGE of ozone-treated and modified atmosphere packaged farmed cod fillets. Journal of Food Protection, in press, to be published in Vol. 70.

Paper IV Hovda, M. B., Lunestad, B. T., Fontanillas, R. & Rosnes, J. T.: Molecular characterisation of the intestinal microbiota of farmed Atlantic salmon (*Salmo salar* L.). Aquaculture. Submitted.

These four papers are referred to in the text by their roman numerals.

Contents

ACKNOWLEDGEMENTS **4**

INTRODUCTION **5**

ABSTRACT **7**

LIST OF PUBLICATIONS **9**

CONTENTS **11**

1. INTRODUCTION **13**

1.1 MICROBIAL DIVERSITY 13

1.2 MOLECULAR METHODS DESCRIBING MICROBIAL DIVERSITY 13

1.3 PRIMERS FOR BACTERIAL DIVERSITY EXAMINATION 14

1.4 DENATURING GRADIENT GEL ELECTROPHORESIS (DGGE) 16

1.5 DGGE OF BACTERIA FROM FOODS AND FISH PRODUCTS 19

1.6 FARMED FISH 20

2. AIMS OF THE STUDY **23**

3. SHELF-LIFE EXTENSION AND PREDICTION OF FARMED FISH PRODUCTS **24**

3.1 PRODUCT DETERIORATION 24

3.2 BACTERIAL SPOILAGE OF FISH 25

3.3 QUALITY PARAMETERS 28

3.4 MODIFIED ATMOSPHERE PACKAGING 30

3.5 OZONE TREATMENT 32

4. METHODOLOGICAL CONSIDERATIONS FOR THE MOLECULAR METHODS **34**

4.1 LIMITATIONS OF MOLECULAR METHODS AND DGGE 34

4.2 BACTERIAL DETECTION LIMITS 37

5. BACTERIAL FLORA OF FARMED FISH **40**

5.1 DETECTION OF THE MICROFLORA OF FARMED COD AND HALIBUT 40

5.2 DNA EXTRACTION FROM THE FISH MATRIX AND CULTIVATED BACTERIA 45

5.3 *SHEWANELLA* SPECIES IN FARMED FISH DURING STORAGE 46

5.4 DETECTION OF INTESTINAL FLORA OF FARMED SALMON 48

5.5 ENHANCED SHELF-LIFE BY IMPROVED HYGIENIC HANDLING 50

5.6 CONCLUDING REMARKS 52

6. FURTHER PERSPECTIVES 54

REFERENCES 55

1. Introduction

1.1 Microbial diversity

Researchers worldwide have been interested in the differences in microbial composition of various environmental samples. The knowledge about bacterial diversity is useful for understanding the nature of the sample to be studied. Studies of the microflora and its relation to ecosystems have traditionally focused on the cultivable fraction of the bacteria present. It is, however, well known and established within environmental ecology research that only a minor proportion, typically 1 %, of the total bacteria present are cultivable on agar (Amann *et al.*, 1995). Important questions to be raised when studying bacterial floras of environmental samples are how bacterial communities respond to environmental changes, and how the microflora interact and are dependent on the species in the composition.

Traditional analyses of microbiota use cultivation on specific or non-specific growth agars. These methods include colony isolation, phenotypic characterisation, including morphology, and biochemical testing. The weaknesses of phenotypic methods include poor reproducibility and discriminatory power, laboriously investigations, and the ambiguity of some techniques caused by complex growth conditions. Alternatively, genotypic methods, studying the bacterial DNA can be introduced. Genotypic techniques have also limitations, such as costly equipment and procedures, and there is often a need for databases for analyses.

1.2 Molecular methods describing microbial diversity

Molecular methods provide an outstanding tool for detection, identification and characterisation of microorganisms found in environmental samples, foods and other complex ecosystems. Applications of culture independent molecular methods are

needed to improve our understanding of the total microbiota, as the conventional culture-based methods are selective and do not cover the entire microbial diversity of complex environments.

Brosius *et al.* (1978) described the complete nucleotide sequence of the 16S ribosomal DNA (rDNA) from *Escherichia coli*. Thereafter, researchers have been able to design primers suitable for detection of single bacterial species, as well as universal primers intended for population analyses. The 16S rDNA has several attributes that make it suitable for bacterial identification. In particular, 16S rDNA occurs in all bacteria and consists of both variable and conserved regions that can be used for species differentiation. Genes that encode the 16S rDNA can be sequenced to identify an organism's taxonomic group and determine relationships between organisms. In addition, the 16S rDNA is described as molecular chronometers. The 16S rDNA has advantages as a chronometer as it shows a high degree of functional constancy, is large enough for adequate amounts of information, and consists of many domains (Woese, 1987). Furthermore, the 16S rDNA can be easily, and thereby rapidly, sequenced. Online electronic databases with large amounts of available sequences, e.g. the EMBL database from the European Bioinformatics Institute (http://www.ebi.ac.uk/embl/), and the BLAST database at NCBI (http://www.ncbi.nlm.nih.gov/BLAST/), allows direct comparison of the achieved sequences. When unknown bacterial populations and species are to be identified, 16S or 23S rDNA sequencing provides powerful tools with high discriminatory power (Vandamme *et al.*, 1996).

1.3 Primers for bacterial diversity examination

Examination of the bacterial diversity in a food sample can be performed by PCR amplification of the bacterial DNA. One of the most common target regions for PCR amplification is the 16S rDNA, and several primers have been employed to amplify variable regions of the rDNA. Universal primers amplifying one or more

hypervariable regions of the 16S rDNA can be used to detect the dominant bacteria in a sample. The review of Ercolini (2004) summarises primers targeting the different variable regions of the 16S rDNA and their application in food products. Recently, Cocolin *et al.* (2007) published a study where common primers used for profiling bacteria in foods were evaluated and the DGGE conditions and fluorescence *in situ* hybridisation were optimised. The 16S rDNA primers can be universal, targeting theoretically all bacteria from a sample, or they can be species-specific and detect specific bacterial groups such as lactic acid bacteria (LAB). When used on bacterial samples from food, the variable region V3 is extensively used.

The heterogeneity of 16S rDNA, resulting in multiple copies of the sequence, is one of the disadvantages when using it as a target region for amplification (Nübel *et al.*, 1996). The average number of 16S rRNA genes per genome is reported to be 4.1 for the domain *Bacteria* (Klappenbach *et al.*, 2001). Schmalenberger *et al.* (2001) found that the heterogeneity varied between the different variable regions on 16S rDNA. For the regions V2-V3, they found an average of 2.2 bands per organism, evaluated as single-strand-conformation polymorphism (SSCP) bands. For the V4-V5 region, 1.7 bands were detected, whereas 2.3 bands were found in the V6-V8 region.

Other target regions or genes have been suggested to overcome the disadvantages of 16S rDNA. The RNA polymerase beta subunit gene (*rpo*B) appears to be present in only one copy, and has shown a high level of discrimination between species for some groups (Dahllöf *et al.*, 2000; Qi *et al.*, 2001). However, the use of *rpo*B presents a taxonomic disadvantage as the database of the sequence is less documented than that of 16S rDNA. During the last few years, the *rpo*B gene has been used to study specific bacteria such as LAB (De Angelis *et al.*, 2007; Rantsiou *et al.*, 2004; Renouf *et al.*, 2006a; 2006b) and *Yersinia* spp. (Cocolin & Comi, 2005), in foods and food products. Another approach for studying diversity uses group-specific primers or amplification of bacterial functional genes. Functional genes are especially suitable when investigating structure-function relationships (Dahllöf, 2002).

1.4 Denaturing Gradient Gel Electrophoresis (DGGE)

A large number of molecular methods have been developed for examination of microorganisms in complex samples. Denaturing gradient gel electrophoresis (DGGE) is a widely used fingerprinting method for detection of the bacterial population and the diversity in a sample. In food related research, DGGE has been used with success for several products. Fischer and Lerman (1983) were the first to describe the theoretical aspects of this method. The principle of DGGE is the separation of DNA fragments with differences in the base sequence, and the ability of the double-stranded DNA to melt, i.e. partially revert from double to single-stranded DNA, in a polyacrylamide gel, with an electric current. The DGGE separates DNA fragments of the same length, based on differences in the GC content and GC distribution of the DNA fragments. Molecules with different sequences have different melting behaviours in a polyacrylamide gel containing a gradient of DNA denaturants, such as a mixture of urea and formamide. The DNA sequence consists of "melting domains", defined as stretches of base-pairs with an identical melting temperature, which is sequence-specific (Muyzer & Smalla, 1998). Once the melting temperature of the lowest melting domain is reached at a particular position in the denaturing gradient gel, the fragment becomes partially melted and the molecule becomes branched. This will result in a decreased migration through the gel (Figure 1). Based on this principle, a sample containing many different bacteria, all with different melting domains, will result in many bands on the gel. Comparing different samples will result in different profiles, reflecting the bacterial diversity of the sample. To prevent the complete dissociation of the double-stranded DNA, a 30-50 base pair GC-rich sequence is attached to the 5'-end of one of the primers (Sheffield *et al.*, 1989). This GC-clamp acts as a melting restrictive domain (Figure 1).

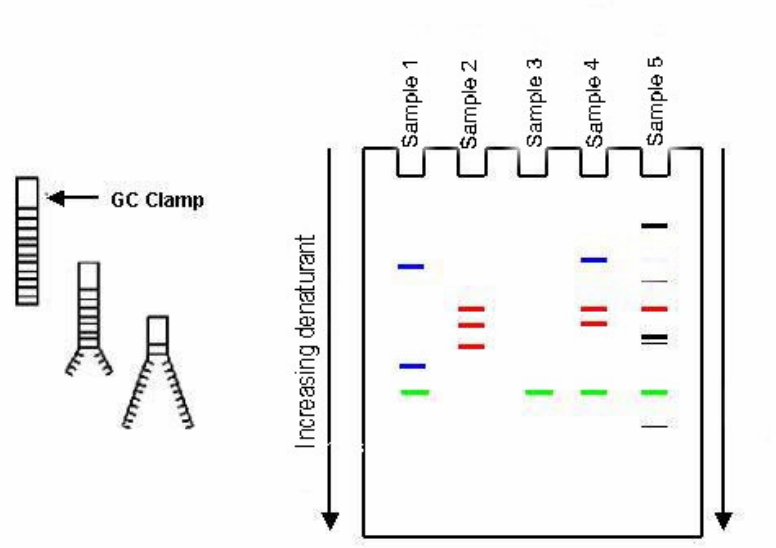

Figure 1. The principle of denaturing gradient gel electrophoresis (DGGE). Double stranded DNA fragments (amplicons) of equal length, obtained by PCR, are separated on a polyacrylamide gel with a denaturing gradient. The increasing gradient of denaturants causes the double-stranded DNA to melt and thus, separate as the PCR products move through the gel. The GC-clamp attached to the 5'-end of the PCR fragment prevents the amplicons from complete denaturation. After DGGE separation, each band on the gel will theoretically represent DNA fragments from specific bacteria having different base pair compositions.

For initial fingerprinting analysis, the DGGE gel can be used directly. The bacterial profiles from the gel are also useful when analysing multiple samples over time, and to reveal profile differences. Time studies can also be achieved when samples taken at different time points are compared on the same gel.

To identify the origin of DNA in gel bands of special interest, the bands can be recovered from the gel and sequenced. By sequencing the band, the bacteria present in the sample can be determined, based on the DNA sequence information. Figure 2 gives an overview of the process flowsheet, from the point at which the fish is sampled, to when the bacterial diversity is identified.

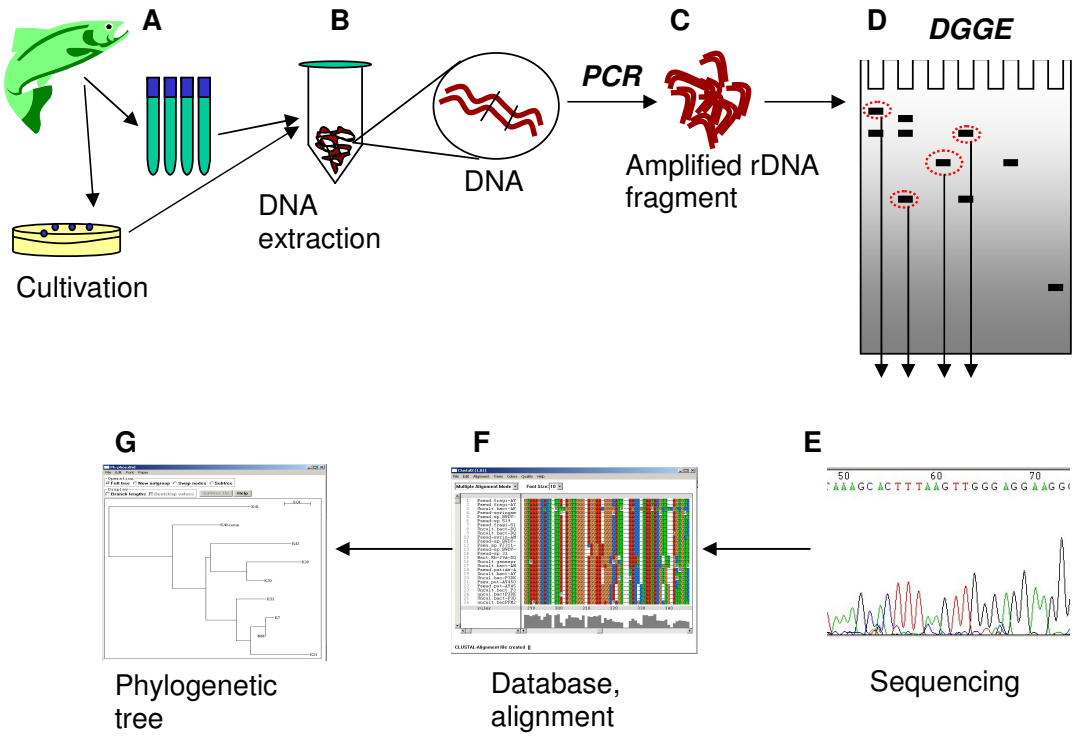

Figure 2. Flowsheet of the process form fish sampling to bacterial detection and identification. Samples from the fish are taken directly for DNA extraction or for cultivation on growth media (A). In B, bacterial DNA is extracted using standard procedures, or kits, before the DNA is amplified using PCR (C). The PCR products are separated on a denaturing gradient gel (D), and bands of interest are excided and sequenced (E). For further comparison of the bands, the sequences can be aligned in suitable programs, such as ClustalX (F), and a phylogenetic tree can be made to display similarities graphically (G).

Using the 16S fragment of the rRNA gene, one can describe both cultivable and uncultivable bacteria by their phylogenetic relationship. The DGGE approach represents a rapid and reproducible method of studying population dynamics, and is well-suited for time interval studies, e.g. shelf-life studies (Cocolin *et al.*, 2001; Giraffa & Neviani, 2001). Applying this method, information about the bacterial profiles of the sample can be achieved within 24 hours (Figure 2, A-D) (Temmerman *et al.*, 2004).

1.5 DGGE of bacteria from foods and fish products

Overall, DGGE is one of the most used molecular methods for studying bacterial diversity and microbial changes in food products. Both the total microbial composition and specific bacteria have been analysed by this technique for several foods.

One of the first paper published using this cultivation-independent method on food discussed the distribution of microorganisms in Mexican pozol, which is fermented maize dough (Ampe *et al.*, 1999). In this study, lactic acid bacteria (LAB) were identified as the predominant bacteria present in the samples, and the authors obtained information about the possible biological role of LAB and the dynamic changes during fermentation. They demonstrated that the ecology of fermented foods cannot be effectively studied by cultivation methods alone, and concluded that cultivation-independent methods should be used in such studies. After this publication describing the use of DGGE for foods, numerous papers have been published using this method. Most of these works have been within the research area of fermented food and detection of LAB (Fontana *et al.*, 2005; Rantsiou *et al.*, 2004). Since the first publication, various food products have been studied using the DGGE approach e.g. fermented Italian sausage (Cocolin *et al.*, 2001), Argentinean sausage (Fontana *et al.*, 2005), ham and Viennese sausage (Takahashi *et al.*, 2004), asparagus (Yergeau *et al.*, 2005), Mozzarella cheese (Coppola *et al.*, 2001), Stilton cheese (Ercolini *et al.*, 2003), Spanish blue-veined Cabrales cheese (Florez & Mayo, 2006), mineral water (Dewettinck *et al.*, 2001), wine (Lopez *et al.*, 2003) and farmed cod and halibut (**Paper I**-**III**), among others. Despite the wide application of DGGE to describe food microbiology and the monitoring of bacterial changes during packaging and storage, there are few papers discussing the use of this technique for fish and fish products.

Yang *et al.* (2007) studied DGGE and subsequent sequencing to detect the bacterial community of the skin, intestine, liver, and ovary of puffer fish (*Takifugu obscurus*).

The authors used the variable V3 16S rDNA region as the target, and detected a wide variety of bacteria in the various parts of the fish. Huber *et al.* (2004) also used DGGE to detect the microflora of fish intestine. Another approach using DGGE with respect to fish and seafood was the detection of the bacterial diversity in marine hatchery (Schulze *et al.*, 2006). Furthermore, DGGE has been used to study the bacterial community associated with Atlantic halibut larvae (Jensen *et al.*, 2004) and for the early life stage of Coho salmon (Romero & Navarrete, 2006).

Another aspect where DGGE-based methods have been shown to be useful is in product authentication in situations of claimed commercial frauds. Some producers substitute the product partially or entirely with species of lower commercial value. This illegal activity has been seen for Atlantic salmon, where rainbow trout has been substituted, and for cod (*Gadus morhua*) being substituted with other cod-fish. Not only is this practice illegal, but other important issues to consider include allergic reactions to specific species, as well as the ethical and religious concerns. PCR and DGGE have been used as a method to differentiate between species of cod-fish (Comi *et al.*, 2005) and to discriminate Atlantic salmon from rainbow trout (Zhang *et al.*, 2007). Differentiation between fish species may be based on differences in the cytochrome *B* gene. The authors used DGGE analysis in addition to other molecular-based techniques, but did not provide consistent conclusions. While Comi *et al.* (2005) found DGGE to have the best discriminative level for detection between cod-fish, Zhang *et al.* (2007) suggested using a combined method of DGGE, amplified fragment length polymorphism (AFLP) and a species-specific sequence characterised amplified region (SCAR) marker.

1.6 Farmed fish

The aquaculture industry is probably the fastest growing food-producing industry in the world. Today, approximately 50 % of all fish produced for consumption is aquacultured (FAO, 2007). Atlantic salmon (*Salmo salar*) is the main species for

Norwegian aquaculture production, accounting for 80 % of the total fish farmed (FAO, 2007). Lately, interest has become oriented towards new species such as Atlantic cod, Atlantic halibut, wolf-fish, turbot, Artic char, lobster, red king crab, blue mussels, and sea urchin (Directorate of Fisheries, 2007). The production of these species is increasing and some are now in the process of being commercialised. As new farmed fish species, they are important for the Norwegian farming industry. This industry is known for its high-quality products. To ensure this position, research and knowledge about the raw material composition, quality parameters, storage conditions, and bacterial and chemical degradation has to be gained for the new species.

The interest in farming has increased as a result of variations in wild catches from year to year, and the quality differences in wild caught fish. These differences are due to seasonal variations, different handling, fishing gear, and fishing ground. The time between catch and processing will, in addition, strongly influence the quality. Compared to wild caught fish, farmed fish have several advantages as a raw material, showing a more uniform quality. Farming also provides controlled feeding, slaughtering and processing, and enables the possibility to trace the whole valued chain to the market.

Atlantic cod is an important species from the northern Atlantic, both economically and socially. As decreasing catches and high prices are reported for wild caught cod, farming becomes more interesting for the aquaculture industry. Furthermore, focus has been drawn towards ecologically and environmentally friendly production. As a result of this, the term “sustainable seafood” has been introduced, indicating usage of 100 % sustainable natural fish feed, such as off-cuts of herring and mackerel already caught for human consumption (Dybdal, 2007).

Only some investigations compare wild and farmed fish of the same species. These studies conclude that there is difference in quality between wild and farmed fish, as observed for Atlantic halibut (Olsson *et al.*, 2003), Murray cod (De Silva *et al.*,

2004), and Atlantic cod (Herland *et al.*, 2007). Herland *et al.* (2007) studied farmed and wild Atlantic cod and found the microbial counts in farmed cod to be significantly lower than for wild. The authors also observed differences in other quality parameters, such as lowered trimethylamine oxide (TMAO) content in farmed cod and a different body composition of farmed cod compared to wild. Other attributes have also been studied, including the higher condition factor, smaller head and liver for farmed Atlantic cod (Gildberg, 2004) and higher carbohydrate level and lower pH in the muscle (Rustad, 1992). Farmed halibut has been reported to have lower pH and higher fat content compared with wild (Olsson *et al.*, 2003).

The observed differences between farmed and wild caught fish, makes the evaluation of bacterial composition of farmed fish important. Introduction of molecular-based techniques, in addition to cultivation-based methods, are suitable for such analysis.

2. Aims of the study

The aims of this work were to:

1. Apply molecular methods as an analytic tool for detection and identification of the general bacterial flora and specific spoilage bacteria of farmed fish.

2. Evaluate the effect of improved hygienic handling and processing of farmed fish using molecular methods.

This was achieved by:

1. Introduction of the molecular methods PCR, DGGE and sequencing to detect and characterise the microflora of farmed Atlantic halibut and cod.

2. Characterisation and detection of the microflora of farmed Atlantic halibut and cod, stored under modified atmosphere (**Paper I** and **II**).

3. Evaluation of the hygiene enhancing effects of ozone pre-treatment, before packaging and storage of farmed cod (**Paper III**).

4. Comparison of the intestinal bacterial flora using molecular-based methods and traditional cultivation-based methods (**Paper IV**).

3. Shelf-life extension and prediction of farmed fish products

3.1 Product deterioration

In all ecosystems, the growth and metabolic activity of microorganisms are important and crucial factors for the turnover of organic and inorganic material. Bacterial growth will occur in all foods, except for sterile products, and may result in product spoilage over time. Food spoilage is defined as changes that make a product unacceptable for human consumption. For example, such changes can include visible bacterial growth, slime formation, physical damage or off-odour. The process collectively known as food spoilage is a very complex event, in which a combination of microbial and biochemical or chemical activities interact.

The parameters responsible for microbial spoilage in foods can be divided into four groups (Huis in't Veld, 1996):

i) Intrinsic parameters

This parameter includes physical, chemical, and structural properties of the food itself, such as water activity, pH, redox potential, available nutrients, and natural antimicrobial substances.

ii) Extrinsic parameters

Environmental factors such as storage time, temperature, humidity, and the composition of the storage atmosphere.

iii) Modes of processing and preservation

Processing can change the characteristics of the food product, and thereby the microorganisms associated with the product.

iv) Implicit parameters

These are mutual factors that synergistically or antagonistically influence microbial growth. Growth of one bacterial sub-population may affect other sub-populations in a food product.

It is important to stress that the parameters are influenced by the effects of the others. The overall effect, as a combination of parameters, is generally much higher than the perceived effect of each individual parameter.

3.2 Bacterial spoilage of fish

The quality of fresh fish and seafood products is rapidly reduced as a consequence of various microbial, biochemical and chemical breakdown processes. The initial quality loss is mainly due to the *post mortem* autolytic activity and chemical degradation processes, such as lipid oxidation. The rate of quality loss depends directly on the nature of the fish species in question, as well as handling and storage conditions. In the mid and later stages of product shelf-life, the microbial contribution to quality reduction increases (Huss, 1995).

Analyses of the microbial load and diversity are used to determine the amount of specific spoilage bacteria in a sample as well as the total bacterial count. Bacterial detection on agar media may reduce the bacterial diversity, as agar media are selective by their composition, and by the incubation conditions, such as atmosphere and temperature.

The bacterial spoilage flora contributes to the degradation of fish by producing off-odour and off-flavour. For marine fish stored aerobically on ice, the bacterial flora is well studied, and dominated by *Pseudomonas* spp. (Gram & Huss, 1996), in addition to *Shewanella putrefaciens*, *Shewanella baltica*, *Shewanella hafniensis*, and *Shewanella morhuae* (Gram *et al.*, 1987; Satomi *et al.*, 2006; Vogel *et al.*, 2005). From storage in modified atmosphere enriched by CO_2, *Photobacterium phosphoreum* have been reported as the most important spoilage bacterium (Dalgaard *et al.*, 1993), whereas *Brochothrix thermosphacta* (López-Gálvez *et al.*, 1995; Pournis *et al.*, 2005) represented the main spoilage bacterium in fish from the Mediterranean.

Knowledge of spoilage organisms and their specific activity in various fish species at different storage conditions has led to more precise shelf-life predictions and facilitated modelling of spoilage (Gram & Dalgaard, 2002). A mathematical model: "The Seafood Spoilage and Safety Predictor (SSSP)" has been developed by Dalgaard *et al.* (2002) for estimation of the remaining shelf-life. This software uses both kinetic models for growth of specific spoilage microorganisms, and empirical relative rates of spoilage models to determine the shelf-life based on the initial amount of *P. phosphoreum* in the sample.

The most important spoilage bacteria of marine fish from cold waters, and spoilage bacteria identified in the present work are described in the following.

Photobacterium phosphoreum

P. phosphoreum is a Gram-negative rod with bioluminescent ability. It belongs to the *Vibrionaceae* family. *Photobacterium* spp. are common in the marine environment and present in the intestinal contents of marine animals. The bacterium is cold tolerant and thus able to grow at low temperatures (4 ^{o}C), but not at higher temperatures (> 40 ^{o}C). In addition, Na^+ ions are required for growth (Krieg & Holt, 1984). The fish spoilage potential of *P. phosphoreum* is, to a large extent, due to the ability of the bacterium to grow in high CO_2 concentrations, and its active reduction

of trimethylamine oxide (TMAO) to trimethylamine (TMA) (Gram & Huss, 1996). This TMA production results in off-odour and off-flavour during storage (Gram & Dalgaard, 2002). *P. phosphoreum* is a large bacterium (5 µm) and produces a relatively high amount of TMA per cell (Dalgaard, 1995b). The bacterium is regarded as the main spoilage bacterium in fish products stored under high CO_2 concentrations e.g. in modified atmosphere (Gram & Huss, 1996).

Shewanella spp. and S. putrefaciens

The bacterium *Shewanella* is a facultative anaerobe Gram-negative, oxidase- and catalase- positive rod in the *Shewanellacea* family. *S. putrefaciens* is regarded as a specific spoilage bacterium of marine fish from temperate water, stored aerobically in ice (Gram *et al.*, 1987; Gram & Huss, 1996). This species is capable of anaerobic respiration using TMAO as the terminal electron acceptor (Dalgaard *et al.*, 1993; Jørgensen *et al.*, 1988), and production of H_2S from the sulphur containing amino acid L-cysteine (Jørgensen & Huss, 1989). Production of H_2S results in a foul off-odour. The former group of *S. putrefaciens* is known for being phenotypically heterogeneous. Members of this group have recently been reclassified, based on 16S rRNA gene sequencing, into several species including some new strains. *S. baltica*, *S. hafniensis* and *S. morhuae*, among others, were found in ice-stored fish after reclassification (Satomi *et al.*, 2006; Vogel *et al.*, 2005).

Traditionally, the detection of the spoilage bacteria *S. putrefaciens* has been performed on Iron agar supplemented with L-cysteine, where the bacterium form black colonies. From previous analyses of ice-stored fish fillets, the majority of black colony forming bacteria on Iron agar were found to be *S. putrefaciens* (Dalgaard *et al.*, 1993; Gram *et al.*, 1987), although formation of H_2S can also occur among members of the family *Vibrionaceae* (Gram *et al.*, 1987; Lund *et al.*, 2000).

Pseudomonas

Pseudomonas is a large and poorly defined group of microorganisms. The genus is in the group of aerobe Gram-negative, catalase- and oxidase-positive rods. Many species have a psychrophilic nature and are regarded as part of the natural flora of fish. The species can form aldehydes, ketones, esters and sulphides following food spoilage, causing odours described as fruity, rotten and sulfhydryl-like (Lund *et al.*, 2000). *Pseudomonas* is sensitive to CO_2 in concentrations as low as 20 % (Eyles *et al.*, 1993), and the removal of oxygen, as under vacuum packaging.

Brochothrix thermosphacta

This bacterium is a Gram-positive, non-sporing, non-mobile and facultative anaerobe rod. It has a growth temperature range between 0 and 30 ^{o}C, and the ability to grow in high CO_2 concentrations. The bacterium is found during spoilage of modified atmosphere packaged fish and meat (Borch *et al.*, 1996; Lund *et al.*, 2000; Pournis *et al.*, 2005; Stamatis & Arkoudelos, 2007). *B. thermosphacta* gives a sour odour due to lactic acid production under oxygen-free atmospheres, whereas production of acetoin-diacetyl occurs in oxygen rich atmospheres (Pin *et al.*, 2002). The bacterium produces short fatty acids under aerobic conditions, however lower amounts are reported in tuna than in meat (López-Gálvez *et al.*, 1995).

3.3 Quality parameters

As a fresh and slightly processed product, the fish needs to be of good quality to be regarded as a foodstuff. Shelf-life and quality descriptions are based on a combination of several factors.

Microbial analyses

The available literature on bacterial spoilage and deterioration of fish during storage is ample. Depending on the fish species and the storage conditions, different bacterial groups contribute to the quality loss to a varying degree. Microbial analyses have

traditionally been performed using general or selective media for enumeration of total viable counts or specific bacteria. Detection of bacteria on fish from the Northern Atlantic typically involves media for determination of psychrotrophic bacteria, H_2S producing bacteria, and for total viable counts (Table 1). Using such media, one can detect the most important spoilage flora of the fish, and estimate the remaining shelf-life.

Table 1. Commonly applied agar media used for enumeration and detection of bacteria associated with fish spoilage.

Bacterium	**Medium**	**Reference**
P. phosphoreum	Plate Count Agar, (PCA), modified by addition of 1 % NaCl	Nordic Committee on Food Analysis, 2000
	Long and Hammer	van Spreekens, 1974
S. putrefaciens	Iron agar[1]	Gram *et al.*, 1987
Aerobic plate count	Iron agar[2]	Gram *et al.*, 1987
	Long and Hammer	van Spreekens, 1974
Pseudomonas	Pseudomonas agar base, (C-F-C)	Mead & Adams, 1977
B. thermosphacta	Streptomycin thallous acetate actidione agar, (STAA)	Gardner, 1966
Lactic acid bacteria	Man-Rogosa-Sharke, (MRS)	Baird *et al.*, 1987

[1]*S. putrefaciens* is counted as black colonies.
[2]Aerobic plate count is the total number of black and white colonies.

Sensory evaluation

For fish as a food, the quality understanding is very much dependent on the consumer opinion. After storage, the fish must possess an acceptable sensory quality making it suitable for consumption. The sensory evaluation of fresh and stored seafood is based on different methods to describe the product, such as QIM (Martinsdottir *et al.*, 2001) and the Torry scale (Shewan *et al.*, 1953). When new species and products are introduced, thorough investigation and sensory characterisation must be performed. Even though the microbiological quality can be acceptable, other attributes or

characteristics can make the product appear spoiled or not suitable for consumption. For farmed Atlantic halibut and wild and farmed cod an adjusted QIM has been developed for sensory assessment (Cardenas Bonilla *et al.*, 2007; Esaiassen *et al.*, 2007; Guillerm-Regost *et al.*, 2006).

Chemical analyses and other attributes

Huss (1995) summarises chemical attributes contributing to the fish spoilage. Analyses of the total volatile basic amines (TVB) or the total amount of volatile nitrogen (TVN) are the most widely used methods. The TVB and TVN include TMA (produced by spoilage bacteria), dimethylamine (produced by autolytic enzymes during frozen storage), ammonia (produced by the deamination of amino-acids and nucleotide catabolites), and other volatile basic nitrogenous compounds. Although such analyses generally are easily performed, they only reflect the later stages of spoilage (Huss, 1995). For chilled stored lean fish, the most important chemical degradation is caused by degradation of sulphur containing amino acids and TMAO to TMA. Most of the volatile compounds found in spoiled fish are produced by bacteria, including TMA, aldehydes, ketones, esters, hypoxanthine, volatile sulphur compounds and other low molecular weight compounds (Lund *et al.*, 2000).

In addition to the above mentioned quality parameters, colour evaluation, drip-loss, water content and physical properties, as pH and texture, are used to describe the quality of the products.

3.4 Modified atmosphere packaging

Modified atmosphere (MA) packaging is a mild preservation method and has been shown to extend the shelf-life of many seafood products. Preservation using MA has been known for more than 100 years, but not commercially used until the latter part of the 20th century (Brody, 1998). In fish products, MA packaging has been studied since the 1930s (Coyne, 1932; 1933; Killeffer, 1930). During the last decades, MA

packaging of fish and seafood has been well studied and documented (Sivertsvik *et al.*, 2002; Stammen *et al.*, 1990).

Different foods may need different gas mixtures to provide optimal bacterial inhibition. Thus, various packaging conditions are reported for fish products, e.g. cod (Sivertsvik, 2007) and Mediterranean mullet (Pournis *et al.*, 2005), and it has been reviewed by Sivertsvik (2002). In addition to various gas compositions and concentrations, various types of pre-MA treatments and storage conditions have been examined and validated. Shelf-life studies on MA-packaged wild fish have shown the importance of temperature, production hygiene and gas composition, including the CO_2 concentration, in the development of specific spoilage organisms. In addition, the microbial load, fat content and gas-to-product-volume ratio in the package play an important role in optimising the shelf-life extension of MA packaging.

During MA storage of fish, CO_2 is the most important gas due to its inhibitory properties. The gas is known to inhibit growth of many Gram-negative and Gram-positive bacteria responsible for fish spoilage. The Gram-negative bacteria *Pseudomonas fluorescens*, *P. phosphoreum*, *S. putrefaciens*, and *Aeromonas hydrophila* were more inhibited by the CO_2, when compared to the Gram-positive bacteria *Lactobacillus sake*, *B. thermosphacta* and *Bacillus circulans* (Devlieghere & Debevere, 2000). The high sensitivity to CO_2 observed for *P. phosphoreum* is in contrast to the findings of Dalgaard (1995a), who found *P. phosphoreum* to be highly resistant to CO_2. For marine fish packaged with high CO_2 concentration and stored at low temperatures (< 4 ^{o}C), *P. phosphoreum* has been identified as the main organism responsible for spoilage (Dalgaard *et al.*, 1997).

3.5 Ozone treatment

The food industry is constantly looking for new and improved methods of shelf-life extension, and ways to improve and control product characteristics. Such methods include, e.g. UVC light treatment, salting and brining, super chilling, soluble gas stabilization (SGS), high pressure processing, and ozone treatment. These techniques use different approaches to achieve an increased product quality and extended shelf-life. A combination of these technologies can provide the greatest degree of product quality improvement.

Ozone (O_3) has been deemed "Generally Recognized as Safe" (GRAS) for several food applications (Graham, 1997), and has been widely used as a disinfectant for food products. Ozone is an effective antimicrobial agent that reverts within minutes to oxygen, giving no significant toxic residues in the environment following its use. Furthermore, ozone does not generate any halogenated and potentially carcinogenic by-products making its use more environmentally friendly than chlorine (Kim *et al.*, 1999). The effect of ozone includes bacterial disinfection, virus inactivation and removal of discolouration, odour and taste. The most common use of ozone as a bactericide is on drinking water (von Gunten, 2003a; 2003b). The bactericidal effect has been tested for preservation of foods such as meat, poultry products, eggs, fruits and vegetables, as reviewed by Kim *et al.* (1999). Ozone treatment is not universally beneficial, and excessive use of ozone may promote oxidative food spoilage (Rice *et al.*, 1982). Table 2 summarises the use of ozone on fish and fish products. The effect of ozone treatment on shelf-life extension and product quality was evaluated by chemical and sensory analyses, in addition to microbial counts (Table 2).

Despite the variable effect of ozone on fish and fish products (Table 2), the efficacy of ozonated water against pure cultures of food related microorganisms has shown that Gram-positive and Gram-negative bacteria were killed at sufficient doses (Restaino *et al.*,
1995).

Table 2. Effect of ozone treatment on bacteria on fish and fish products.

Product	**Ozone treatment**	**Result**	**Reference**
Cod	NA[1]	No effect	Ravesi *et al.*, 1987
Salmon	0.6-1.5 ppm	No effect on *Listeria innocua*	Crapo *et al.*, 2004
Salmon (whole)	0.6-1.1 ppm, 40 min	Reduction of *L. monocytogenes*	Holm *et al.*, 2005
Cold smoked salmon-trout	0.1 ppm, 20 min	No effect on *L. innocua*	Vaz-Velho *et al.*, 2006
Fresh Jack mackerel	0.6 ppm, 30-60 min	2-3 log reduction on skin	Haraguchi *et al.*, 1969
Horse mackerel mince	NA ppm, 10-20 min	Increased sensory score, lowered pH, undesirable gel strength and oxidation of fish oil	Chen *et al.*, 1997
Tilapia	6 ppm, 1 hr	Increased sensory score and shelf-life of 12 days	Gelman *et al.*, 2005
Scad	0.27 ppm (gas)	Increased sensory score. Bacterial count reduction of 1 log/cm^2	da Silva *et al.*, 1998
Catfish	10 ppm	Increased shelf-life of 25 %	Kim *et al.*, 2000
Shrimp-meat extract	5 ppm	<1 log reduction on aerobic plate count	Chen *et al.*, 1992
Shucked mussels	1 ppm, 60-90 min	Increased shelf-life of 35 %	Manousaridis *et al.*, 2005

[1]NA = data not available.

4. Methodological considerations for the molecular methods

4.1 Limitations of molecular methods and DGGE

All kinds of methods have specific limitations that need careful evaluation regarding analytic procedures and interpretation of results. Sampling and sample handling are known to produce biases.

The first obvious source of variability for molecular methods is the extraction of bacterial DNA from a complex food matrix, or from a mixture of cultured bacteria. Foods represent a complex matrix, including various proteins, fats, enzymes, polysaccharides, making it a difficult product for analyses. These substances, in addition to various other unknown substances, may interfere and act as inhibitors in the following analyses. DNA extraction methods need to be optimised in order to gain a concentrated and pure product, and a high yield suitable for subsequent analyses. Rudi *et al.* (2004) collected bacterial cells from MA-packaged salmon and coalfish before DNA extraction with a commercial kit. The pre-treatment included dilution of the fish muscle and gentle centrifugation in order to capture bacterial cells from the matrix. In the current work, this extraction method was adapted and used for cod and halibut (**Paper I**-**III**). The method was found appropriate, as it already had been developed and tested for fish samples. After the pre-treatment the cells were lysed and DNA collected. To assess the performance of the method, the waste supernatant was grown on solid agar, and the bacteria enumerated. Results showed that less than 1 % of the total bacterial amount was lost during preparation.

The next possible source for biases may be the PCR reaction itself, with numerous pitfalls, but also possibilities for application improvements. The main issue associated with the analysis of food samples is the presence of substances in the DNA mixture,

caused by the insufficient purification of the target DNA. These substances may inhibit the PCR amplification of DNA (Wilson, 1997). To minimize the risk of incomplete PCR amplification, well established and verified PCR conditions and procedures were used in the present studies (**Paper I**-**IV**).

The fragments studied by DGGE are limited to a length of 500 base pairs (bp), caused by the decreased resolution of DNA in the gel (Myers *et al.*, 1985). This represents a limiting factor when sequencing is to be performed, or if probes are to be designed. Using the universal primers spanning the V3 16S rDNA region as a target, a product of approximately 150-200 bp is obtained. This is a relatively short sequence for database comparison, although the V3-region is known to have a high grade of resolution and to be highly variable (Øvreås, 2000). Hence, it is not always possible to differentiate within the same genus. In the present work, detection of *S. putrefaciens* in farmed cod (**Paper II**) was based on sequencing the partial 16S V3 sequence. This result is in contrast to the findings of Vogel *et al.* (2005) who found *S. baltica* in marine fish, based on 16S sequencing of a 1400 bp product. The difference in the observed results may be due to the difference in the length of sequence being analysed, or the fact that different *Shewanella* species are found in different environments.

The choice of primers is crucial, and several studies have shown that amplifying different 16S variable regions may lead to different results in the observed species composition of a sample (Cocolin *et al.*, 2001; Dewettinck *et al.*, 2001; Ercolini *et al.*, 2003). Bottled water was investigated using two different primer sets, where only one of the primer sets gave a detectable PCR-product when visualized on agarose gel (Dewettinck *et al.*, 2001). Differences in the community structure were also obtained when analysing Stilton cheese using the 16S V3-region and the V4-V5-regions as targets (Ercolini *et al.*, 2003). The inconsistency among the results was explained due to different preferential amplification between the two pairs of primers used. The universal primers used in this work were selected based on literature studies on

analysis of fish products and other foods, and from discussions with experienced DGGE users.

Heterogeneous sequencing, giving rise to more than one band on DGGE and thereby overestimating the community diversity, is another problem arising when using the 16S rDNA as a target. DGGE profiles of *S. putrefaciens* (CCUG 13.452 = ATCC 8071) and *P. phosphoreum* (CCUG 12.228) pure cultures resulted in four and five distinct bands, respectively (Figure 2, **Paper I** and Figure 1, **Paper II**). These bacteria have been used throughout the experiments as internal standards and positive controls (**Paper I**-**IV**). The heterogeneity was also observed in the performed experiments, where multiple DGGE bands were assigned the same bacterium, with identical association number in BLAST (Figure 2, **Paper I** and Figure 1, **Paper II** and **III**). The pure cultures of *S. putrefaciens* and *P. phosphoreum* were bidirectionally sequenced with the primers covering the *E. coli* base positions 27 to 1491. Our in-house *S. putrefaciens* strain was assigned as *S. putrefaciens* gene for 16S rRNA. *P. phosphoreum*, on the other hand, was found to be *P. phosphoreum* and *P. "kishitanii clade"* 16S rRNA gene when comparing the sequence result with sequences in BLAST. *P. "kishitanii clade"* and *P. phosphoreum* is known for its sequence similarity (Ast & Dunlap, 2005; Dunlap & Ast, 2005). Although *P. "kishitanii clade"* is most often found in light organs of deep-sea fish, it has been detected on the skin of some fish (Ast & Dunlap, 2005), in addition to cod flesh (**Paper II**).

A problem when using universal primers is the complex binding pattern and the ability of some bands to possibly represent multiple species (Temmerman *et al.*, 2004). A consequence of this gel co-migration is that a DGGE single band does not always represent a single bacterial strain (Sekiguchi *et al.*, 2001). Furthermore, different 16S regions and DGGE conditions can result in different resolutions of the separation (Muyzer & Smalla, 1998).

Quantification of PCR products and analysis of the DGGE band intensity may yield additional information on the abundance of the different species, though this should be regarded with caution. The band intensity may reflect the relative amount of particular bacteria or a bacterium for which the PCR amplification is favoured. Nevertheless, it is believed that the detected bands are from the predominant species of a bacterial community, and that the appearance or disappearance of bands in the DGGE profiles indicates an increase or decrease in the numbers of these bacteria (Ferris & Ward, 1997).

Although the method of DGGE is often used, there are some disadvantages that can reduce the usefulness for community analysis. Despite these limitations, DGGE is highly preferred and considered as one of the few techniques allowing a fast and reproducible microbial analysis of bacterial communities (Cocolin *et al.*, 2001; Schäfer & Muyzer, 2001; Temmerman *et al.*, 2004).

4.2 Bacterial detection limits

One of the major problems and concerns for any quantitative bacteriological analysis is the detection limit. A key question is: how many bacteria are needed for detection? The sensitivity of PCR-DGGE is based on the PCR reaction and its ability to amplify bacterial DNA, or product DNA of interest from a complex sample. To get the best possible results, the product must be as pure and as concentrated as possible. Theoretically, one cell in a 10 µl sample added to a PCR reaction of 100 µl total volume, corresponding to 100 cfu/ml, can be amplified by PCR. Generally, the sensitivity in food samples is reduced due to a wide range of inhibitory substances (Wilson, 1997; section 4.1). Detection limits in the range of 10^2-10^5 cfu/ml have previously been reported for bacteria in foods (Silvestri *et al.*, 2007; Wilson, 1997). The PCR-DGGE approach was used with success for mixed bacterial population samples from fermented sausage containing 10^4 cfu/g (Cocolin *et al.*, 2001). Whilst the detection limits for *E. coli*-containing mineral water have been indicated to be in

the range from 10^4 to 10^8 cfu/ml (Dewettinck *et al.*, 2001). Other authors have found it possible to identify constituents which represent only 1 % of the total bacterial population (Muyzer *et al.*, 1993). In fact, the detection limit is suggested to be species-, and perhaps strain-, dependent, especially when using the so-called "universal bacterial primers".

The fact that PCR does not distinguish between alive and dead cells is both an advantage and disadvantage. PCR amplification is dependent on intact nucleic acid, rather than viable or non-viable cells (Josephson *et al.*, 1993). Hence, positive PCR amplification and the presence of a PCR product do not imply that the target organisms were viable, as PCR can detect viable but non-culturable (VBNC) and dead cells. As a consequence, the PCR amplification may result in a false positive result. Direct extraction and PCR amplification of mRNA can by-pass the problem of viability, although mRNA is unstable and has other disadvantages (Josephson *et al.*, 1993). During the storage experiments in **Paper I**-**III**, bacterial DNA from dead cells may contribute to false positive results. Despite the possibility of false positives, the predominant population will represent the cultivable bacteria during storage. In fact, the bacterial profile of DGGE will be represented by bacterial DNA from the dominant, viable species rather than the dead cells.

Detection limits of spoilage bacteria

For the spoilage bacteria *S. putrefaciens* and *P. phosphoreum* shelf-life rejection limits at log 8 cfu/g (Jørgensen *et al.*, 1988) and log 7 cfu/g (Dalgaard *et al.*, 1993), respectively, have been proposed. For the aerobic plate counts (APC), the maximum level set for human consumption is approximately log 6 cfu/g (ICMSF, 1986). However, this limit does not necessarily represent spoilage. The method of PCR-DGGE will theoretically, based on previous discussion, be able to detect the spoilage flora at shelf-life rejection. Storage of halibut (**Paper I**) revealed a product of high quality with a low bacterial load. The APC after 5 days was log 1.3-3.3 cfu/g depending on MA or air storage. Even with such low bacterial number, the extraction method and PCR gave adequate material for DGGE analysis, resulting in DGGE

profiles with clear and distinct bands. These findings proved the method suitable for the extraction of the bacterial DNA present in the samples. The results for cod (**Paper II** and **III**), which had a higher initial bacterial count, showed that DGGE profiles were detected during the entire storage experiment. MA storage of cod gave an initial bacterial count of approximately log 4 cfu/g. According to Muyzer *et al.* (1993), bacterial populations that make up at least 1 % or more of the total population may be identified by DGGE. Microorganisms with abundance above 1 % will, in a sample consisting of log 4 cfu/g, represent 100 cfu/g. During storage and bacterial growth, the detection limit will refer to log 5 cfu/g at log 7 cfu/g. From a consumer and shelf-life point of view, the DGGE method is adequate for detection of the bacterial flora. Furthermore, the spoilage flora of *S. putrefaciens* and *P. phosphoreum* will be detected before the spoilage rejection limit of log 6 cfu/g APC.

During storage, the bacterial flora will be represented by the species able to grow in the fish product at the given storage conditions. For fish samples, this mainly represents the spoilage flora. However, pathogens may consist in low numbers in the sample, and thereby not be detected by the method. The focus of this work is solely the bacterial diversity of products, and a comprehensive discussion on health risks from pathogens is not included. It is important to note that psychrotrophic pathogens, such as *Listeria monocytogenes*, *Aeromonas hydrophila*, and psychrotrophic non-proteolytic *Clostridium botulinum* type E, are known from fish processing and may grow at temperatures $\leq 4\ ^{o}C$. *Burkholderia* sp., which was found in **Paper I**, has to our knowledge not been associated with a health risk from fish species in temperate waters. *Bacillus cereus* was found initially on ozone-treated cod (**Paper III**), but the DGGE band representing this bacterium disappeared during storage. In MA, hazardous levels of botulinum toxins are formed after 3-4 weeks at $4\ ^{o}C$ (Sivertsvik *et al.*, 2002). Furthermore, *Listeria monocytogenes* and *Aeromonas hydrophila* may be a hazard when fish is stored at $4\ ^{o}C$ (Huss *et al.*, 2003) and should therefore be studied further using longer shelf-lives.

5. Bacterial flora of farmed fish

5.1 Detection of the microflora of farmed cod and halibut

Characterisations of the microbial flora, primarily detected as the spoilage flora, of fish caught in the Northern Atlantic have been performed by many researchers (Table 3). During the last decade, as the farming industry has increased and as new farmed species are commercialised, knowledge about the microflora on these products have to be gained.

Enumeration and characterisation of the bacterial flora of fish, especially the spoilage flora, are commonly examined by cultivation and thereafter physiological and biochemical characterisation. Traditional methods have been found to be inadequate because of non-specific bacterial detection and the use of costly and time consuming techniques. The lack of selective media for some important fish spoilage bacteria, like *S. putrefaciens* and *P. phosphoreum*, illustrates the challenges encountered with traditional methods for examining bacterial diversity. Quantification of *P. phosphoreum* can, however, be determined by a conductance method (Dalgaard *et al.*, 1996). For the detection of sulphide-producing bacteria, and mainly *S. putrefaciens*, an easy and rapid method has been developed (Skjerdal *et al.*, 2004). The Colifast test measures the level of sulphide-producing bacteria in a sample, by monitoring the colour change in the growth media as a function of time.

Identification of the bacterial composition of MA-packaged farmed fish is of interest as MA packaging is an important shelf-life extending method used for the retail market. Shelf-life studies, where bacterial changes during storage are monitored, can give valuable information about the bacteria responsible for product spoilage. Certain bacterial species are known to be involved in spoilage and responsible for most of the spoilage in air and MA-packaged products. These bacteria have not been thoroughly

investigated for farmed fish species. Furthermore, molecular-based techniques are widely used when studying other foods. However, their use is not as common in the study of the bacterial flora of fish and fish products.

Gram and Huss (1996) reviewed and discussed spoilage bacteria of fish and fish products up to the middle of the 1990s. Table 3 summarises publications from the last 10 years discussing bacterial spoilage of air stored and MA-packaged fresh seafood products. However, the table only focuses on fish from the Northern Atlantic and mostly on farmed fish.

Table 3. Spoilage bacteria detected in marine seafood products during storage in air or MA. The table summarise farmed fish and fish from the Northern Atlantic, during the last 10 years.

Bacterium	**Fish and fish product**	**Reference**
P. phosphoreum	Farmed cod	Esaiassen *et al.*, 2007[1];
		Herland *et al.*, 2007[1];
		Sivertsvik, 2007;
		Paper II[2] and **III**[2]
	Wild cod	Dalgaard *et al.*, 1997;
		Herland *et al.*, 2007
	Farmed halibut	Rotabakk *et al.*, 2008;
		Paper I[2]
	Farmed salmon	Sivertsvik *et al.*, 2003
	Coalfish	Dalgaard *et al.*, 1997;
		Rudi *et al.*, 2004[2]
	Farmed wolf-fish	Rosnes *et al.*, 2006
	Redfish	Dalgaard *et al.*, 1997
	Trout	Dalgaard *et al.*, 1997
	Plaice	Dalgaard *et al.*, 1997

Table 3. Continued.

Bacterium	Fish and fish product	Reference
S. putrefaciens	Wild cod	Boskou & Debevere, 1997; Herland *et al.*, 2007; Olsson *et al.*, 2007
	Farmed cod	**Paper II** and **III**
	Farmed salmon	Sivertsvik *et al.*, 2003
	Farmed halibut	Guillerm-Regost *et al.*, 2006
	Wild haddock	Olsson *et al.*, 2007
S. baltica	Marine fish (cod, flounder, plaice)	Vogel *et al.*, 2005[2]
	Farmed cod	**Paper III**
Shewanella spp.	Marine fish (cod, flounder, plaice)	Satomi *et al.*, 2006[2]; Vogel *et al.*, 2005
Pseudomonas spp.	Salmon	Rudi *et al.*, 2004
	Farmed cod	**Paper II** and **III**
	Farmed halibut	**Paper I**
Brochothrix thermosphacta	Salmon	Rudi *et al.*, 2004
	Farmed salmon	Olofsson *et al.*, 2007[2]
	Farmed halibut	Rotabakk *et al.*, 2008; **Paper I**
Other spoilage bacteria	Coalfish	Rudi *et al.*, 2004
	Salmon	Rudi *et al.*, 2004

[1]*P. phosphoreum* was detected by a conductance test.
[2]With the exceptions of Olofsson *et al.* (2007), Rudi *et al.* (2004), Satomi *et al.* (2006), Vogel *et al.* (2005), and **Paper I**-**III**, all papers used microbial analyses to detect *P. phosphoreum* and the H_2S producing bacteria *S. putrefaciens*.

To characterise the bacterial flora in **Paper I**-**III**, PCR-DGGE followed by sequence analysis of the V3-region gel bands were performed. The results displayed different bacterial profiles during storage and between the storage variants, and showed that the DGGE profiles consisted of different bacteria. Using *S. putrefaciens* and *P. phosphoreum* as internal standards (**Paper I**-**IV**), bands from different gels can be compared and manually normalised. The profiles of *P. phosphoreum* and *S. putrefaciens* have also been used as a "ruler" within the gels to determine eventually "smiley" gels.

The product spoilage of cod in MA with high CO_2 concentrations is caused by the presence of *P. phosphoreum* (Dalgaard *et al.*, 1997). Traditionally *P. phosphoreum* is detected by cultivation on plate count agar (PCA) supplemented with NaCl, as *P. phosphoreum* is salt requiring. However, PCA is not a species-specific media, and supports growth of many bacteria. The findings in **Paper I**-**III** revealed that the predominant bacterial flora detected from PCA plates consisted of a more diverse bacterial composition than previously reported for fish (Gram & Huss, 1996). The sequencing detected *Photobacterium* spp., *Pseudomonas* spp., *Brochothrix thermosphacta*, *Serratia* sp., *Yersinia* sp., *Micrococcus luteus*, *Shewanella* spp. and some bacteria determined as uncultivable (**Paper I**-**III**). However, both cod and halibut were dominated by the spoilage bacteria *P. phosphoreum* and *Pseudomonas* (**Paper I**-**III**).

Recent shelf-life studies on farmed cod, using microbial and chemical analyses, have suggested MA packaging with CO_2 and O_2 as an optimal gas mixture (Sivertsvik, 2007). However, until now no proper information about which bacteria that were inhibited, and thereby, causing this extended shelf-life has been available. The microbial analysis (APC and psychrotrophic counts) gave a lower bacterial count in CO_2/O_2 storage, when compared to CO_2/N_2. The extended shelf-life was based on a combination of the quality parameters; sensory, microbial and chemical analyses (Sivertsvik, 2007). With regards to farmed cod (**Paper II**), no differences between the two storage atmospheres were found by cultivation. However, different

microfloras were found in the products having different gas mixtures when the bacterial composition was described by PCR-DGGE. The DGGE and sequencing enabled the examination of the bacterial diversity and population shift, as a result of packaging. The observed shelf-life extension of CO_2/O_2 storage was described by the bacterial diversity, where *Pseudomonas* spp. dominated in the packages during storage.

The microflora of farmed Atlantic halibut (*Hippoglossus hippoglossus*) has, to my knowledge, not previously been sufficiently studied. In the storage of halibut (**Paper I**), differences in the microbial enumeration on PCA were found between the two gas mixtures CO_2/N_2 and CO_2/O_2. DGGE was used to detect and display the changes in the bacterial profiles of the various gas compositions. Based on sensory and microbial analyses, the CO_2/O_2 gas mixture was suggested as the better mixture for halibut storage (**Paper I**). During CO_2/O_2 storage of halibut, there was a clear shift in the DGGE bands during the storage period (Figure 2, **Paper I**). Sequence analyses revealed a change in the bacterial composition during storage from *P. phosphoreum* to *Pseudomonas*, *B. thermosphacta* and *Serratia* sp. Detection of *P. phosphoreum* during the entire CO_2/N_2 storage can explain the somewhat lower shelf-life of this gas composition. *B. thermosphacta* has not been regarded as part of the spoilage flora for MA-packaged marine fish, although it is known to be a part of the spoilage flora of meat and seafood. This bacterium was, however, found in MA-packaged halibut (**Paper I**). The genus *Brochothrix* is known to grow in both CO_2 and CO_2/O_2 enriched atmospheres. Lately, *B. thermosphacta* has been found in the initial flora of fresh cold-smoked Norwegian salmon (Olofsson *et al.*, 2007), but not during storage in vacuum packaging where *Lactobacillus* spp. and *Photobacterium* spp. dominated. The growth potential of *B. thermosphacta* in a mixture of other spoilage bacteria has been studied by Russo *et al.* (2006). The authors found that bacterial competition might take place when *B. thermosphacta* and other spoilage bacteria grow on the same media. The growth of *B. thermosphacta* was especially inhibited in the presence of LAB.

The general advantage using bacterial DNA isolated directly from a fish matrix without prior cultivation is that it represents a more rapid technique, giving a more complex profile compared to cultivation-based methods (Amann *et al.*, 1995; Masco *et al.*, 2005). Unfortunately, the method of DGGE does not allow quantification of the bacteria present. To gain sufficient information both cultivation and molecular analyses should be performed. A combination of the methods ensures quantification and bacterial identification.

5.2 DNA extraction from the fish matrix and cultivated bacteria

Comparing the DGGE profile for DNA extracted directly from the fish matrix with that of a DNA sample taken from a bacterial population obtained after cultivation on agar, is of special interest to this work. Theoretically, one would expect a more diverse community composition in samples taken directly from the muscle. Since different bacteria have different growth requirements and characteristics, such as the inability of certain bacteria to grow on the media and growth conditions used, culture-based methods can be biased. Moreover, some bacterial species may outgrow others.

In **Paper I**-**III**, a more diverse bacterial composition was generally found in samples where direct DNA extraction was conducted, compared to what was found after cultivation and DNA extraction. Results from the cod storage experiments showed initially seven bacterial species using direct DNA extraction, compared to only two found after cultivation (**Paper II**). The bacteria found on the fish at the beginning of storage may represent the bacterial diversity in the water environment of the fish. These can be introduced onto the flesh when filleting the fish.

Analyses of the salmon gut content revealed great differences between direct DNA extraction and cultivation prior to DNA extraction (**Paper IV**). Analysis from 16S V3

sequencing identified the genus *Lactobacillus*, *Lactococcus* and *Bacillus* as the predominant bacteria in the salmon intestine, based on direct bacterial DNA extraction, whereas the genus *Pseudomonas*, *Acinetobacter*, *Vibrio* and *Janthinobacterium* were found after cultivation. The bacterium *P. phosphoreum* was detected in both extraction methods. The differences found in the salmon intestine are further discussed in section 5.4.

Cultivation and subsequent sequencing of isolates were found to give a more diverse bacterial composition when analysing cold-smoked salmon, compared to direct extraction and cloning (Olofsson *et al.*, 2007). However, the cloning technique was needed to identify new species of *Photobacterium* in the analysed samples. These findings are in contrast to Rudi *et al.* (2004) who found the microbial diversity in coalfish and salmon to be more complex than previously suggested, based on sequencing.

5.3 *Shewanella* species in farmed fish during storage

Formation of black colonies on Iron agar can occur among members of the family *Vibrionaceae* (Gram *et al.*, 1987; Lund *et al.*, 2000). Analyses of Atlantic salmon intestine, combined with comparison of phenotypic characteristics and 16S sequence analysis, detected the pure cultured black Iron agar isolates as *Vibrio* sp. based on 16S rDNA sequencing (**Paper IV**). Recently, black colonies on Iron agar from the belly flap area of Danish marine fish were assigned as *Shewanella* species (Satomi *et al.*, 2006; Vogel *et al.*, 2005). The authors used the primer set "27f and 1512r", which was found capable of identifying a wide variety of bacterial taxa (Weisburg *et al.*, 1991). The primer set used in **Paper IV** ("27f and 1491r") has been found to extend the species' diversity compared to the 27f and 1512r primer set (Weisburg *et al.*, 1991).

A main concern regarding the spoilage potential of *Shewanella* spp. is the ability to produce ammonia, sulphides and TMA, and thereby sour odour and product spoilage. Species in the genus *Shewanella*, including *S. putrefaciens*, *S. baltica*, *S. hafniensis* and *S. morhuae* are found as spoilage bacteria in air stored fish (Gram *et al.*, 1987; Satomi *et al.*, 2006; Vogel *et al.*, 2005). *S. putrefaciens* is commonly found on marine fish (Table 2), besides wild caught sea salmon (Hozbor *et al.*, 2006) and MA-packaged Mediterranean mullet (Pournis *et al.*, 2005). Farming is expected to inhibit the bacterial growth, and *S. putrefaciens* has been found in low amounts, or totally absent in farmed Atlantic cod (Esaiassen *et al.*, 2007; Herland *et al.*, 2007; Sivertsvik, 2007), rainbow trout (Chytiri *et al.*, 2004) and wolf-fish (Rosnes *et al.*, 2006). In MA-packaged farmed halibut the genus *Shewanella* were found only sporadically on Iron agar during storage (**Paper I**), which is in agreement with the results of Rotabakk *et al.* (2008). In **Paper II**, where the bacterial diversity was monitored on post-*rigor* filleted farmed cod, *S. putrefaciens* was detected in low numbers on Iron agar during MA storage. Sequencing of DGGE bands detected *S. putrefaciens* in samples when DNA was extracting directly from the fish fillet. Contrary to these storage results, no growth of *S. putrefaciens* was observed for farmed pre-*rigor* cod (Esaiassen *et al.*, 2007; Sivertsvik, 2007). Pre-*rigor* filleting can have a positive affect on the quality of the raw material, resulting in reduced bacterial growth during storage. The reasons for the lack of *S. putrefaciens* are not known, but assumptions include the effects of controlled farming conditions, feeding regimes, hygienic handling, and the reduced time between slaughtering and processing, in addition to different farming locations and year cycle variations.

In **Paper II**, *S. putrefaciens* was detected in samples from direct DNA extraction, but not from cultivation. The results in **Paper III** show that *S. putrefaciens* and *S. baltica* were present in samples from Long and Hammer medium. The detection of *Shewanella* spp. in **Paper II** and **III** was based on sequence analyses of DGGE bands. No differences in the detection of *Shewanella* spp., as a function of the gas mixtures, were observed during this work. Moreover, ozone treatment did not inhibit or affect the growth of these species.

5.4 Detection of intestinal flora of farmed salmon

The incentive for studying the intestinal content of farmed salmon was to compare results obtained using different methods (**Paper IV**). The DGGE profiles and sequence analyses gave different results when DNA from cultured bacterial populations was compared to DNA extracted directly from stool samples. These findings display one of the major problems regarding cultivation-based methods, and especially for diverse samples containing a wide variety of bacteria. Bacteria which cause spoilage of fish are often introduced onto the flesh when filleting the fish. Sea water, the skin, and the intestinal content can all contribute to such contamination. Increased knowledge of the bacterial flora of the intestine can give valuable information about the origin of the bacteria, and thus the potential sources of microbial contamination of the fish flesh.

To compare different methods, cultivation and molecular analyses were performed on the same samples obtained from the salmon intestine (**Paper IV**). Pure colonies were described by microscopy and studied using a limited number of biochemical tests, such as the Gram-, catalase- and oxydase-reactions, and the API system. The results in **Paper IV** showed that it was not possible to identify any of the tested isolates with satisfactory discrimination, or to classify the isolates into taxonomic groups, using the selected tests. For further biochemical and phenotypic characterisation, optimisation must be carried out, as discussed by Hansen and Sørheim (1991), Ringø and Olsen (1999), Satomi *et al.* (2006), and reviewed by Popovic *et al.* (2007). Sequencing of the 16S rDNA was applied to determine the genotype of the isolates. The 16S rDNA sequencing of pure culture isolates were in accordance with the V3-region results of DGGE bands obtained after cultivation (**Paper IV**). Both methods detected *Vibrio* spp. and *P. phosphoreum* as part of the predominant microflora of the hind-gut. The V3-region, of about 200 bp, is considered to be a relatively short sequence when used in comparative studies. However, it was proven to be adequate for the determination of bacteria from cultivated samples, as discussed in section 4.1. Disadvantages of the cultivation-based method were found when the bacterial

compositions of samples collected directly from the intestinal matrix were compared to cultivated samples. Cultivation favours certain bacteria, and can bias the result compared to direct analyses. This result is also partly observed in **Paper I-III**, where **Paper II** concludes that low bacterial diversity exists among the cultured samples, and shows the advantages of using DNA from direct extraction. The findings in **Paper IV** show that the PCR-DGGE approach can be used to determine the bacterial flora of complex samples.

The intestine of aquatic animals are known to harbour LAB (Ringø, 2004), a heterogeneous group of bacteria with different habitats and phenotypic characteristics (Lund *et al.*, 2000). In intestines of fish LAB is found in various levels (Ringø, 2004). Generally, LAB from fish is known to be slow growing, and therefore, the recommended incubation conditions include low temperatures for up to 4 weeks on agar-media (Ringø & Gatesoupe, 1998). As reported in **Paper IV**, *Lactobacillus* spp. and *Lactococcus* sp. were only detected by sequencing of samples obtained by direct extraction of bacterial DNA, and not from cultivation at 15 ^{o}C for 1 week. It can be assumed that this was caused by the slow growth, and detection on growth media can be lower than the actual bacterial number.

The detection of LAB was limited to salmon intestine (**Paper IV**), as these bacteria were not found on cod or halibut flesh (**Paper I-III**). These bacteria are commonly found on fish from tropical waters (reviewed by (Gram & Huss, 1996)), and on lightly preserved fish products, such as cold-smoked fish and MA-packaged fish products (Gonzalez-Rodriguez *et al.*, 2002; Olofsson *et al.*, 2007; Paludan-Müller *et al.*, 1998; Rudi *et al.*, 2004).

5.5 Enhanced shelf-life by improved hygienic handling

Enhanced shelf-life, by inhibition of the spoilage flora, was investigated using ozone treatment of farmed Atlantic cod, prior to MA packaging (**Paper III**). The effects of ozone treatment on the bacterial population in the cod during chilled storage were examined. Ozone has recently been used in combination with other preservation techniques, such as MA for preservation of fruits and vegetables (An *et al.*, 2007; Beltran *et al.*, 2005; Zhang *et al.*, 2006). No papers addressing this combined approach for farmed fish have been found. By combining ozone treatment and MA packaging, an increased shelf-life and improved quality for the products was, however, expected. Furthermore, we anticipated DGGE profile differences among the fish spoilage organisms as a function of the O_3 treatment.

When combining different treatments, it is important to have rapid and precise methods to detect changes in the microbial community. Detection of the bactericidal effect of ozone treatment is commonly performed by microbiological enumeration on agar media. As previously discussed, such media may be selective and not able to display microbial changes in the products due to ozone treatment. PCR-DGGE was applied in **Paper III** to determine the effect of ozone treatment. The DGGE technique has been used to measure the effect of ozone treatment on the microbiota of ozone-treated drinking water (Fonseca *et al.*, 2001).

Does ozone treatment affect growth of* S. putrefaciens *and* P. phosphoreum *in cod?

During this thesis, discussions and detection of the spoilage bacteria *Shewanella* spp. and *P. phosphoreum* have been emphasised. One central issue is whether ozone treatment does influence the growth of *P. phosphoreum* and *S. putrefaciens*, as well as giving a reduction in the total number of bacteria in cod. *P. phosphoreum* is not considered a spoilage bacterium, other than in cold water aquaculture. Thus, no publications discussing the effect of ozone on *P. phosphoreum* have been found. Determination of psychrotrophic bacteria, often regarded as *P. phosphoreum*, showed

a 0.8-1.5 log cfu/g reduction in ozone-treated MA-packaged cod, compared to MA-packaged cod (Figure 2A, **Paper III**). However, the observed effect was not significant (P = 0.21). As for the genus *Photobacterium*, there was a minor, 0.2-0.5 log cfu/g, reduction of H_2S producing bacteria during storage of ozone-treated MA cod, compared to the MA cod (Figure 2B, **Paper III**). However, no statistical significance (P = 0.69) was observed. Previously, ozone treatment, vacuum packaging, and storage of aquacultured mussels resulted in a decreased formation of H_2S producing bacteria enumerated on Iron agar (Manousaridis *et al.*, 2005). The authors reported decreased bacterial counts of log 1.1-2.5 cfu/g, as an effect of ozone treatment. Furthermore, analysis of H_2S producing bacteria on ozone-treated scad (*Trachurus trachurus*) gave significantly lower bacterial growth as a function of ozone treatment, represented as > 1 log cfu/g reduction (da Silva *et al.*, 1998). Exposure of gaseous ozone (0.27 ppm) on agar plates incubated with *S. putrefaciens* resulted in growth inhibition and an antimicrobial effect (da Silva *et al.*, 1998).

Both growth on agar media and molecular analyses of the ozone-treated cod in **Paper III** detected the bacterium *Shewanella*. 16S rDNA sequence analysis showed that both *S. putrefaciens* and *S. baltica* were present in the cod. The finding of *S. baltica* is in agreement with Vogel *et al.* (2005). No inhibitory effect of ozone was observed on the bacterial growth of *Shewanella* or by detection using DGGE and sequencing.

Paper III concludes that no observable effect of ozone treatment was found. Sequence analysis of DGGE bands detected *P. phosphoreum*, *Pseudomonas* spp., *S. baltica* and *S. putrefaciens* as the predominant bacteria in all samples, regardless of whether or not ozone treatment has been employed. This result is in agreement with previous publications, where the ozone treatment alone has variable effects when used on products with a high organic content, such as foods (Kim *et al.*, 1999).

5.6 Concluding remarks

The molecular approach is much more than just a method for detecting the bacteria present in a sample. It can provide a new tool when studying the bacterial diversity during a storage experiment. For further experiments on MA-packaged halibut or cod, bacterial profiles obtained from **Paper I** and **II** can be used as references, and thereby, indicate the bacteria present in the sample.

The method of PCR-DGGE and sequencing is known to amplify and display the predominant bacteria in samples, and allows detection of the entire predominant bacterial composition in a sample. The conventional, culture-based methods used for detection of fish spoilage flora, are not always species-specific. The results presented show that there can be more spoilage bacteria as part of the flora of farmed Atlantic halibut and cod than previously assumed. Detection and characterisation of the bacterial composition of farmed halibut, during MA packaging is of special interest since there are, to my knowledge, few other reports available.

The main conclusions are summarised in the following:

- DNA extraction directly from the fish flesh, or the intestine, showed a more diverse microflora than for DNA isolated after prior cultivation.
- DGGE is a rapid method for investigation of bacterial profiles in complex sample. Within 48 hours the profiles of unknown samples can be obtained, using the methodical conditions described in this work.
- Farmed Atlantic halibut and cod were dominated by the microflora of *Photobacterium* spp., *Pseudomonas* spp., *Brochothrix thermosphacta*, *Serratia* sp., *Yersinia* sp., *Micrococcus luteus*, and *Shewanella* spp. during modified atmosphere storage.

- Sequence analysis revealed differences in the bacterial composition during storage, which was not detected by agar plating techniques alone.
- The ozone treatment of farmed Atlantic cod did not affect the microflora.
- In order to obtain quantitative results, agar plating techniques have to be performed.
- A combination of molecular techniques and microbiological analyses should be applied to obtain the most representative picture of the microorganisms present in a fish sample.

6. Further perspectives

To continue the present work several approaches and topics can be studied more in detail.

- The first obvious attempt is to introduce molecular methods to quantify the bacterial numbers in foods in a rapid and precise manner. One possible method is real-time PCR, quantifying the entire bacterial community of the samples. In addition, quantification of the spoilage bacteria *Shewanella* spp. and *P. phosphoreum* by real-time PCR would give valuable information.

- Determination of the bacterial detection limits. Theoretically, the predominant bacterial flora is identified using the PCR-DGGE approach. Some publications discuss this detection limit for specific food applications and in relation to specific bacteria. An interesting task for further work would be to determine the bacterial detection limit for the fish spoilage flora, and in different fish species.

- Another valuable discussion is regarding inhibitory substances in the fish. Such substances can cause problems during bacterial DNA extraction and amplification. More research can be directed towards bacterial DNA extraction from fish as a matrix.

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The reference of Huss *et al.*, 2003 was not included in the reference list as the thesis was submitted, but it is now included.

Paper I

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Characterisation of the dominant bacterial population in modified atmosphere packaged farmed halibut (*Hippoglossus hippoglossus*) based on 16S rDNA-DGGE

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Abstract

It is not well understood why Atlantic halibut (*Hippoglossus hippoglossus*) has longer shelf-life than most other white fish species. Our approach was to examine the microbiological diversity of the spoilage microbiota during modified atmosphere (MA) packaging of farmed Atlantic halibut. Portions were packaged with gas mixtures of CO_2:N_2 and CO_2:O_2 (50%:50%) and with air as a reference. The packages were stored at 4 °C and samples were taken 6 times during the 23 days of storage. Analyses with molecular techniques (PCR-DGGE) determined profiles of the bacterial populations in the various samples and sequencing detected the bacterial species present. In addition, samples were analysed for microbial, chemical and sensory parameters. The shelf-life was 10–13 days when stored in air and between 13 and 20 days for MA packages, with oxygen-enriched packages suggested as the better gas mixture, based on microbial growth and sensory scores. From sequence analyses of the bacterial population *Photobacterium phosphoreum* and *Pseudomonas* spp. were found to dominate in the halibut. *Brochothrix thermosphacta* was found in most samples at the end of the storage period. *Shewanella putrefaciens* was found sporadically and in low concentrations based on microbial methods, but not detected by PCR-DGGE.

Keywords: Farmed halibut; MA packaging; Spoilage; PCR-DGGE; Microbial population

1. Introduction

Atlantic halibut (*Hippoglossus hippoglossus*) is a promising species for coldwater aquaculture, and an important food fish of excellent flavour associated with a long shelf-life compared to other white fishes as cod, saithe and wolffish. Previous studies have reported halibut to be well suited for ice storage (Ruff et al., 2002; Guillerm-Regost et al., 2006). The factors responsible for this long shelf-life are not fully known, but during substantial research, factors as fish size and dietary fat content (Nortvedt and Tuene, 1998; Ruff et al., 2002), water holding capacity (Olsson et al., 2003b) and seasonal variations (Olsson et al., 2003a) have been suggested to contribute. For improved shelf-life in retail packages, storage in vacuum or modified atmosphere (MA) is likely to be beneficial.

Compared to ice storage in air, MA packaging inhibits growth of the specific spoilage organisms (SSO) which supports bacterial degradation by producing off-odour and off-flavour. From shelf-life studies of MA packaged farmed cod and salmon (Dalgaard et al., 1993; Sivertsvik et al., 2002, 2003) it is known that *Shewanella putrefaciens* and *Pseudomonas* spp. are inhibited in MA, resulting in a prolonged shelf-life. *Photobacterium phosphoreum* is more CO_2 tolerant and may cause deterioration in MA (Dalgaard et al., 1993). The main spoilage microbiota for halibut is, however, not fully characterised. In order to

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develop high-quality products it is important to know the microbiota, and to have rapid and precise methods to detect this microbiota.

Applications of culture independent molecular methods are needed to improve our understanding of the total microbial microbiota, as conventional culture-based methods are selective and do not provide the entire microbial diversity of complex environment (Amann et al., 1995). DNA extracted from a microbial population can be used to identify the genetic diversity of the dominant populations by PCR and denaturing gradient gel electrophoresis (DGGE) (Muyzer et al., 1993). Using the 16S fragment of the rRNA gene one can describe both cultivable and unculturable bacteria by phylogenetic relationship. PCR and DGGE are commonly used in environmental microbial ecology and have recently been implemented in studying foods as reviewed by Ercolini (2004). Universal primers amplifying a hypervariable region of the 16S rDNA are used to detect the dominant bacteria in the samples. Separation of DNA fragments on gel is based on sequence differences, and bacteria can subsequently be identified by sequencing. This is a reliable and fast method to study the variation of dominant bacteria and it is well suited for time interval studies, and to characterise complex microbial populations (Giraffa and Neviani, 2001).

To our knowledge, there are no reports using the PCR-DGGE technique to compare the predominant bacterial composition and diversity in farmed Atlantic halibut. In the present study our aim was to explain the shelf-life and quality of farmed halibut (stored in different atmospheres) by means of the PCR-DGGE method in combination with sensory, chemical and traditional microbiological analyses.

2. Materials and methods

2.1. Raw material and sample preparation

Commercial farmed Atlantic halibut (*H. hippoglossus*) of 6–8 kg were obtained from Marine Harvest (Hjelmeland; Norway). The fish were gutted and stored on ice during shipment to Norconserv (Stavanger, Norway). The fish were filleted, deskinned and packaged at Norconserv 1 day after slaughtering.

2.2. Packaging materials

Portions of 150 g were packaged in 415 ml high-density polyethylene (HDPE) semi-rigid trays (no. 523, Polimoon, Kristiansand, Norway). The air was evacuated and a food grade gas mixture (50% CO_2 and 50% N_2 or O_2, AGA, Oslo, Norway) was introduced into the package before heat-sealing (lidding film: 15 my PE/74 my PA, Dynoseal ST 1575, Polimoon) on a semi-automatic packaging machine (Dyno VGA 462, Polimoon). The gas volume-to-product (G/P) ratio was approximately 2:1. The air-stored portions were wrapped with non-barrier cling film (PVC-film, Linpac plastics pontivy SA, Noyal-Pontivy, France). Packages of both MA and air were stored at 4.0 °C in chill cabinets (Porkka CM710, Huurre Group, Hollola, Finland). Duplicate samples were taken for analyses 1, 5, 9, 13, 20 and 23 days after packaging.

2.3. Cultivation and isolation of bacteria from fish matrix

Samples of 10 g fish muscle were homogenised in 90 ml peptone water (0.9% NaCl (w/v) and 0.1% peptone (w/v)) for 30 s in a Stomacher 400 Laboratory Blender (AJ Steward Company LTD, London, England). Total viable counts were measured as aerobic plate counts (APC), where aliquots from suitable dilutions were added to melted and temperate (45 °C) iron agar with an overlay (Iron Agar Lyngby, Oxoid CM 964, Basingstoke, England). The agar was supplemented with 0.04% L-cysteine, and incubated at 20 ± 1 °C for 3 days. Black colonies were counted as H_2S-producing bacteria and APC were counted as the total of black and white colonies. The content of psychrotrophic bacteria was determined by a spread plate count method with plate count agar (PCA, Merck, Darmstadt, Germany) added 1% NaCl, in order to support growth of the salt requiring *P. phosphoreum*. Plates were incubated at 7 ± 1 °C for 10 days. Average results of duplicate measurements are presented as log colony forming units (cfu) per gram muscle.

After counting, the psychrotrophic bacteria from PCA spread plates were collected, using an inoculation loop, washed twice with 1 × PBS (137 mM NaCl, 2.7 mM KCl, 0.9 mM KH_2PO_4 and 6.4 mM Na_2HPO_4 [pH 7.4]) and frozen at −20 °C. The pellets were thawn immediately prior to DNA extraction, and the cell pellet DNA was purified using a commercial extraction kit. To ensure extraction of Gram-positive bacteria DNA, a lysozyme lysis was performed in advance, in accordance with the manufacturer's recommendations (DNeasy Tissue Kit, Qiagen, Hilden, Germany).

2.4. Extraction of total DNA from fish matrix

Duplicate surface samples of 10 g muscle were aseptically removed by making a 0.2–0.5 cm deep cut of approximately 20 cm^2 and diluted 1:10 in peptone water, homogenised for 2 min in a Stomacher 400 Laboratory Blender (Colworth, AJ Steward Company LTD, London, England) before 50 ml of the suspensions were frozen. Bacterial DNA was extracted in accordance with the method of Rudi et al. (2004), and DNA was purified using the DNeasy Tissue Kit with the Gram positive bacteria modification.

2.5. PCR protocol

Universal primers for the domain *Bacteria* were used for amplification of the hypervariable V3 region on 16S rDNA (Table 1). The forward primer included a 40 base GC clamp (Sheffield et al., 1989). Bacterial DNA extracted from fish matrix or cell pellet, 2.5 µl, was used as a template

Table 1
PCR primers and their position used for DGGE analysis and sequencing (Muyzer et al., 1993; Øvreås et al., 1997)

Primer[a]	Position[b]	Sequence	Target
BA338f[c]	338–357	5′ACT CCT ACG GGA GGC AGC AG	*Bacteria*, 16S rDNA V3-region
UN518r	518–534	5′ATT ACC GCG GCT GCT GG	Universal, 16S rDNA V3-region
GC clamp		5′CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G	

[a]f, forward primer; r, reverse primer.
[b]The numbering of position is based on *E. coli* 16S rRNA.
[c]The GC clamp was attached to the 5′ end of the BA338f primer.

in a PCR reaction containing a final concentration of 0.5 μM of each primer (Eurogentec, Ougrée, Belgium and MWG-Biotech, Ebersberg, Germany), 0.5 U DyNAzyme II Polymerase (Finnzyme, Espoo, Finland), 0.1% BSA (Sigma, Germany) and 0.2 mM of each dNTP (Fermentas, Lithuania) in a total reaction volume of 25 μl. The reaction was performed on a Mastercycler personal (Eppendorf, Germany) using the following conditions: 92 °C for 2 min and than 30 cycles of 92 °C for 1 min, 55 °C for 30 s and 72 °C for 1 min. The reaction was terminated with an extension step of 6 min at 72 °C. PCR products were examined and verified on 2% agarose gels (SeaKem Le agarose, BMA, Rockland, USA) and visualised by ethidium bromide staining. The gels were photographed under UV light in a Bio-Rad GelDoc 2000 system (Bio-Rad Laboratories Inc., USA).

2.6. DGGE analysis and identification of bands

DGGE was performed with the V20-HCDC system (Scie-Plas Limited, Southham, England) using 10 μl of the PCR product on a 0.75 mm thick 8% (w/v) polyacrylamide gel, with a denaturing gradient ranging from 30% to 55%. The electrophoresis was run in 0.5 × TAE buffer (Eppendorf AG, Hamburg, Germany) at 60 °C for 10 min at 20 V and further 18 h at 70 V. After electrophoresis the gel was stained for 1 h with 1 × (final concentration) SybrGold (Molecular Probes, Eugene, USA) in 1 × TAE buffer, rinsed in water and the bands were visualised under UV light as described above. DNA fragments to be nucleotide sequenced were excised with sterile pipette tips and transferred to 30 μl sterile water. The DNA was allowed to diffuse into the water at 4 °C overnight. From the eluate 5 μl was used as a template and re-amplified using PCR as described above. The PCR product was re-run on DGGE to confirm that it migrated as a single band, to the same position. Single and correct positioned products were cleaned by E.Z.N.A. Cycle-Pure Kit (Omega Bio-Tek, Doraville, USA), according to the manufacturer's recommendations. Sequencing was performed at the University of Bergen Sequencing Facility (Bergen, Norway) with an ABI PRISM 3700 DNA Analyser (Applied Biosystems, Warrington, UK). The partial sequencing was performed using the forward primer BA338f without the GC clamp and the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Warrington, UK). Searches in BLAST from GenBank were used to find the closest known relatives to the partial 16S rDNA sequences (140–210 bp) (Altschul et al., 1997). Sequences with 97% or higher identity were considered to represent the same species.

2.7. Chemical analyses

Total volatile bases (TVB-N), trimethylamine (TMA) and trimethylamine oxide (TMAO) was determined using a Conway microdiffusion method (Conway and Byrne, 1933), as described by Rosnes et al. (2006).

2.8. pH measurements

The pH of the fish tissue was determined in triplicate, using a pH meter (Beckman 72, Beckman Instruments Inc., Fullerton, USA) on homogenised muscle (25 g) added 25 ml 0.1 M KCl (ISO, 1993).

2.9. Sensory analysis (Quality Index Method)

The sensory evaluations were assessed using the Quality Index Method (QIM) described by QIM Eurofish (Martinsdottir et al., 2001), modified and adapted for halibut. The QIM-score was based on appearance, colour, texture and odour of raw products. The panel gave demerit scores of 0–2 points for the different attributes. The odour was evaluated as sea fresh, fishy or sour, giving 0, 1 or 2 points, respectively. The other attributes evaluated were colour (0: homogeneous white—2: yellow, translucent), appearance (0: transparent—2: dull) and texture (0: firm—2: very soft). The QIM-score was the sum of the scores given by the sensory panel on the individual quality parameters on a scale from 0 to 8. Coded trays of raw halibut were evaluated in duplicate by a trained sensory panel consisting of 4 experienced evaluators.

2.10. Gas analysis

The headspace gas composition in the MA packages was determined in quadruplicate by analysing an aliquot (20 ml) of the headspace gas in the trays using an oxygen

and carbon dioxide analyser (Checkmate 9900 Analyzer, PBI-Dansensor, Ringsted, Denmark).

2.11. Statistical analysis

Analysis of variance (ANOVA) was performed with Minitab 14 (Minitab Inc., US) using General Linear Model with Tukey's HSD test at level $P<0.05$ (95%). The analyses determined the main effects of the experimental variables (time and packaging method) on the responses.

3. Results and discussion

3.1. Microbiological analyses

Growth started immediately in air, but had a lag phase of about 5 days (CO_2:N_2) and 13 days (CO_2:O_2) in MA for both psychrotrophic counts (PC) (Fig. 1) and APC. Psychrotrophic bacteria are defined as bacteria capable of growing at 0 °C and with a growth optimum exceeding 25 °C (Huss, 1995). International fish trade organisations use APC as an indicator for high-quality shelf-life (not necessarily spoilage), and log 6 cfu/g is often used as a maximum level for fish sold for human consumption (ICMSF, 1986). Application of this limit would give a high quality shelf-life of about 10 days in air storage and 20 days in CO_2:N_2, whereas the CO_2:O_2 did not reach this limit during the 23 storage days (Table 2).

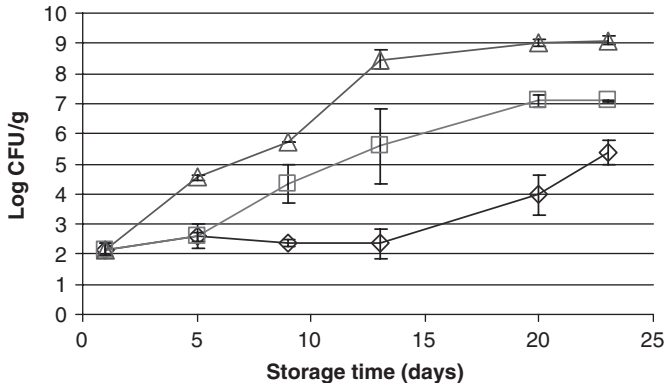

Fig. 1. Growth of psychrotrophic bacteria (PC) in farmed halibut as a function of storage time. Halibut samples were stored for a total of 23 days after packaging. △ = air, □ = CO_2:N_2 and ◇ = CO_2:O_2.

For fish stored at high CO_2 concentrations, *P. phosphoreum* is regarded as the main spoilage organism, e.g. on salmon (Emborg et al., 2002) and cod at 0 °C (Dalgaard et al., 1993; Dalgaard, 1995). Spoilage of chilled, CO_2 packaged fish is found at a level of log 7 cfu/g *P. phosphoreum* (Dalgaard et al., 1993). Assuming that the major part of the psychrotrophic bacteria are *P. phosphoreum*, this level was reached for air packaged halibut after about 10 days, and for CO_2:N_2 packages first at day 20. CO_2:O_2 packages had a lag phase of 13 days and the bacterial number were below the rejection limit the entire storage period (Fig. 1). A significant difference was found between different gas compositions (Table 2).

S. putrefaciens is a main spoilage organism on marine fish during cold storage in air (Gram et al., 1987), causing production of off-flavouring volatile compounds such as ammonia, TMA and sulphides (Dalgaard, 2000). It forms characteristic black colonies in Iron Agar. Of the halibut samples ($n = 32$) only 6 formed black colonies above the detection level ($\geqslant 10$ cfu/g). Growth was detected sporadically and irrespectively of the parallels and atmosphere, and the bacterial number varied between log 1 ($n = 3$) and log 4 ($n = 1$). Log 8 cfu/g are often set as a spoilage limit

Table 2
Effect[a] of storage time (days) and gas mixture on aerobic plate count (APC) (log CFU/g), psychrotrophic count (PC) (log CFU/g), quality index method (QIM) score (0–8), trimethylamine oxide (TMAO), trimethylamine (TMA) and total volatile bases (TVB-N) (mg-N/100 g fish)

	APC	PC	QIM	TMAO	TMA	TVB-N
Storage time (days)						
5	1.9^{A} ($n = 6$)	3.2^{A} (6)	2.2^{A} (24)	13.2^{A} (6)	0.2^{A} (6)	16.1 (6)
9	3.3^{AB} (6)	4.1^{AB} (6)	2.3^{A} (24)	13.2^{A} (6)	0.3^{A} (6)	16.3 (6)
13	4.4^{B} (6)	5.5^{BC} (6)	3.8^{B} (24)	11.3^{AB} (6)	1.9^{AB} (6)	18.1 (6)
20	6.3^{C} (6)	6.7^{CD} (6)	4.8^{BC} (18)[c]	9.7^{AB} (6)	3.1^{AB} (6)	23.5 (6)
23	7.0^{C} (6)	7.2^{D} (6)	5.2^{C} (18)	6.3^{B} (6)	5.8^{B} (6)	32.8 (6)
Effect of time (*P*-value)	<0.001	<0.001	<0.001	0.003	0.022	0.072
SEM	0.4	0.3	0.3	1.2	1.2	4.5
Gas mixture						
Air	7.1^{A} (10)	7.4^{A} (10)	5.2^{A} (36)	7.8^{A} (10)	5.6^{A} (10)	31.7^{A} (10)
50% CO_2:50% N_2	4.3^{B} (10)	5.3^{B} (10)	3.3^{B} (36)	11.0^{AB} (10)	1.0^{B} (10)	16.1^{B} (10)
50% CO_2:50% O_2	2.4^{C} (10)	3.3^{C} (10)	2.5^{C} (36)	13.4^{B} (10)	0.2^{B} (10)	16.4^{B} (10)
Effect of gas mixture (*P*-value)	<0.001	<0.001	<0.001	0.001	0.001	0.005
SEM	0.3	0.3	0.2	0.9	1.0	3.5
R^2(adj)	0.88	0.88	0.55	0.54	0.47	0.37

Values are given as least squares means[b]. Number (n) of samples per sampling point is given in brackets.
[a]Effect of treatment (*P*-value), ANOVA (GLM) and Tukey's pairwise comparison test.
[b]Means with different upper case superscripts in the column are significant different by ANOVA (GLM) and Tukey's pairwise comparison test.
[c]At day 20 and 23 there were only 3 evaluators for the samples.

(Jørgensen et al., 1988), and consequently our results suggests other organisms to be involved in spoilage.

3.2. Molecular analysis

Molecular analysis using PCR-DGGE followed by sequencing enabled characterisation of the bacterial population in farmed halibut, and also monitoring of the profile changes during storage (Table 3). This approach allows detection of the dominant bacteria present in the samples by sequencing the interesting gel bands. Farmed halibut is a new and not well-characterised species, and to our knowledge there is no information on the bacterial microbiota causing deterioration during storage.

Excised DGGE bands were compared with BLAST references based on the phylogenetic relationship of the ~180 bp partial 16S rDNA sequence of the hypervariable V3 region. The V3 region is regarded as a good choice when it comes to length and species–species heterogeneity (Coppola et al., 2001; Ercolini, 2004), the region is also considered to be highly variable and have a high grade of resolution. The primers used in this experiment have previously been used with success to analyse the bacterial diversity in other foods, e.g. Cabrales cheese (Florez and Mayo, 2006), fermented sausages (Fontana et al., 2005) and meat products (Takahashi et al., 2004). Only two reports have discussed the use of these primers to study fish, Jensen et al. (2004) on halibut larvae and in MA packaged farmed cod (Hovda et al., 2006).

3.2.1. PCR-DGGE profiles of bacteria isolated from cell pellets

Air storage of farmed halibut, where oxygen could pass freely through the cling film, resulted in a product that rapidly deteriorated (10 days) as evaluated by microbial and sensory methods. The bacterial profile of air stored samples changed during the storage period (Fig. 2). The main spoilage organisms of coldwater fish *P. phosphoreum* and *Pseudomonas* spp. were detected as part of the observed bacteria population (Table 3).

Seven bands were found in the CO_2:O_2 stored samples during the first 9 days. At day 23 the profile was completely changed and contained 10 "new" bands (Fig. 2). From sequence analyses *P. phosphoreum* represented the bands in the first period, in addition to an uncultured bacteria clone spb33, found the first 9 days. Moreover, at the end of the storage period the dominating bacteria were the aerobic bacteria *Pseudomonas* spp., *Serratia* sp. and probably *Brochothrix thermosphacta* with 95% similarity (Table 3).

The CO_2:N_2 samples had a similar bacterial profile as the CO_2:O_2 during the first days of storage, but changed during storage. This atmosphere gave, however, a more diverse composition than CO_2:O_2 during storage (Table 3).

B. thermosphacta and *Pseudomonas* spp., known as common spoilage bacteria in raw and processed meat stored in air or MA (Borch et al., 1996), were found in air and CO_2:N_2 packages after 13 days. These bacteria are also associated with spoilage of e.g. ice stored Sea bass and rainbow trout (Chytiri et al., 2004; Paleologos et al., 2004), and MA packaged salmon and Mediterranean mullet (Rudi et al., 2004; Pournis et al., 2005).

3.2.2. PCR-DGGE profiles of bacteria extracted directly from fish matrix

Samples with CO_2:O_2 gave a distinct profile of 11 bands the first 13 days of storage, before a change at day 20 (data not shown). Bacteria present the first 20 days represented *P. phosphoreum*, *Micrococcus luteus* and different *Pseudomonas* species (Table 3). In addition two of the sequenced bands were assigned as eukaryotic 18S rDNA sequences from fish, with similarity of 100% and 95%. Only two 18S rDNA sequences of halibut are available from the BLAST database, obtained from a pooled cDNA library of halibut anterior kidney, liver and spleen (Park et al., 2005). Our sequences did not match any of these, but due to lack of information it is not possible to omit an origin from halibut. The primers we used have been shown to amplify halibut 18S rDNA, and to avoid this the annealing temperature is suggested to be raised to 65 °C (Jensen et al., 2004). After 23 days there were only 2 bands present in the profile, representing *Staphylococcus* sp. and presumably *B. thermosphacta* (Table 3).

The bacterial diversity in nitrogen-enriched packages was more complex than that of the CO_2:O_2 samples, and contained in addition *B. thermosphacta* (day 13–20) and *P. phosphoreum* (day 23), whereas *Pseudomonas* spp. were not detected at days 13–20. Products stored in air had bacterial profiles unlike the MA packaged samples, and the number of bands increased during storage. However, sequencing did not show any other species than found in the MA samples, except the presence of *Burkholderia* sp., a potential pathogen, at day 23 (Table 3).

It is surprising not to find *S. putrefaciens* which is one of the well-defined spoilage organisms on lean fish stored in air, and responsible for the sour odour. *S. putrefaciens* is commonly found on wild caught salmon (Hozbor et al., 2006) and MA packaged Mediterranean mullet (Pournis et al., 2005). Lately Vogel et al. (2005) identified *S. baltica* as the most important H_2S-producing species during ice storage of Danish marine fish. On farmed fish, i.e. cod (Sivertsvik, 2007; Hovda et al., 2006), wolf-fish (Rosnes et al., 2006) and the present study *S. putrefaciens* seems to be absent, and to our knowledge there are no reports explaining this observation. This supports the iron agar results, where H_2S-producing bacteria were found only sporadically and in low numbers, and with variations between the parallels. Reasons for this may be associated with the controlled farming conditions, including feeding regimes, hygienic handling and reduced time between slaughtering and processing.

This work discusses the shelf-life based on spoilage, i.e. microbial counts and sensory evaluation. A comprehensive discussion on health risk from pathogens is not included since the present work does not contain data on pathogenic

Table 3
The table shows the results after sequencing of the dominant bands from the bacterial profiles of Fig. 2, in the different storage variants and during the storage period

Storage variant	Air (cell pellet)	CO_2:O_2 (CP)	CO_2:N_2 (CP)	Air (fish)	CO_2:O_2 (fish)	CO_2:N_2 (fish)
After 5–9 days	Uncultured bacterium clone spb33b1 (#8) *Pseudomonas* sp. (#15) *Pseudomonas putida* (#19) *P. phosphoreum* (#12)	Uncult. bacterium clone spb33b1 (#20–21) *P. phosphoreum* (#22–24)	Uncult. bacterium clone spb33b1 (#20–21) *P. phosphoreum* (#22–24)	Uncult. bacterium clone spb33b1 *Pseudomonas* sp. *P. phosphoreum*	*Pseudomonas sp.* *Pseudomonas putida* *P. phosphoreum* *Micrococcus luteus*	*Pseudomonas sp.* *Pseudomonas putida* *P. phosphoreum* *M. luteus*
After 13–20 days	*Pseudomonas putida* (#14,19) *Pseudomonas sp.* (#15,18) *P. phosphoreum* (#13) Uncult. bacterium isolate DGGE *B. thermosphacta*[a]	*P. phosphoreum* (#22–24)	Uncult. bacterium clone spb33b1 (#20) *Pseudomonas* spp. (#2) *B. thermosphacta*[a] *Serratia* sp. *Yersinia aleksiciae* (#7)	Uncult. bacterium clone spb33b1 *Pseudomonas* sp. Uncult. bacterium isolate DGGE	*Pseudomonas sp.* *Pseudomonas putida* *P. phosphoreum* *M. luteus*	*B. thermosphacta*[a] *M. luteus*
After 23 days	Uncult. bacterium clone spb33b1 (#8)*Pseudomonas putida* (#14,19)*Pseudomonas* spp. (#15,16,18)*B. thermosphacta*[a]Uncult. bacterium (#17)	*Pseudomonas* spp. (#25–27) *B. thermosphacta*[a] (#28) *Serratia* sp. (#29-30)	*Pseudomonas fluorescens* (#1) Uncult. bacterium clone MAR-47 (#3) *Pseudomonas sp.* (#2, 4) *B. thermosphacta*[a] (#28) *P. phosphoreum* (#5)	Uncult. bacterium clone spb33b1 *Burkholderia* sp. *Pseudomonas* sp. Uncult. bacterium isolate DGGE *B. thermosphacta*[a] *Serratia* sp. (#6) *Yersinia aleksiciae* (#7) *Y. aldovae*	*Staphylococcus sp.* *B. thermosphacta*[a]	*Staphylococcus sp.* *B. thermosphacta*[a] *P. phosphoreum* *P. phosphoreum*

The associated bands from Fig. 2 and Table 4 are shown in brackets. Only sequences with similarity >97% are included in the table.
[a]The similarity observed for sequences assigned as *B. thermosphacta* was 95–96%.

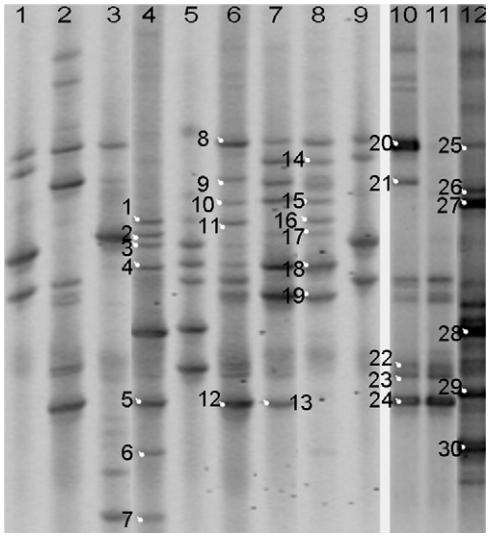

Fig. 2. DGGE bacterial profile of halibut (cell pellet) stored in different gas mixtures and in air, for 23 days. *Shewanella putrefaciens* (lanes 1 and 9) and *Photobacterium phosphoreum* (lane 5) were used as internal standards. Profiles of CO_2:N_2 packaged samples were taken after 5 (lane 2), 13 (lane 3) and 23 (lane 4) days. The lanes 6–8 represent air stored samples and lanes 10–12 CO_2:O_2, taken at the same time points. The identification of the bands (1–30) are shown in Table 4.

growth. However, psychrotrophic pathogens as *Listeria monocytogenes*, *Aeromonas hydrophila* and psychrotrophic non-proteolytic *Clostridium botulinum* type E are known from fish processing and may grow at temperatures $\leqslant 4\,^{\circ}C$. *Burkholderia* sp. has to our knowledge not been associated with a health risk from fish species in temperate waters. In MA hazardous levels of botulinum toxins are formed after 3–4 weeks (Sivertsvik et al., 2002). Furthermore, *Listeria monocytogenes* and *Aeromonas hydrophila* may be a hazard on stored fish at $4\,^{\circ}C$ (Huss et al., 2003) and should be further explored at extended shelf-lifes.

It is important to note that not all bands in the profiles have been sequenced and analysed, due to problems making them appear as a single band. Hence some biologically significant bacteria may remain undiscovered. Some analysed bands had sequence similarity of less than 97%, and were thereby not assigned as the same species. Limitations of the method include the heterogeneity where the observed bands in the profiles does not necessarily correspond to different bacteria strains. It is well established that some bacteria have heterogeneous copies of rDNA operons (Nübel et al., 1996). In pure culture of *S. putrefaciens* (CCUG 13.452) and *P. phosphoreum*

Table 4
16S rDNA sequence similarities to closest relatives of DNA recovered from the respective bands in the DGGE gel, Fig. 2

Band no.	Closest relative in GenBank database (accession number)	Similarity (%)
1	*Pseudomonas fluorescens* (AJ971392.1)	99
2	*Pseudomonas* sp. (AB079096.1)	96
3	Uncultured bacterium clone MAR-47 (AY842561.1)	98
4	*Pseudomonas* sp. (AM114526.1)	99
5	*Photobacterium phosphoreum* (AY780010.1)	98
6	*Serratia* sp. (AM048794.1)	100
7	*Yersinia aleksiciae* (AJ627597.1)	100
8	Uncultured bacterium clone spb33b11 (DQ321621.1)	97
9	*Pseudomonas pseudoalcalignenes* (AY789572.1)	83
10	*Photobacterium phosphoreum* (AY780010.1)	89
11	Uncultured *Vibrio* sp. clone (AY702295.1)	84
12	*Photobacterium phosphoreum* (AY780010.1)	96
13	*Photobacterium phosphoreum* (AY780010.1)	100
14	*Pseudomonas putida* (AY450555.1)	100
15	*Pseudomonas* sp. (AM111035.1)	100
16	*Pseudomonas* sp. (DQ137854.2)	100
17	Uncultured bacterium clone MAR-47 (AY842561.1)	99
18	*Pseudomonas* sp. (AJ864857.1)	99
19	*Pseudomonas putida* (AY450555.1)	99
20	Uncultured bacterium clone spb33b11 (DQ321621.1)	98
21	Uncultured bacterium clone spb33b11 (DQ321621.1)	97
22	*Photobacterium phosphoreum* (AY577825.1)	94
23	*Photobacterium phosphoreum* (AY780010.1)	94
24	*Photobacterium phosphoreum* (AY780010.1)	97
25	*Pseudomonas* sp. (AB121752.1)	97
26	*Pseudomonas* sp. (AY574283.1)	96
27	*Pseudomonas* sp. (AM111028.1)	97
28	*Brochothrix thermosphacta* (AY543029.1)	95
29	*Serratia* sp. (AM048794.1)	95
30	*Serratia* sp. (AM048794.1)	97

(CCUG 16.288) both showed 4 and 5 main bands (Fig. 2). Also in the MA packaged samples this heterogeneity is clearly seen, represented by 3 bands identified as *P. phosphoreum* (Table 4).

Comigration and issues related to one band representing more than one bacterium was evaluated by Li et al. (2006). These migration problems were also observed in this study where similarly migrating bands represented different bacteria depending on the sample source (data not shown). By altering the resolution capability and denaturing conditions (different temperature, time and denaturing percentage) one can improve the band separation.

3.5. Sensory characteristics

Both CO_2:N_2 and CO_2:O_2 stored halibut had a low sensory score and good quality until day 13 and a shelf-life around 20 days based on the QIM score and the observed sour odour and the dull appearance (Table 2). Samples stored in air had a shelf-life up to 13 days set by the sour odour and the soft texture. This prolonged shelf-life is, in fact, supported by the observed low TMA production, low microbial growth and low pH. Comparable results were observed for storage of whole halibut, which had a constant muscle quality, at the best score, for 14 days (Guillerm-Regost et al., 2006). As there is little experience using QIM for halibut, the QIM scheme used was not ideal to define the quality and shelf-life of farmed halibut. This has to be taken into account when results from the sensory evaluation are analysed, and the scheme has to be further optimised based on sensory panel experiences.

3.6. Chemical analyses

The amount of TMAO and TMA in Atlantic halibut fillets were 12.3 and 1.1 mg N/100 g fish, respectively, 1 day after filleting. TMAO is an important osmoregulator in marine fish found at low levels in salmon (Rosnes et al., 1997), while high levels (55–102 mg N/100 g) are reported in cod, red fish and haddock (Oehlenschläger, 1992). Many bacteria are able to produce TMA, mainly responsible for the fishy odour, by using TMAO as a terminal electron acceptor in the metabolism. No degradation of TMAO was seen and no TMA was produced in any storage variants during the first 9 days of storage (Table 2). A clear increase in TMA production was seen in air from day 9 to a final amount of 14.4 mg TMA-N/100 g fish. In cod 30 mg TMA-N/100 g is set as the limit for sensory rejection (Dalgaard et al., 1993). Oxygen-enriched packages produced no TMA and in CO_2:N_2 the production of TMA started after 20 days and ended at 3.2 mg TMA-N/100 g. In fact, this lack of TMA production indicates that TVB-N may explain the off-odour. The production of total volatile bases started after 9 days in air and exceeded 30 mg TVB-N/100 g after about 17 days (Table 2). As MA packaging did not produce any TVB-N, and the samples had an initial concentration of 15.2 mg TVB-N/100 g throughout the storage period, Table 2 gives the result of TVB-N production in the air stored products. The European Commission (1995) has set critical limits of 25, 30 and 35 mg TVB-N/100 g for certain fish species. The results showed that off-flavour in spoiled Atlantic halibut were not caused by a single chemical degradation product alone. The main effects (Table 2) showed that TVB-N agreed with the observed shelf-life and better described differences between the packaging methods than TMA.

There were significant differences in the pH between the MA and air stored samples, but no specific trend in pH as a function of storage time (data not shown). pH varied between 6.0 and 6.9 during air storage, between 6.0 and 6.4 in CO_2:O_2 and 6.0–6.2 in CO_2:N_2 storage. The low pH in the MA packaged samples can explain the absence of *S. putrefaciens*, according to the results of Boskou and Debevere (1998) that found a prolonged lag phase below pH 6.2. MA packaging resulted in a CO_2 concentration in the head space above 20% of the entire period (data not shown), a level often regarded as necessary to obtain sufficient bacterial inhibition (Devlieghere and Debevere, 2000). MA packaging significantly improved the shelf-life compared to air storage. There were also significant differences between the two gas mixtures, where oxygen is suggested to give the most shelf-life extension and also a lower bacterial diversity based on the results of PCR-DGGE analyses.

4. Conclusion

Our findings revealed that halibut as a fresh product has properties that makes it well suited for MA packaging and has a shelf-life ranging from 13 to 20 days at 4 °C. There was a significant difference between CO_2:N_2 and CO_2:O_2 packaging, where oxygen enrichment was suggested to be the better gas mixture. The PCR-DGGE technique was useful to identify a broad range of bacteria from stored MA packaged farmed halibut. Combining the results obtained from PCR-DGGE, microbial counts and sensory analyses enabled us to determine the shelf-life of the product. The main bacterial microbiota in both MA packaged and air stored farmed halibut were found to be *P. phosphoreum*, *Pseudomonas* spp. and *B. thermosphacta*. *S. putrefaciens* was not detected by molecular methods during this experiment, confirming the plate count results and other reports on farmed fish species. These results can be used in order to optimise MA packaging as a mild preservation method for Atlantic halibut (*Hippoglossus hippoglossus*).

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Paper II

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Characterisation of the bacterial flora of modified atmosphere packaged farmed Atlantic cod (*Gadus morhua*) by PCR-DGGE of conserved 16S rRNA gene regions

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Abstract

The present article describes the use of broad-range molecular analyses to characterise the microbial population of farmed Atlantic cod (*Gadus morhua*) packaged for the retail market. Cod was filleted *post rigor*, packaged in air or in modified atmosphere (MA) (50% CO_2:50% N_2 or 50% CO_2:50% O_2) and stored at 0 °C for 11 days. To determine the community profiles of the samples the variable V3-region of the bacterial 16S rRNA gene were amplified by PCR, before the PCR products were separated by denaturing gradient gel electrophoresis (DGGE). From sequence analyses *Pseudomonas* spp. were found to be the predominant bacteria in oxygen enriched atmospheres, whereas the spoilage bacteria *Photobacterium* sp., *Shewanella putrefaciens* and *Pseudomonas* spp. dominated in CO_2:N_2 and air packaged samples. Additional microbial analyses by cultivation methods observed highest bacterial numbers in air stored samples, and both MA mixtures gave growth inhibition when measuring aerobic plate count, psychrotrophic bacteria and H_2S-producing bacteria. The results show that PCR-DGGE can be applied to examine bacterial diversity and population shifts among different MA-packaged products.

Keywords: Farmed cod; MA packaging; PCR; DGGE; Bacterial community

1. Introduction

Marine fish products deteriorate rapidly *post mortem* as a consequence of various microbial and biochemical breakdown processes. The rate of quality loss depends directly on the nature of the fish species in question, as well as the handling and storage conditions. The quality of wild caught cod may vary considerably, due to seasonal variations, different handling, fishing gear and fishing ground. The time between catch and processing will in addition strongly influence the quality. Compared to wild caught fish, farmed fish have several advantages as a raw material. Wild caught cod is known to have a different body composition than farmed cod, with a higher condition factor, smaller head and larger liver (Gildberg, 2004). There is also observed a higher carbohydrate level and lower pH in the muscle (Rustad, 1992). Shelf life studies on MA-packaged wild fish have shown the importance of temperature, production hygiene and gas composition on the development of specific spoilage organisms (SSO) (Gram and Huss, 1996). The bacterial flora of wild cod stored aerobically on ice is well studied and dominated by *Pseudomonas* sp. (Gram and Huss, 1996), *Shewanella baltica*, *Shewanella hafniensis* and *Shewanella morhuae* (Vogel et al., 2005; Satomi et al., 2006), rather than *Shewanella putrefaciens*, which has previously been considered, in many studies, as the main spoilage organism (Gram et al., 1987). In modified atmosphere (MA) packaging with high CO_2 concentration, the CO_2 tolerant bacterium *Photobacterium phosphoreum* has been identified as the main organism responsible for spoilage (Dalgaard et al., 1997).

Knowledge about spoilage organisms and their specific activity in various fish species at different storage conditions has led to more precise shelf life predictions and facilitated modelling of spoilage (Gram and Dalgaard, 2002). Thus, in

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order to develop new high quality products it is important to have rapid and precise methods to analyse changes in the microbial community as a function of hygienic handling, packaging and storage. Publications during the last decade tend to present investigation of the whole microbial community, rather than sub populations, by using culture-independent methods (Muyzer and Smalla, 1998; Torsvik et al., 1998). Identification of the bacterial flora based on conserved genomic regions coding for the 16S rRNA gene by the polymerase chain reaction (PCR) and denaturing gradient gel electrophoresis (DGGE) is a widely used method in environmental microbiology (Muyzer et al., 1993; Øvreås, 2000; Schäfer and Muyzer, 2001) as well as in the food industry (Ercolini, 2004; Nakano et al., 2004; Takahashi et al., 2004). Recently the method has been used to study the bacterial flora of MA-packaged Atlantic halibut (Hovda et al., 2007) and Atlantic halibut larvae (Jensen et al., 2004). Rudi et al. (2004) described the bacterial community in MA-packaged salmon and coalfish using the PCR and terminal restriction fragment length polymorphism (T-RFLP) technique. Culture independent methods involving the 16S rRNA gene fragment can be used to describe both cultivable and unculturable bacteria in a population. The target sequence for PCR amplification is the hypervariable V3-region on the 16S rRNA gene. Separation of DNA fragments on a gel is based on sequence differences, and subsequent sequencing and phylogenetic relationship can determine the predominant bacteria population in a sample. To our knowledge there are no available studies which have used PCR-DGGE to describe the predominant bacteria composition and diversity in cod. PCR-DGGE is a reproducible and reliable method for fingerprinting the microbial community in a sample, directly after DNA extraction.

The aim of this work was to evaluate PCR-DGGE as a molecular approach to analyse the predominant microbial community on farmed cod. Moreover, we wanted to study how this method can be used as a tool to differentiate microbial development occurring during storage in different atmospheres.

2. Materials and methods

2.1. Raw material, preparation and storage

Farmed Atlantic cod (*Gadus morhua*) was obtained from the commercial fish farm Fjord Marin Helgeland AS (Brønnøysund, Norway). Whole, gutted fish with a weight of 2.1 to 2.9 kg were stored and shipped on ice to Norconserv (Stavanger, Norway). *Post rigor* cod were filleted and cut into single portions of 150±5 g, without skin and bone, 5 days after slaughtering. Fillets were individually packaged in high density polyethylene (PE) semi rigid trays (no. 523, volume: 415 ml, Polimoon, Kristiansand, Norway; oxygen transmission rate (OTR)=3.2 cm^3 d^{-1} atm^{-1} $tray^{-1}$, CO_2 transmission rate=14.0 cm^3 d^{-1} atm^{-1} $tray^{-1}$, at 23 °C, 0% RH). The packages were evacuated and a food grade gas (50% CO_2 and 50% O_2 or N_2, AGA, Linde Gas, Stavanger, Norway) was introduced into the package before heat sealing with a laminate cover film (15/70 μm PA/PE, Polimoon, ibid.; OTR=30 cm^3 m^{-2} d^{-1} atm^{-1}) on a semi-automatic tray sealer (Dyno 460 VGA, Polimoon, ibid.). The gas volume-to-product (g/p) ratio was approximately 2:1. Control fillets were stored in high density PE trays wrapped in cling film with low barrier properties to allow air to penetrate (PVC-film, Linpac plastics Pontivy SA, Noyal-Pontivy, France). The packages were stored in chill cabinets at 0 °C for a total of 11 days, were day 0 was defined as the day of packaging (5 days after slaughtering). Samples were taken at day 0, 4, 7, 9 and 11.

2.2. Gas measurement

The gas composition (O_2 and CO_2, %) in the packages was measured in triplicate using an oxygen and carbon dioxide analyser (Checkmate 9900 Analyzer, PBI-Dansensor, Ringsted, Denmark). A 20 ml aliquot of the gas was collected through a syringe from the headspace after intrusion of the top foil and analysed. Before intrusion of the syringe, a foam rubber septum (Nordic Supply, Skodje, Norway) was added to the top foil to avoid introduction of false atmosphere into the gas analyser.

2.3. Cultivation and isolation of bacteria from fish matrix

Duplicate samples of 10 g were collected by slicing through the fillet, diluted 1:10 in peptone water (1 g/l Bacto peptone (Merck 1.07224, Darmstadt, Germany) and 8.5 g/l NaCl (p.a.)) and homogenised for 30 s in a Stomacher 400 (AJ Steward Company LTD, London, England). The aerobic plate count (APC) and number of H_2S-producing bacteria were determined from appropriate 10-fold dilutions added to melted and temperate (45 °C) Iron Agar Lyngby (IA, Oxoid CM 964, Basingstoke, England) supplemented with 0.04% L-cysteine (p.a.), and incubated for 3 days at 20±1 °C. Black colonies were counted as H_2S-producing bacteria and the total numbers of black and white colonies were determined as the APC. Psychrotrophic bacteria were determined by a spread plate count method on solid Plate Count Agar (PCA) (Merck 1.05463, Darmstadt, Germany) with 1% NaCl (p.a.), which support growth of *P. phosphoreum*. Plates were incubated aerobically for 10 days at 7±1 °C. Colonies were counted, and colony forming units (CFU) per gram sample were calculated.

After counting, the psychrotrophic bacteria from PCA spread plates were collected, using an inoculation loop, washed twice with 1× PBS (137 mM NaCl, 2.7 mM KCl, 0.9 mM KH_2PO_4 and 6.4 mM Na_2HPO_2 [pH 7.4]) and frozen at −20 °C. The pellets were thawed immediately prior to DNA extraction, and cell pellets DNA were purified using DNeasy Tissue Kit (Qiagen, Hilden, Germany) or E.Z.N.A. Tissue DNA kit (Omega Bio-tek, Doraville, USA).

2.4. Extraction of total DNA from fish matrix

Duplicate surface samples of 10 gram muscle were aseptically removed by making a 0.2–0.5 cm deep cut of approximately 20 cm^2 and DNA was extracted in accordance with the method of Rudi et al. (2004). Briefly the method consisted of 1:10 dilution of the samples in peptone water, homogenising for 2 min in a Stomacher 400 (Colworth, AJ Steward Company LTD, London, England) before 50 ml of the suspensions were

frozen. For bacterial extraction the tubes were thawed and diluted 1:2 with peptone water before centrifugation for 2 min at 700 rpm (Sorvall RC 5C Plus, Sorvall Products, Newtown, USA). The supernatants were removed, and collected, until approximately 10 ml was left. 90 ml peptone water was added to the fish suspensions and the centrifugation repeated. The supernatants were added to the first supernatants, and centrifuged for 15 min at 13 000 rpm. Pellets were diluted in 10 ml TE-buffer pH 8 (10 mM Tris–HCl (p.a.) and 1 mM EDTA (p.a.)), and centrifuged for 10 min at 9000 rpm. Pellets were diluted in 5 ml TE-buffer and DNA was purified using DNeasy Tissue Kit (Qiagen, Hilden, Germany) or E.Z.N.A. Tissue DNA kit (Omega Bio-tek, Doraville, USA) following the manufacturer's instructions.

2.5. Bacterial control strains

P. phosphoreum (CCUG 12.228) and *S. putrefaciens* (CCUG 13.452) from the culture collection of Gothenburg, Sweden, were used as reference strains. The DNA was extracted using DNeasy Tissue Kit (Qiagen, Hilden, Germany).

2.6. PCR protocol

Universal primers for the domain Bacteria were used for amplification of the variable V3-region on 16S rRNA gene. For PCR the forward primer BA338f (5′ACT CCT ACG GGA GGC AGC AG) (Øvreås et al., 1997) included a 40 base GC clamp at the 5′ end (5′CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G) (Sheffield et al., 1989), and the reverse primer was UN518r (5'ATT ACC GCG GCT GCT GG) (Muyzer et al., 1993). Bacterial DNA extracted from fish matrix or cell pellet, 2.5 µl, were used as template in the PCR reaction containing a final concentration of 0.5 µM of each primer (Eurogentec, Ougrée, Belgium and MWG-Biotech, Ebersberg, Germany), 0.5 U DyNAzyme II Polymerase (Finnzyme, Espoo, Finland), 0.1% BSA (Sigma, Germany) and 0.2 mM of each dNTP (Fermentas, Lithuania) in a total reaction volume of 25 µl. The reaction was performed on a Mastercycler personal (Eppendorf, Germany) using the following conditions: 92 °C for 2 min and than 30 cycles of 92 °C for 1 min, 55 °C for 30 s and 72 °C for 1 min. The reaction was ended with an extension step of 6 min at 72 °C. PCR products were examined and verified on 2% agarose gels (SeaKem Le agarose, BMA, Rockland, USA), visualized by ethidium bromide staining. The gels were photographed under UV light in a Bio-Rad GelDoc 2000 system.

2.7. DGGE analysis

The DGGE-unit V20-HCDC (Scie-Plas Limited, Southham, England), a vertical dual heater system, was used for DGGE analysis of the PCR 16S rRNA gene products. Electrophoresis was performed on a 0.75 mm thick 8% (w/v) polyacrylamide gel in 0.5× TAE (40 mM Tris–Acetate and 1 mM EDTA [pH 8.3]), using 10 µl of the PCR product. The polyacrylamide gels (37.5:1 acrylamide:bisacrylamide (Bio-Rad, Hercules, USA)) were made with a denaturing gradient ranging from 30 to 60%. The denaturant (100%) corresponds to 40% formamide (BDH Electran, England) deionised with Dowex 1-X8 mixed-bed resin (BDH Electran, England) and 7 M Urea (BDH Electran, England). Electrophoresis was run in 0.5× TAE buffer at 60 °C for 10 min at 20 V and further 18 h at 70 V. After electrophoresis the gel was stained for 1 h with 1× (final concentration) SybrGold (Molecular Probes, Eugene, USA) in 1× TAE buffer, rinsed in water and visualized under UV light as described previously.

2.8. Sequencing of DGGE fragments

Main fragments were selected for nucleotide sequence determination. Materials from selected bands were excised with sterile pipette tips and transferred to 30 µl of sterile water. PCR fragments recovered in the solution by passive diffusion at 4 °C over night. From the eluate 5 µl was used as template and re-amplified using PCR with the same primers and conditions as described. The PCR product was re-run on DGGE to confirm that it migrated as a single band, to the same position. Single and correct positioned products were cleaned by GenElute PCR Clean-Up Kit (Sigma-Aldrich, St. Louis, USA), according to the manufacturer's recommendations. Sequencing was performed at the University of Bergen Sequencing Facility (Bergen, Norway) with an ABI PRISM 3700 DNA Analyser (Applied Biosystems, Warrington, UK), the forward primer BA338f without the GC clamp and the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Warrington, UK). Searches in BLAST from GenBank were used to find the closest known relatives to the partial 16S rRNA gene sequences (145 bp–190 bp) (Altschul et al., 1997).

2.9. pH measurement

The pH was measured in duplicate directly in cold cod fillet using a pH meter (Orion 410Aplus Benchtop, Thermo Electron Corporation, Beverly, US) equipped with a puncture combination electrode (81-63 ROSS™, Thermo Electron Corporation, Beverly, US).

2.10. Determination of trimethylamine (TMA) and trimethylamine oxide (TMAO)

Trimethylamine (TMA) and trimethylamine oxide (TMAO) was determined using the Conway microdiffusion method (Conway and Byrne, 1933), as described by Rosnes et al. (2006).

2.11. Sensory characteristics (Quality Index Method)

The sensory characteristics of the cod were assessed by a modified Quality Index Method (QIM) described by QIM Eurofish (Martinsdottir et al., 2001), and adapted for cod. The QIM-score was based on appearance, texture and odour of raw products. The panel gave demerit scores of 0 to 2, or 0 to 3 points for the different attributes. The odour was evaluated as sea fresh, neutral, fishy or ammonia/sour, giving 0, 1, 2 or 3

points, respectively. The other attributes evaluated were gaping (0: none–3: severe), colour (0: homogeneous white–2: yellow, translucent), surface (0: dry and shiny–2: dispersed) and texture (0: natural–3: very soft). The QIM-score was the sum of the scores given by the sensory panel on the individual quality parameters on a scale from 0 to 13. Coded trays of raw cod were evaluated in duplicate by a trained sensory panel consisting of 4 panellists.

2.12. Statistical analysis

Analysis of variance (ANOVA) by the general linear model (GLM) were performed with Minitab 14.13 (Minitab Inc., US) using Tukey's HSD test at level $P<0.05$ (95%) to obtain confidence intervals for differences between level means for packaging type and storage time. ANOVA was applied to log transformations of the microbiological counts.

3. Results and discussion

3.1. Microbial diversity during storage

The molecular approach, PCR-DGGE and sequencing, was applied to describe changes in the bacterial populations found on farmed cod stored over a period of time, in different atmospheres (Fig. 1). The universal primer set BA338f and UN518r amplified the hypervariable V3-region on the 16S rRNA gene, and it was used to determine the bacterial profile of the community during storage of cod. The V3-region is considered to have a high grade of resolution and to be highly variable, and it is regarded as a good choice when it comes to length and species–species heterogeneity (Coppola et al., 2001; Ercolini, 2004). These primers have previously been used with success for detection of bacteria from e.g. meat products and food processing plant (Takahashi et al., 2004), Atlantic halibut larvae (Jensen et al., 2004), diary products (Coppola et al., 2001) and mineral water (Dewettinck et al., 2001). We have recently used this method to investigate the bacterial population in MA-packaged farmed halibut (Hovda et al., 2007). We used PCR-DGGE as a tool to explain the observed quality changes during storage based on the bacterial composition. In this paper we have discussed and compared the results from the PCR-DGGE analysis with results obtained by cultivation methods and sensory scores.

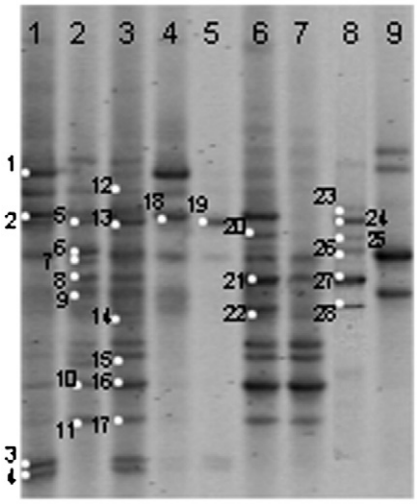

Fig. 1. DGGE bacterial profile of farmed cod stored in different gas mixtures and in air, for 11 days. The initial bacterial population profiles on the fish (day 0) are shown in lane 1 and 2 (different parallels). Samples were taken after 4 and 11 days; air stored samples in lane 3 (4 days) and 6 (11 days), CO_2:O_2 packaged samples (lane 4 and 8) and CO_2:N_2 packaged samples (lane 5 and 7). *Shewanella putrefaciens* (lane 9) was used as internal reference. The identification of the bands (1–28) is shown in Table 1.

Table 1
16S rRNA gene sequence similarities to closest relatives of DNA recovered from the respective bands in the DGGE gel, Fig. 1

Band no.	Closest relative in GenBank database (accession number)	Similarity (%)
1	*Chryseobacterium* sp. (AY751083)	98
2	*Pseudomonas* sp. (DQ200852)	99
3	*Tiedjeia arctica* (DQ107523)	99
4	*Serratia* sp. (AY745744)	98
5	*Gadus morhua* (AF518205)	98
6	*Shewanella putrefaciens* (AY321590)	99
7	Endosymbiont of *Acanthamoeba* sp. (AF366582)	100
8	*Pseudomonas fragi* (AY972397)	99
9	*Shewanella putrefaciens* (AF005255)	99
10	*Photobacterium 'kishitanii clade'* (AY849426)	99
11	*Photobacterium phosphoreum* (AY888016)	94
12	*Carnobacterium piscicola* (AF270795)	97
13	*Pseudomonas fluorescens* (AY730552)	93
14	*Photobacterium phosphoreum* (AJ746359)	96
15	*Photobacterium phosphoreum* (AJ746359)	99
16	*Photobacterium phosphoreum* (AY780010)	96
17	*Photobacterium phosphoreum* (AY780010)	98
18	*Pseudomonas* sp. (DQ200852)	100
19	*Pseudomonas fluorescens* (AY730552)	99
20	*Pseudomonas putida* (AY450556)	100
21	*Pseudomonas putida* (AY450555)	95
22	*Pseudomonas putida* (AY450555)	100
23	*Pseudomonas* sp. (DQ226216)	100
24	*Pseudomonas* sp. (AF388027)	99
25	*Pseudomonas* sp. (DQ173210)	99
26	*Pseudomonas syringae* (AM086227)	98
27	*Pseudomonas* sp. (AY331374)	90
28	*Pseudomonas* sp. (DQ200852)	99

Analysing the bacterial profiles of DNA extracted directly from the fish muscle, it was observed a decrease in the bands for air stored samples, whereas an increase in the MA-packaged samples during storage (Fig. 1). Sequence analyses of 28 bands, included the indicated bands (Fig. 1), detected *Pseudomonas* spp. and *P. phosphoreum* as the dominating bacteria in the MA samples (Table 1). *P. phosphoreum* is regarded as the main spoilage bacteria in chilled stored MA-packaged cod (Dalgaard et al., 1993; Dalgaard, 1995). Initially the bacteria of farmed cod included *Chryseobacterium* sp., *Carnobacterium piscicola*, *Tiedjeia arctica*, *Serratia* sp., *S. putrefaciens*, various *Pseudomonas* and *Photobacterium* (Table 2A). *C. piscicola* has been reported to be part of the microflora of MA-packaged smoked salmon, however the bacterium do not contribute to product spoilage (Paludan-Müller et al., 1998). *Chryseobacterium* sp. and *Serratia* sp., mainly *Serratia liquefaciens*, has been

Table 2
Sequencing of dominant bands in DGGE profiles obtained from direct extraction on DNA from the fish matrix (A) and from psychrotrophic bacteria cultivated on agar plates (B)

A

Day	Day 0	Day 4			Day 11		
Bacteria/storage		Air	CO_2:O_2	CO_2:N_2	Air	CO_2:O_2	CO_2:N_2
Chryseobacterium sp. (#1)	x		x				
Carnobacterium piscicola (#12)	x	x					
Pseudomonas sp. (#2, 18, 23–25, 28)	x		x		x	x	
Gadus morhua (#5)	x						
Shewanella putrefaciens (#6, 9)	x	x	x		x		x
Endosymbiont of *Acanthamoeba* sp. (#7)	x	x					
Pseudomonas fragi (#8)	x	x					
Photobacterium 'kishitanii clade' (#10)	x	x			x		x
Photobacterium phosphoreum (#15, 17)	x	x			x		x
Tiedjeia arctica (#3)	x	x					
Serratia sp. (#4)	x	x					
Pseudomonas fluorescens (#19)		x		x			
Pseudomonas putida (#20, 22)					x		x
Pseudomonas syringae (#26)						x	

B

Day	Day 0	Day 4		Day 11	
Bacteria/storage		Air	CO_2:N_2	Air	CO_2:N_2
Pseudomonas sp.	x	x	x	x	x
Pseudomonas fragi	x	x	x	x	x
Pseudomonas putida	x	x	x		
Photobacterium sp.	x	x	x	x	x
Photobacterium phosphoreum	x	x	x	x	x

The table includes the different storage variants during the storage period. The associated bands from Fig. 1 and Table 1 are shown in brackets (A). Only sequences with similarity >97% are included in the table.

reported as part of the fish microflora (Gonzalez et al., 2000; Olsson et al., 2004), while *Tiedjeia arctica* has been found in tundra and artic environment (Rivkina et al., 2004). Sequencing detected DNA from cod as one of the bands represented in the profiles (Fig. 1, Table 1). The universal primer set BA338f and UN518r has previously been shown to amplify eukaryotic 18S rDNA (Jensen et al., 2004). During air storage the bacterial profile changed to include only the spoilage bacteria *Pseudomonas* spp., *P. "kishitanii clade"*, *P. phosphoreum* and *S. putrefaciens*. MA with CO_2:N_2 inhibited the bacterial growth, and the initial bacterium was *Pseudomonas fluorescens*. A higher bacterial diversity was detected during storage, and *Pseudomonas putida, S. putrefaciens*, *P. "kishitanii clade"* and *P. phosphoreum* were detected as the predominant bacteria in the samples at the end of the trail. Compared to air and CO_2:N_2, the oxygen enriched packages were dominated by *Pseudomonas* spp. during storage (Table 2A). *S. putrefaciens* was found only at day 4 in CO_2:O_2 stored samples and the absence during storage supported earlier findings that this bacterium is inhibited at high O_2 concentrations during cold (7 °C) storage (Boskou and Debevere, 1997). Based on analyses of the V3-region on 16S rRNA gene *S. putrefaciens* was detected as part of the bacterial flora on farmed cod, in contrast to *S. baltica* which was found by Vogel et al. (2005). The differences in the results obtained might be the result of different bacterial population on farmed and wild cod or cod caught at various fishing grounds and during a year cycle (Vogel et al., 2005). The choice of primers and detection area on 16S rRNA gene may also contribute to the observed differences (Schmalenberger et al., 2001). In the study of Vogel et al. (2005) the detected H_2S-producing bacteria were identified based on the sequence analysis of a 1.4 kb nucleotide sequence of the 16S rRNA gene. Our study used the 16S rRNA gene V3-region resulting in a ~180 bp partial sequence.

Photobacterium sp., *P. phosphoreum*, *Pseudomonas fragi*, *P. putida* and *Pseudomonas* sp. were the predominant bacteria in air and CO_2:N_2 stored cod found as psychrotrophic bacteria on spread plates (Table 2B). We found a low bacterial diversity in the cultured samples (15 bands sequenced), and detected the same bacteria as the predominant population in all samples. A general advantage using bacterial DNA extracted directly from fish matrix is more rapid results and a more complex profile compared to cultivation methods (Amann et al., 1995; Masco et al., 2005).

Using the recently described method one can monitor and compare the effect of modified atmosphere storage on the predominant microflora. Another advantage is the possibility to follow community changes over time, where samples taken during storage can be compared and analysed simultaneously. The two reference strains *P. phosphoreum* and *S. putrefaciens* had both multiple profiles, which are caused by sequence heterogeneity as described by Nübel et al. (1996) and Schmalenberger et al. (2001). The reference strains confirm that the primers amplify known spoilage bacteria, and they can be used as a "reference" when bands are compared. Some bands from the DGGE profile were not sequenced, hence some bacteria of possible relevance for quality may remain undiscovered in the farmed cod flora. Other bands had sequence similarity of less than 97%, and were thereby not assigned as the same species. Limitations of this technique include extraction and sample preparation as well as detection limits of the PCR reaction (Wintzingerode et al., 1997). The DGGE approach allows only short sequence fragments (<500 bp) to be used, thereby limiting the amount of sequence information available.

3.2. Microbiological characteristics

The initial APC was log 3.9 CFU/g cod (Fig. 2A). After 11 days in air storage at 0 °C this number had increased to log 9.5 CFU/g. Packaging of cod fillets using CO_2:O_2 or CO_2:N_2

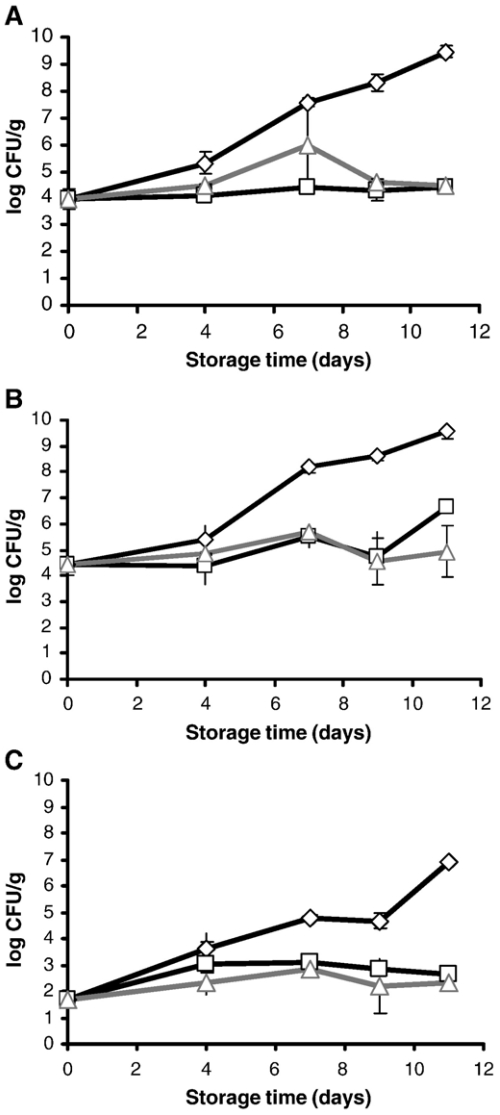

Fig. 2. Bacterial growth in farmed cod during storage. The cod samples were stored for a total of 11 days after packaging. Fig. 2A) aerobic plate counts, B) psychrotrophic bacteria and C) H_2S-producing bacteria. ◇ = air packaged variant, □ = CO_2:N_2 MA packaging and Δ = CO_2:O_2 MA packaging.

(50%:50%) strongly inhibited the bacterial growth ($P<0.001$). After 11 days there were almost no increase in APC, and the counts were approximately log 4.4 CFU/g for both gas mixtures. No differences in bacterial growth between the two atmospheres CO_2:O_2 and CO_2:N_2 were observed (Fig. 2A). APC is used as an indicator for high quality shelf life (not necessarily spoilage), and log 6 CFU/g is used as a maximum level for fish sold for human consumption (ICMSF, 1986). The air stored sample reached this limit after approximately 5 days, whereas the MA packaging extended the microbial shelf life, and gave acceptable microbial levels after 11 days, which is 16 days after slaughtering (Fig. 2A).

Both atmospheres inhibited the growth of psychrotrophic bacteria ($P<0.001$) (Fig. 2B) and H_2S-producing bacteria ($P<0.001$) (Fig. 2C) compared to the air storage. Spoilage of chilled CO_2 packaged fish is found at a level of log 7 CFU/g *P. phosphoreum* (Dalgaard et al., 1993). Using this level and assuming that the major part of the psychrotrophic bacteria are *P. phosphoreum*, this spoilage level was reached after 6 days in air storage and after about 11 days for CO_2:N_2 (Fig. 2B). At the end of the trail oxygen enrichment inhibited growth of psychrotrophes more than the CO_2:N_2 atmosphere, as also observed by Sivertsvik (2007) who found a decreased bacterial growth in 50% O_2 and 50% CO_2. MA also inhibited the growth of H_2S-producing bacteria. Bacterial counts increased from log 1.7 to log 2.6 CFU/g for both gas mixtures after 11 days, and to log 6.9 CFU/g when stored in air. It is well established that CO_2 inhibit growth of strict aerobic bacteria (Dalgaard et al., 1993; Dalgaard, 1995) and H_2S-producing spoilage organisms (Debevere and Boskou, 1996). *S. putrefaciens* is also known to be inhibited by low storage temperature and lowered pH, as discussed in the chemical analyses section. *S. putrefaciens* has been detected in low numbers or reported as absent in other farmed fish e.g. wolf-fish (Rosnes et al., 2006), halibut (Hovda et al., 2007) and *pre rigor* filleted farmed cod (Sivertsvik, 2007).

3.3. Sensory evaluation

The cod fillets were characterised as dry and shiny white, with a sea fresh odour and a firm to elastic texture before packaging, which is 5 days after slaughtering. The first 4 days of storage changed the parameters except the surface, to a less fresh appearance. Further air storage decreased the sensory quality of the fillets, and after 7 days the odour was described as "sour/ammonia", and the fish was considered as spoiled (Fig. 3). MA packaging gave significantly lower QIM-score and better quality for both CO_2:N_2 and CO_2:O_2 packaged samples compared to samples stored in air ($P<0.05$). For packaging in CO_2:N_2 this "sour/ammonia" odour was observed after 11 days. The CO_2:O_2 packaged samples never got this characteristic sour odour and were acceptable even at the end of storage.

3.4. Chemical analyses

Bacteria like *P. phosphoreum*, *S. putrefaciens*, *Vibrio* spp., *Aeromonas* spp. and psychotolerant *Enterobacteriaceae* use

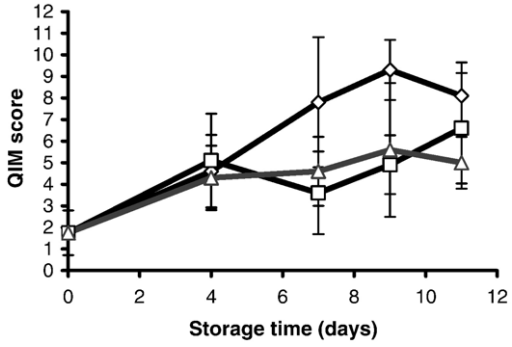

Fig. 3. QIM scores of raw fillets as a function of storage time, for the different gas compositions. ◇ = air packaged variant, □ = CO_2:N_2 MA packaging and Δ = CO_2:O_2 MA packaging.

TMAO as a terminal electron acceptor with TMA as end product in the metabolism (Gram and Dalgaard, 2002). The TMAO and TMA concentrations were measured before storage and after 14 days. The initial TMA concentration (4 mg TMA-N/100 g) increased to 20 mg TMA-N/100 g for CO_2:N_2, and to 5 mg TMA-N/100 g for CO_2:O_2 storage. Air storage was measured after 11 days, and gave 36 mg TMA-N/100 g. The TMAO concentration was initially 26 mg TMAO-N/100 g, and decreased to 23 mg TMAO-N/100 g (CO_2:N_2), 19 mg TMAO-N/100 g (CO_2:O_2) and 6 TMAO-N/100 g (air). In the experiment of Sivertsvik (2007) there were not observed any TMA development in farmed cod stored under CO_2:O_2 (50:50%) at 0 °C. Similar results are found by Boskou and Debevere (1997, 1998), with an optimal inhibitory concentration of 40% O_2 and 60% CO_2 for storage at 7 °C. Our results indicated a reduced TMA production in MA, and especially for enriched oxygen concentrations, which supported previous results. Sequencing data supports these findings where CO_2:O_2 storage detected only the non TMAO reducing *Pseudomonas*, whereas CO_2:N_2 storage included the TMAO reducing bacteria *P. phosphoreum* (Table 2A). At sensory rejection the concentration of TMA in cod is 30 mg TMA-N/100 g (Dalgaard et al., 1993), and about log 7 CFU/g *P. phosphoreum* is needed to produce this concentration (Dalgaard, 1995). Plate count of air stored psychrotrophes, assumed to consist of *P. phosphoreum*, reached this limit after 6 day (Fig. 2B), and could thus explain the observed off-odour.

The high gas to product ratio (2:1) and CO_2 levels above 20% during storage in MA (data not shown) should account for a substantial bacterial growth inhibition in the packaged cod (Devlieghere and Debevere, 2000). In addition, at low storage temperature (0 °C) increased amounts of CO_2 are absorbed in the fish muscle causing lowered pH.

The pH measurements showed a significant difference between the air stored and MA-packaged samples (data not shown). Air storage increased the pH from 6.0 in fresh fish to 6.4 at day 11. In MA the pH was about 6.0 the entire storage period (max = 6.1, min = 6.0). Low pH, i.e. below 6.2, has been shown to prolong the lag phase of *S. putrefaciens* (Boskou and Debevere, 1998). This may be an additional factor for explaining our findings that H_2S-producing bacteria were inhibited in MA-packaged cod, but increased in the air stored samples (Fig. 2C).

4. Conclusion

Our results indicate a shelf life of at least 11 days at 0 °C for MA-packaged farmed cod, which is 16 days after slaughtering. There was a significant difference between storage in air, and CO_2:N_2 and CO_2:O_2, where oxygen was suggested as the better gas mixture. The PCR-DGGE technique allowed identification of the predominant bacterial species and observed the variations in community composition between packaging and storage conditions. Sequence analyses showed that *Pseudomonas* sp. dominated in CO_2:O_2 packaged cod during storage, whereas *Photobacterium* spp., *S. putrefaciens* and *Pseudomonas* spp. dominated in the air and CO_2:N_2 packages. PCR-DGGE and sequencing detected the microbial community differences that were not detectable by the cultivation based methods alone.

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Paper III

Hovda, M. B., Sivertsvik, M., Lunestad, B. T. & Rosnes, J. T. (2007): Microflora assessments using PCR-DGGE of ozone-treated and modified atmosphere packaged farmed cod fillets. Journal of Food Protection, in press, to be published in Vol. 70.

The included **Paper III** is the proof to be published. There are some minor spelling corrections in the published paper, compared to this version.

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Microflora Assessments using PCR–Denaturing Gradient Gel Electrophoresis of Ozone-Treated and Modified Atmosphere–Packaged Farmed Cod Fillets

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ABSTRACT

Denaturing gradient gel electrophoresis (DGGE) of a PCR-amplified 16S rDNA sequence was used to characterize changes in the microbial flora caused by ozone (O_3) treatment of farmed cod (*Gadus morhua*). Portions of cod were produced under controlled conditions, bathed in fresh water supplemented with 2 ppm of O_3 for 30 min, and packaged in modified atmosphere (MA: 60% CO_2 and 40% N_2) before 4°C storage. Control samples were packaged in MA or air, without prior O_3 treatment. Samples were analyzed by PCR-DGGE to determine the predominant bacterial flora and to examine possible differences in the microbial community due to O_3 treatment. The DGGE analysis during the storage period showed that the O_3 treatment produced no significant difference in the microbial flora compared with the controls. Sequencing of 16S rDNA detected the specific spoilage bacteria *Photobacterium phosphoreum, Pseudomonas* spp., *Shewanella baltica,* and *Shewanella putrefaciens* as the predominant bacteria in all samples. PCR-DGGE results were supported by culture and sensory analyses used in predicting product shelf life. Aerobic plate count, H_2S-producing bacteria, and psychrotrophic bacterial counts demonstrated no significant extension of the shelf life of MA-packaged, O_3-treated cod fillets.

Ozone (O_3) is an effective antimicrobial agent that reverts within minutes to oxygen, giving no significant toxic residues in the environment following its use. It has been deemed GRAS (generally recognized as safe) for several food applications *(11).* Ozonated water can be used on food products as a disinfectant, leaving the food products free of disinfectant residues. The use of O_3 in marine-based aquaculture systems has been limited because of the potential of O_3 to form bromate during the oxidation of naturally occurring bromide. Bromate has been demonstrated to be a carcinogen in animals, but there are no data indicating the same effect in humans *(3).* To avoid customer concern about possible carcinogenic by-products, moderate concentrations of O_3 in fresh water can be used.

The bactericidal effect of O_3 on bacteria in food has been tested for preservation of foods such as meat, poultry products, eggs, fruits, and vegetables *(19).* The most common use of O_3 as a bactericide is on municipal and industrial drinking water *(35, 36).* Trials to assess the efficacy of ozonated water against food-related microorganisms have shown that gram-positive bacteria, such as *Listeria monocytogenes, Staphylococcus aureus,* and *Bacillus cereus,* as well as gram-negative bacteria, such as *Pseudomonas aeruginosa* and *Yersinia enterocolitica,* are killed at sufficient doses *(26).* O_3 treatment of <1 ppm for less than 5 min gave growth inhibition of various pathogens *(19).* Previous publications have shown that O_3 alone has variable effects when used on products with a high organic content, such as foods *(19).* However, comparison is difficult because different O_3 concentrations and treatment times, as well as various volumes of food items, have been used. The shelf life extension of seafood based on O_3 treatment shows variable results. Some authors report an extended shelf life and sensory quality *(8, 20, 21),* whereas no or a negative effect is observed by others *(4, 25).* O_3 treatment is not universally beneficial, and excessive use of O_3 may promote oxidative food spoilage *(27).* Thus, producers are encouraged to use additional preservation techniques. By combining different preservation factors, improvements in quality can be achieved. Modified atmosphere (MA) packaging is a mild preservation method, and it has been shown to extend the shelf life of many seafood products. The effects of MA packaging on the shelf life of fish products show that this is dependent on the fish species, the gas composition, and the storage conditions *(30–32).* Spoilage bacteria contribute to degradation of the fish by producing off-odor and off-flavor. *Photobacterium phosphoreum (7), Shewanella putrefaciens, Shewanella baltica, Pseudomonas* sp. *(12, 34),* and *Brochothrix thermosphacta (5)* have been described as the most important spoilage bacteria during fish storage.

When combining different treatments, it is important to have rapid and precise methods to detect changes in the microbial community. DNA extracted from a microbial population can be used to identify the genetic diversity of the dominant populations by PCR and denaturing gradient gel electrophoresis (DGGE) *(22).* This method allows sep-

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aration of DNA fragments with different base sequences and hence provides information about variations in the target region of a bacterial population. The target sequence used for PCR amplification is the hypervariable V3 region on the 16S rRNA gene. Recently, the method has been used to study the bacterial flora of MA-packaged cod *(14).*

The objective of this study was to examine the effects of O_3 treatment on changes in the bacterial population of farmed cod during chilled storage. PCR-DGGE was used to detect the predominant bacteria in a sample representing the bacteria that are not affected by the initial O_3 treatment. As DGGE monitors bacterial profiles, it can be used to study hygienic effects of the O_3 treatment. The PCR-amplified V3 region of the 16S rDNA was analyzed by DGGE to monitor and identify the bacteria able to grow on O_3-treated, MA-packaged farmed cod. The DGGE profiles obtained were compared to determine and describe the effects of O_3 treatment.

MATERIALS AND METHODS

Raw material and packaging. One lot (4 to 5 kg) of commercially farmed, harvested, and processed prerigor cod (*Gadus morhua*) was obtained from Vikenco AS (Aukra, Norway). The fish was produced under controlled conditions, where the fillets were rinsed under water after filleting and kept on ice before and after O_3 treatment. Portions of 400-g skin- and boneless cod fillets were submerged in potable water supplemented with 2 ppm of O_3 for 30 min within 2 to 3 h after filleting. The treatment of cod fillets (10 kg) was conducted in a 500-liter tank with gentle agitation to maximize the O_3 exposure. This exposure was chosen on the basis of previous studies as a treatment suitable for the industry and able to kill food spoilage bacteria. The O_3 was produced by a Corona discharge O_3 generator, 540 W, 50/60 Hz, and O_3 output of 60.1 g/h (SGA Ozone System, Pacific Ozone Technology Inc., Brentwood, Calif.). The O_3 concentrations were measured with a HACH DR 2800 spectrophotometer with AccuVac Ozone Reagent (Hach Company, Loveland, Colo.). The fillets were packaged in 1,800-ml high-density polyethylene semirigid trays (Tray 870, Polimoon, Kristiansand, Norway). The air was evacuated, and a food-grade gas mixture (60% CO_2 and 40% N_2; AGA, Oslo, Norway) was introduced into the package before heat sealing, with a packaging machine (900VG-XL, Polimoon). The air-stored portions were wrapped with nonbarrier cling film directly after filleting. Packages of both MA and air were stored at 4.0°C, and samples were analyzed 1, 6, 11, and 14 days after slaughtering and packaging.

Microbiological analyses. Samples of 10 g of fish muscle were homogenized in 90 ml of peptone water (0.9% NaCl [wt/vol]) and 0.1% peptone [wt/vol]) for 30 s in a Stomacher 400 Laboratory Blender (Seward Ltd., London, UK). Suitable 10-fold dilutions of the fish suspensions were spread in duplicate on plates containing the following media: plate count agar (PCA; Merck, Darmstadt, Germany) containing 1% NaCl, which was used to enumerate psychrotrophic bacteria, including the salt requiring *P. phosphoreum;* iron agar (Lyngby, CM 964, Oxoid, Basingstoke, UK) for aerobic plate counts (APCs) and H_2S-producing bacteria by counting the black colonies; and Long & Hammer Agar (LHA) to determine aerobic count and specific spoilage organisms in fish, as *P. phosphoreum (33).* The PCA plates were incubated at 7°C for 10 days, the iron agar plates were incubated at 20°C for 3 days, and the LHA plates were incubated at 15°C for 7 days. Averages for duplicate plate counts were presented as log CFU per gram of muscle.

DNA extraction. Bacterial DNA was extracted directly from the fish matrix with surface samples of 10 g of muscle, as described previously *(15).* DNA was also obtained from colonies growing on LHA and PCA spread plates. After counting, the bacteria were collected, washed with phosphate-buffered saline (PBS), and frozen at −20°C. The pellets were thawed and suspended in 500 μl of PBS and incubated in boiling water for 20 min and then on ice for 10 min to weaken the bacteria cell wall, before DNA extraction with the DNeasy Tissue Kit (Qiagen, Hilden, Germany) with the gram-positive bacteria modification, as recommended by the manufacturer.

PCR, DGGE, and identification of bands. Bacterial DNA extracted directly from fish and cell pellets was used as the template to amplify the hypervariable V3 region on 16S rDNA, with the universal primers BA338f (5′ACTCCTACGGGAGGCAGC AG) and UN518r (5′ATTACCGCGGCTGCTGG) *(22, 37).* A GC clamp was linked to the forward primer *(28).* After amplification, the PCR products were separated by DGGE on a V20-HCDC system (Scie-Plas Limited, Southham, UK), with denaturing gradient ranging from 30 to 55%, as previously described *(15).* The partial sequencing was performed at the University of Bergen Sequencing Facility (Bergen, Norway) with the forward primer BA338f without the GC clamp and the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Warrington, UK). Searches in BLAST from GenBank were used to find the closest known relatives to the partial 16S rDNA sequences (90 to 200 bp) *(1).* Sequences with 97% or higher identity were considered to represent the same species.

pH measurements. The pH of the fish tissue was determined in duplicate with a pH meter (Beckman 72, Beckman Instruments Inc., Fullerton, Calif.) on homogenized muscle (25 g) in a 1:1 dilution with 0.1 M KCl.

Sensory analyses. The samples were evaluated both as raw and cooked fillets by a descriptive test adopted from Shewan et al. *(29).* Raw assessments of odor and texture were evaluated on a Torry scale for odor scores of 10 (sea fresh) to 3 and below (putrid). The texture range used was 10 (firm) to 4 and below (soft). The sensory evaluation of cooked muscle, 80°C for 8 min in steam, was carried out on fillet portions (15 to 20 mm wide) packaged in cook-plastic pouches (PA/PE 20/50) under slight vacuum (95%). Both odor and flavor of the samples were evaluated on a scale from 10 (fresh seaweedy odor and fresh sweet flavor) to 0 (putrid odor and flavor). Texture assessments, including firmness and juiciness, were on a scale from 10 (firm and juicy) to 0 (very soft and dry). Analyses were carried out in randomized order of coded samples with replicates by a panel of three experienced evaluators.

Statistical analysis. An analysis of variance was performed with Minitab 14 (Minitab Inc., Foster City, Calif.) by the General Linear Model with Tukey's honest significant difference test at the $P < 0.05$ (95%) level.

RESULTS AND DISCUSSION

The cod used in this experiment was filleted prerigor and was of high quality when cut into portions, treated with O_3, and packaged. Molecular analyses by PCR-DGGE monitored the bacterial population and profile changes after O_3 treatment and during storage (Fig. 1). The bacterial pro-

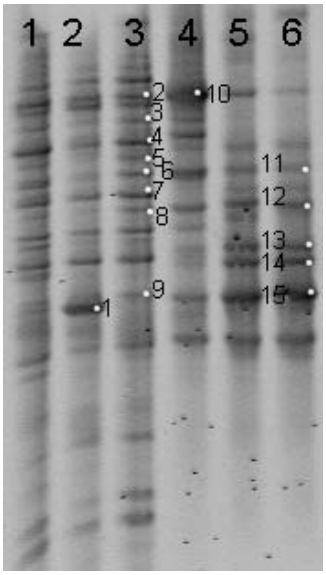

FIGURE 1. *DGGE bacterial profiles of farmed cod fillets at storage day 1 (lanes 1 to 3) and day 14 (lanes 4 to 6). At each time point, samples were taken from air storage (lanes 1 and 4), MA packaging (lanes 2 and 5), and ozone-treated, MA-packaged cod (lanes 3 and 6). The indicated bands (1 to 15) represent the sequenced bands with an identity of ≥97%, with the GenBank accession number in brackets. Bands 1, 13, and 15 identified* Photobacterium phosphoreum *(AY577825), band 2 identified* Pseudomonas *sp. (DQ344864), band 3 identified* Pseudomonas putida *(AY450556), band 4 identified* Flavobacterium *sp. (AY494683), band 5 identified soil bacterium (DQ518553), bands 6 and 11 identified* Pseudomonas *sp. (AJ864857), band 7 identified uncultured bacterium (DQ633741), bands 8 and 12 identified* P. putida *(AY450555), band 9 identified* Bacillus cereus *(DQ841261), band 10 identified* Pseudomonas *sp. (DQ521397), and band 14 identified* P. phosphoreum *(AJ746359).*

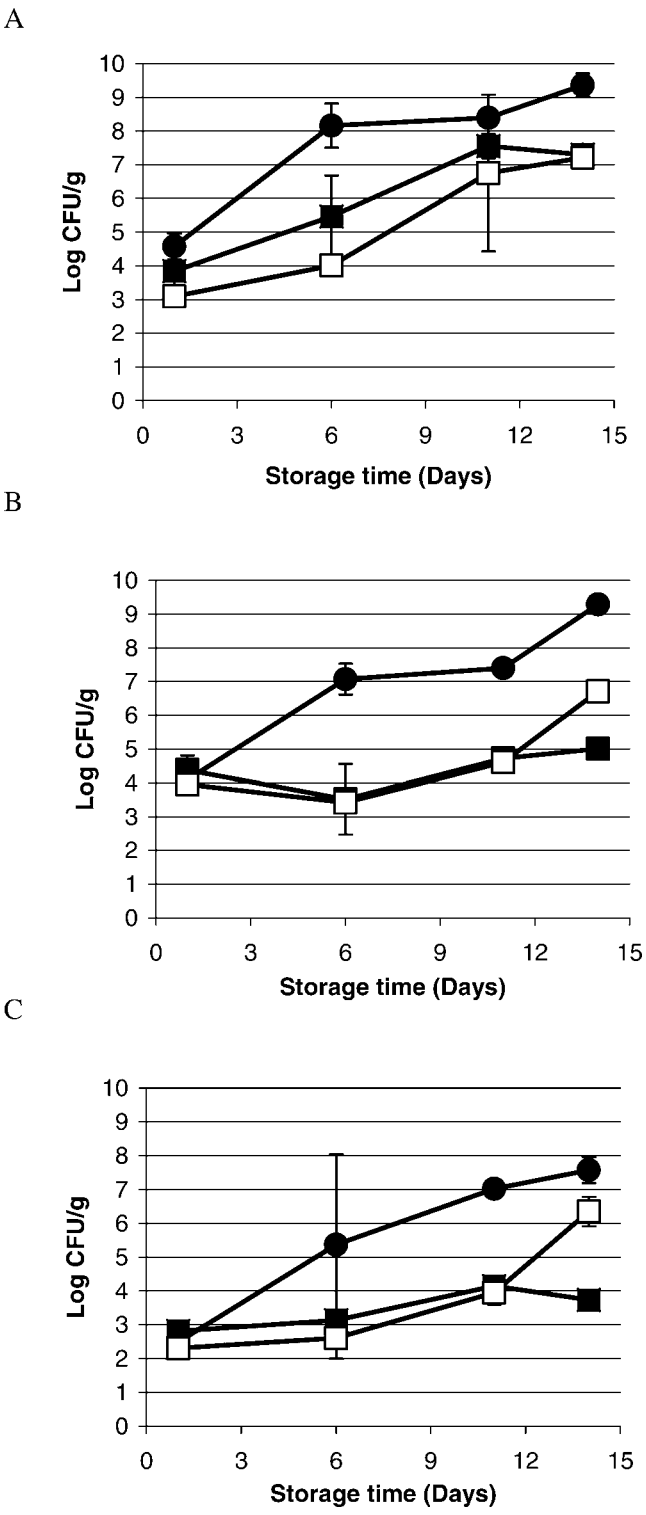

FIGURE 2. *Microbial growth of psychrotrophic bacteria on PCA (A), aerobic plate counts (APCs) on iron agar (B), and H_2S-producing bacteria as black colonies on iron agar (C) on cod stored in air (—●—), MA (—■—), and ozone-treated MA (—□—).*

file obtained directly from the fish muscle at the beginning of the storage represented a wide variety of bacteria, including *P. phosphoreum, Pseudomonas* spp., *Flavobacterium* sp., and *B. cereus.* No bacterial differences were observed between the treatments after day 1. *Flavobacterium* is widely found in marine waters, and *Flavobacterium psychrophilum* is considered a fish pathogen *(23).* During storage, the diversity decreased, and MA-stored samples, with and without ozonization, showed similar DGGE profiles (Fig. 1). The 16S rDNA analyses identified *Pseudomonas* spp. and *P. phosphoreum* as the predominant bacteria during storage. These bacteria have previously been found to be an important part of the spoilage microflora of MA-packaged fish *(7, 14, 24).* Our PCR-DGGE result, showing that the O_3 treatment had no detectable effect on the microbial community composition, was supported by findings from three types of bacteriological plate counts (Fig. 2A through 2C). This study is in agreement with previous studies, showing minor effects of O_3 when applied on fish muscle *(6, 8).* Güzel-Seydim et al. *(13)* tested the potential of O_3 to reduce the bacterial load of five different food components. They reported that the food components had significant effects on the bactericidal power of O_3. These effects must be considered when designing processes that will rely on O_3 for bacterial destruction.

Analyses of the predominant bacteria obtained from cultivation on LHA showed no significant differences between the different storage conditions, which indicated little

effect of the O_3 treatment (data not shown). Gel analyses showed that the main bands were represented in all samples throughout the storage period. Sequencing detected *Pseudomonas* spp., *P. phosphoreum,* and *Psychrobacter* sp. as the bacteria present on the spread plate samples. Although *Pseudomonas* and *P. phosphoreum* are known spoilage bacteria, the spoilage potential of *Psychrobacter* is not fully known *(9).* Furthermore, *S. baltica* and *S. putrefaciens* were found on air-stored and O_3-treated MA-packaged cod. This result supports the result of da Silva et al. *(8),* who found no effect of the ozonization on the growth of *Pseudomonadaceae* or H_2S-producing bacteria on cod, except after 8 days, when there was a 1-log reduction of H_2S-producing bacteria. The detection of *Shewanella* spp. supported the microbial plate counts, although the growth of H_2S-producing bacteria occurred in all packaging variants (Fig. 2C). Cultivated bacteria from PCA plates gave profiles and sequencing results similar to LHA plates (data not shown). The aim of this study was to investigate and detect potential changes in the bacterial flora of O_3-treated fish. Previous studies have shown that pure cultures of *S. putrefaciens* and *Pseudomonas putida* were inhibited when exposed to O_3 *(8).* In the present study, we found that O_3 treatment did not change the bacterial flora compared with untreated samples and that the same predominant bacteria were found during the storage of cod. This is also supported by the study of Ravesi et al. *(25),* in which O_3 treatment of fresh cod had no effect on the shelf life.

On the basis of microbial counts, the O_3-treated MA-packaged cod had a shelf life of about 10 to 13 days. There was no significant effect of O_3 treatment compared with control MA-packaged cod as expressed by microbial plate counts. The psychrotrophic counts increased for both air-stored and MA-packaged products after day 1. There were, however, significantly ($P < 0.05$) lower bacterial numbers in the MA packages (Fig. 2A). Psychrotrophic bacterial growth on PCA-added salt is assumed to consist mainly of *P. phosphoreum,* a spoilage bacterium during MA packaging. Measured as APC, the microbial growth started immediately in air but had a lag phase of about 9 days for MA-packaged products (Fig. 2B). An APC of 6 log CFU/g is often used as a maximum level for fish sold for human consumption *(17).* Application of this limit would give a high-quality shelf life of about 13 days for O_3-treated cod, whereas MA packaging without O_3 treatment was below this limit (Fig. 2B). *S. putrefaciens* and the recently described *S. baltica* are the main spoilage bacteria in air- and ice-stored fish, e.g., cod, plaice, flounder *(16, 34),* causing off-odor by the production of H_2S and trimethylamine. MA packaging inhibited the bacterial growth of H_2S-producing bacteria the first 11 days. On day 14, the O_3-treated cod was 6.5 log CFU/g versus 4 log CFU/g for MA-packaged cod fillets without O_3 treatment (Fig. 2C). During air storage, the growth of H_2S-producing bacteria increased from 3 to 8 log CFU/g, where 8 log CFU/g often is used as a spoilage limit *(18).* O_3 treatment of cod muscle showed no marked differences on the bacterial growth of APC and psychrotrophic bacteria. Variations in the muscle pH may affect the bacterial growth. Farmed cod is known to have a lower pH than wild-caught cod, and previous studies have reported a pH of around 6.0 for farmed cod *(14).* A pH below 6.2 has been shown to prolong the growth lag phase of *S. putrefaciens (2).* We observed a pH of 6.3 ($\pm$0.1),

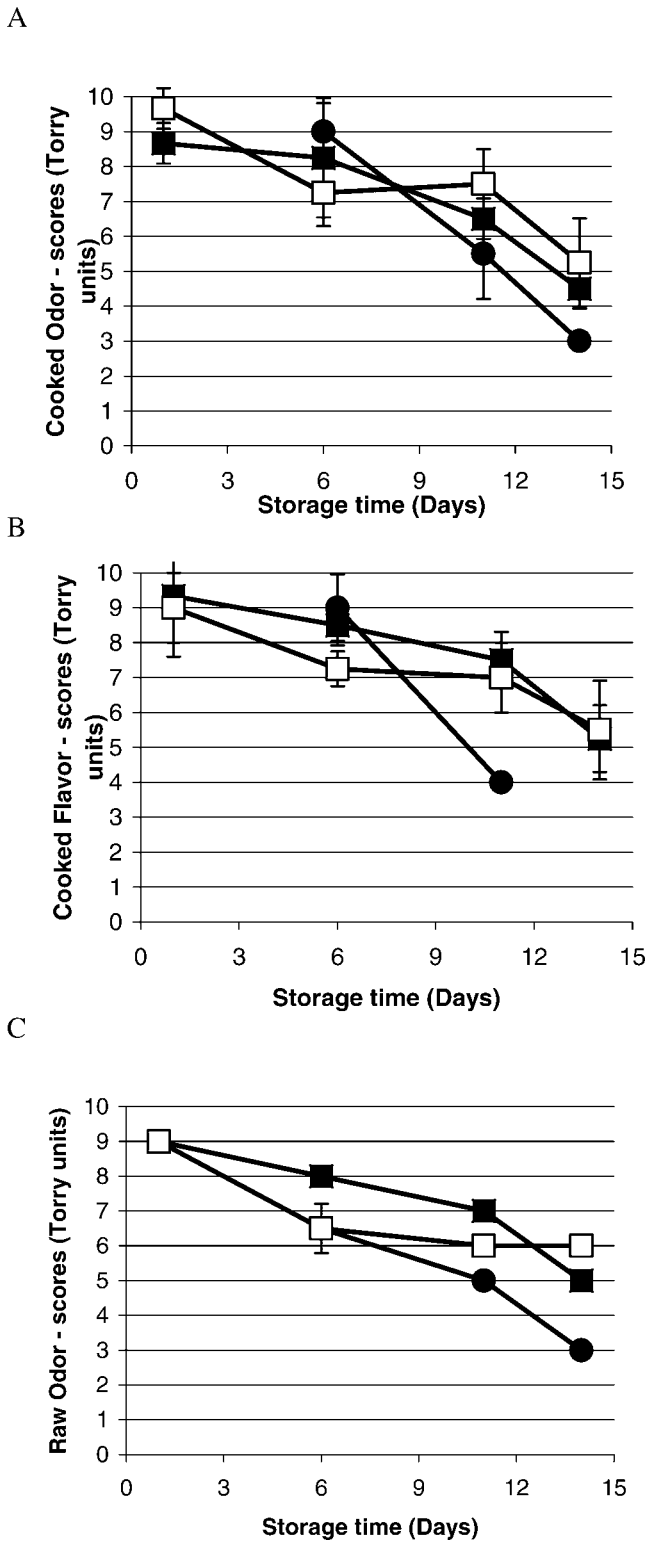

FIGURE 3. *Reductions in the sensory scores for odor (A) and flavor (B) in cooked cod and raw odor (C). Air storage (—●—), MA (—■—), and ozone-treated cod packaged in MA (—□—). The Torry scale of 10 represents a fresh seaweedy odor and fresh sweet flavor, and 0 represents a putrid odor and flavor.*

and there were no differences in the pH as a function of storage or treatment.

During O_3 treatment in a water bath, the fillets appeared whiter and softer than nontreated cod. These differences in surface appearance disappeared after 1 day of storage. A score of 5 was used as the lower limit of acceptability when analyzing the sensory quality during storage. One day after filleting, MA-packaged samples with and without ozonization had a sea fresh and firm raw odor and texture, a seaweedy and sweet cooked odor and flavor, and a firm and elastic texture. During air storage, the fish deteriorated rapidly, and after about 10 days, the air-stored cod was unacceptable based on cooked odor and flavor characteristics (Fig. 3A through 3B). Raw assessment found a firm texture above the acceptance level throughout storage for the MA-packaged cod fillets, whereas air storage had an acceptable score for 12 days. For O_3-treated cod fillets, the raw odor decreased rapidly the first 6 days, giving the characteristic trimethylamine and NH_3 odor. MA-packaged cod without O_3 treatment had a better odor the first 11 days (Fig. 3C). At the rejection level, the shelf life of MA-packaged cod was 12 to 14 days, and there were no differences between O_3-treated and nontreated samples (Fig. 3A through 3C). We observed no marked difference between O_3-treated and nontreated fish stored under MA, and this is in agreement with previous experiments on the ozonization of tilapia *(10).*

In conclusion, 2 ppm of ozonated water for 30 min had no additional effect on the main spoilage flora or the shelf life of MA-packaged farmed cod fillets. The shelf life extension compared with air storage was caused by MA packaging and not the O_3 treatment.

ACKNOWLEDGMENTS

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Paper IV

Hovda, M. B., Lunestad, B. T., Fontanillas, R. & Rosnes, J. T.: Molecular characterisation of the intestinal microbiota of farmed Atlantic salmon (*Salmo salar* L.). Aquaculture. Submitted.

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Molecular characterisation of the intestinal microbiota of farmed Atlantic salmon (*Salmo salar* L.)

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Abstract

In this study, the intestinal microbiota of farmed Atlantic salmon (*Salmo salar* L.) was examined with traditional culture-based techniques and by molecular analysis of the 16S rDNA. The aim of the study was to investigate and compare the results obtained by molecular-based methods and culture-based methods. Samples were collected from the fore-, mid- and hind-gut, respectively. PCR and denaturing gradient gel electrophoresis (DGGE) analyses were performed on DNA extracted directly from the gut content and from bacteria cultivated on Tryptic Soy Agar (TSA). Population fingerprints of the predominant microbiota were generated by DGGE analysis of universal V3 16S rDNA PCR amplicons, and distinct bands from DGGE were sequenced. Results show that the salmon intestine was dominated by *Lactobacillus* spp., *Lactococcus* sp., *Bacillus* sp., *Photobacterium phosphoreum*, *Acinetobacter* sp., *Pseudomonas* sp. and *Vibrio* sp. Molecular analyses of samples from direct DNA extraction enabled detection of lactic acid bacteria. Prior cultivation detected *Vibrio* sp., *Acinetobacter* sp. and *Pseudomonas* sp., which were not detected by the direct analyses. In addition to the PCR-DGGE approach, 50 TSA isolates from the hind-gut were pure cultured and identified by partial sequencing of the 16S rDNA. *Vibrio* spp. and *P. phosphoreum* dominated among these isolates. The data provided demonstrate the advantage of the PCR-DGGE molecular approach for studying the intestinal microbiota of fish.

Keywords: DGGE, Gut microbiota, 16S rDNA, Sequencing, Cultivation

1. Introduction

The functions of the fish intestinal microbiota are not as well studied as for humans and homoeothermic animals, and appear to be simpler (Ringø et al., 1995). However, recent papers find a more diverse microbiota and suggest this statement to be revised (Ringø et al., 2006b; Bakke-McKellep et al., 2007; Kim et al., 2007). The microbial floras of the fish intestine have been shown highly dependent on the bacterial colonization during early development, environmental conditions and dietary changes (Ringø et al., 1995, 2006a; Ringø and Birkbeck, 1999; Olafsen, 2001). In addition to digestion, absorption and metabolism, the intestine act as a main infection route for fish pathogenic bacteria (Ringø et al., 2003; Brikbeck and Ringø, 2005). The interest in gut microbiota investigations of farmed Atlantic salmon is based on the need for a better understanding of how the environment and diet may influence on the bacterial composition.

In the review by Ringø et al. (1995) the culturable intestinal microbiota of salmonids was reported to consist of Gram-negative bacteria, where *Acinetobacter* spp., *Enterobacteriaceae*, *Aeromonas* spp., *Flavobacterium* spp. and *Pseudomonas* spp. were the most common bacteria, in addition to the Gram-positive *Lactobacillus* spp. Applying molecular-based methods *Carnobacterium*, *Shewanella*, *Citrobacter*, *Clostridium* and *Mycoplasma* have also been identified in the intestinal flora of rainbow trout and salmon (Spanggaard et al., 2000; Holben et al., 2002; Huber et al., 2004; Pond et al., 2006).

Traditionally population analyses of the intestinal microbiota of fish have been carried out using conventional culture-based techniques, including cultivation on selective or non-selective media followed by isolation and phenotypic characterisation. Instead of these traditional culture-based methods, molecular methods based on PCR and 16S rDNA

sequencing can be applied, and this approach is highly accepted in the study of microbial ecology. Several authors have used such methods in the study of fish intestine (Ringø et al., 2000, 2006a; Holben et al., 2002; Verner-Jeffreys et al., 2003; Huber et al., 2004; Martin et al., 2006; Pond et al., 2006; Kim et al., 2007). Applications of culture-independent molecular methods are needed to improve our understanding of the total microbial microbiota, as conventional culture-based methods are time consuming and selective, and do not provide the entire microbial diversity of complex environments. DNA extracted from a microbial population can be used to identify the genetic diversity of the dominant populations by PCR and denaturing gradient gel electrophoresis (DGGE) (Muyzer et al., 1993). Using the 16S fragment of the rRNA gene one can describe both cultivable and uncultivable bacteria by phylogenetic relationship. PCR and DGGE are commonly used in environmental microbial ecology, in studying foods (for a review (Ercolini, 2004)) and for analyses of gut microbiota (Simpson et al., 1999; Huber et al., 2004; Mättö et al., 2005; Vanhoutte et al., 2005; Kim et al., 2007). Universal primers amplifying a hypervariable region of the 16S rDNA are frequently used to detect dominant bacteria in the samples. The target sequence for PCR, in the present study, was the V3-region on the 16S rDNA, which is known to have a high grade of resolution and to be highly variable (Øvreås, 2000). After PCR amplification, the separation of the DNA fragments in the gel is based on differences in the GC content and GC distribution in the bacterial DNA. Bacterial DNA from excised gel bands can subsequently be identified by sequencing. PCR-DGGE is a reliable and rapid method to study the variation of dominant bacteria and to characterise complex microbial populations (Giraffa and Neviani, 2001).

The purpose of this work was to use PCR, DGGE and sequencing to describe the intestinal microbiota of farmed Atlantic salmon. In addition, we wanted to compare standard isolation

and characterisation methods, with PCR-DGGE and 16S rRNA gene sequence analyses for the bacteria in the fore-gut, mid-gut and hind-gut.

2. Materials and methods

2.1 Sample collection

Atlantic salmon (*Salmo salar* L.) were raised at the Skretting Research Station in Lerang (Norway) in two sea cages (I and II), from July 2007 until October 2007. The fish were fed to satiation with two diets (9 mm commercial diet, Skretting AS), based on fish meal, fish oil, and extracted soybean meal, in addition to wheat, rapeseed oil, and vitamin premix. At the end of the feeding period, and 4-6 hrs after feeding, 5 fish from each cage with an average weight of ~1.8 kg were killed using high doses of anaesthesia (Tricaine methanesulfonate, Finquel MS 222, Argent Chemical Laboratories). The gut was divided into fore-gut, mid-gut and hind-gut. The fore-gut refers to: the proximal portion of the digestive system including the esophagus and the stomach, the mid-gut: intermediate portion of the digestive system including the pyloric caeca and small intestine, and the hind-gut: distal portion of the digestive system that corresponds to the large intestine. The gut contents, of 5 fish from each cage, were squeezed out, and the gut rinsed three times using ~3 ml peptone water (1 g/l Bacto peptone (Merck) and 8.5 g/l NaCl) to ensure capturing of adherent bacteria, as recommended and described by Ringø et al. (2006b). The hind-gut samples were divided into the squeezed (non-adherent bacteria) and the washed fraction (adherent bacteria). Since the hind-gut were expected to contain the most stabile microbial community, a more throughout examination of this intestinal segment were conducted.

2.2 Cultivation and phylogenetic characterisation

The intestinal content were homogenised for 3 min in a Stomacher 400 Laboratory Blender (AJ Steward Company LTD, London, England), and appropriate 10-fold dilutions were spread and cultivated on Tryptic Soy Agar (TSA, Oxoid CM0131, Basingstoke, England). The plates were incubated at 15 ^{o}C for one week, in order to determine the aerobic plate count of the cultivable bacteria present. The presences of H_2S-producing bacteria in the hind-gut were determined using Iron Agar Lyngby media (Oxoid CM 964, Basingstoke, England), incubated for three days at 20 ^{o}C. Black colonies on the agar were counted as H_2S-producing bacteria. Cultivated bacteria from TSA were collected, washed with 1x PBS and frozen. The pellets were thawed immediately prior to DNA extraction using the E.Z.N.A. Tissue DNA kit (Omega Bio-tek, Doraville, USA). To ensure extraction of DNA from Gram-positive bacteria, a lysozyme treatment was performed in advance.

For phenotypic characterisation, 100 selected hind-gut bacteria colonies from TSA and 20 colonies from Iron Agar were isolated and subcultured, on their respective media, until purity was achieved. The isolates were characterised by microscopy, Gram-, catalase- and oxidase-reactions. Pure cultures were stored at -70 ^{o}C using the Microbank system (Pro-Lab Diagnostics, Richmond Hill, Canada). Based on these preliminary tests, a total of 19 TSA isolates were biochemically examined using API 20E for detection of *Enterobacteriaceae* and other Gram-negative rods, and API 20NE for non-fastidious and non-enteric Gram-negative rods in accordance with the recommendations from the producer (Biomerieux, Marcy I'Etoile, France).

2.3 Direct DNA extraction from intestine samples

In order to ensure detection of non cultivable bacteria, DNA was purified directly from the intestine samples. Extraction was performed using QIAamp DNA Stool Mini kit (Qiagen,

Hilden, Germany). This kit is designed for stool samples, but the obtained DNA concentration was low when measured as the absorbance at 260 nm. Therefore, the following PCR reaction was preformed twice to increase the amount of DNA for subsequent analysis.

2.4 16S rDNA PCR and DGGE

Universal primers for the domain *Bacteria* were used for amplification of the variable V3-region on 16S rDNA. Primers for PCR were the forward primer BA338f (5'ACT CCT ACG GGA GGC AGC AG) including a 40 base GC clamp at the 5' end, and the reverse primer UN518r (5'ATT ACC GCG GCT GCT GG). The PCR and DGGE were performed as described by (Hovda et al., 2007b). Briefly the method consists of PCR amplification before separation of the amplicons on DGGE, with a gradient ranging from 30 to 55 %. The gel was run for 10 min at 20 V and further 18 h at 70 V at 60 ^{o}C. The DNA fragments to be nucleotide sequenced were excised, amplified using the forward primer BA338f without the GC clamp, and sequenced at the University of Bergen Sequencing Facility (Bergen, Norway). Searches in BLAST from GenBank were used to find the closest known relatives to the partial 16S rDNA sequences (117-162 bp).

2.5 Sequencing of the 16S rDNA from pure cultures

Out of the 100 TSA isolates, 50 were selected for sequencing, in addition to 18 of the Iron Agar isolates. The isolates were amplified using PCR and primers corresponding to the nucleotides 27-1491 on the 16S rRNA gene. PCR were performed using 5 µl thawed pure culture, diluted 1:100, as template. To each PCR tube, finale concentrations of the following reagents were included; 0.5 µM of the primers 27f (5'AGA GTT TGA TCM TGG CTC AG) and 1491r (5'GTT TAC CTT GTT ACG ACT T) (MWG-Biotech, Ebersberg, Germany), 0.1 mM of each dNTP (Fermentas, Lithuania), 1x buffer, 0.5 U DyNAzyme II Polymerase

158 (Finnzyme, Espoo, Finland) and distilled water to a final volume of 50 µl. The PCR cycling
159 was performed using the following conditions: 94 ^{o}C for 10 min and than 35 cycles of 94 ^{o}C
160 for 30 sec, 53 ^{o}C for 30 sec and 72 ^{o}C for 90 sec, before 7 min at 72 ^{o}C. Purified isolate
161 amplicons were then fully bidirectional sequenced. The phylogenetic relationships of
162 organisms covered in the present study were determined by comparison of individual rRNA
163 gene sequences to those published in the BLAST database.
164
165 **3. Results**
166 *3.1 Bacterial enumeration*
167 The average bacterial numbers in the different parts of the gut were log 3.9 cfu/g digesta in
168 the fore-gut, and log 3.7 cfu/g digesta in the mid-gut. The squeezed part of the hind-gut
169 consisted of log 6.4 cfu/g digesta, whereas the washed fraction of log 4.8 cfu/g (Table 1).
170 H_2S-producing bacteria, counted as black colonies on Iron Agar, were determined for the
171 mixed sample of squeezed and washed hind-gut, giving a bacterial number of log 5.4 cfu/g
172 digesta.
173
174 *3.2 Characterisation of pure cultures*
175 From the hind-gut TSA plates 100 colonies were randomly collected irrespectively of colour
176 and shape, for further characterisation and identification. The subcultured and purified
177 colonies were tested with Gram-, catalase-, oxidase-reactions. In addition, 19 colonies were
178 tested using API strips (20E and 20NE), but this did not identify any of the isolates with a
179 satisfactory discrimination. From the descriptive tests and analyses it was not possible to
180 classify the isolates into taxonomic groups, hence API failed to identify the isolates.
181

Out of the 100 TSA isolates, 50 were phylogenetic analysed by sequencing the 16S rRNA gene. The sequencing assigned isolates as *Vibrio* spp. (25 isolates), *Photobacterium phosphoreum* (23 isolates), *Psychrobacter glacincola* (AJ312213) (1 isolate) and *Brevundimonas* sp. (DQ177489) (1 isolate). *Vibrio* was found to dominate in gut from sea cage I, whereas *P. phosphoreum* in the gut from sea cage II. Within the Iron Agar isolates, 18 colonies were selected for sequencing. Sequencing resulted in 12 colonies representing *Vibrio* sp. (DQ146979 and AM159569), all from sea cage I, and 6 colonies assigned as *P. phosphoreum*, from sea cage II.

3.3 Intestinal bacterial profiles using DGGE

PCR and DGGE were used to visualize the bacterial diversity, and changes between different parts of the intestine. The bacterial profiles of the gut microbiota achieved by direct DNA extraction are shown in Fig. 1. It was observed a change in the bacterial flora towards more bands in the hind-gut. In order to determine to which bacterial group the particular excised bands could be ascribed, DGGE bands were sequenced. Partial sequencing detected *Lactobacillus* spp., *Lactococcus* sp., *P. phosphoreum*, *Bacillus* sp. and an unidentified bacterium as the predominant microbiota of the salmon intestine (Table 1). Although an increase in DGGE bands was observed through the gut, the results show no increased microbiota diversity. In the hind-gut, *P. phosphoreum* accounted for three bands, which is caused by the heterogeneity of 16S, and also previously observed for the bacterium (Hovda et al., 2007a, 2007b).

Cultivation on TSA enabled growth of aerobic and facultative anaerobic bacteria, and DGGE visualized the predominant bacterial profiles obtained from the cultivated samples. Different bacterial DGGE profiles were observed between samples from the two cages (Fig. 2). In fish

from cage I the bacterial profiles changed towards the hind-gut, and had fewer gel bands than fish from cage II. The bacterial profiles of fish from sea cage II were similar for the mid- and hind-gut samples, whereas the fore-gut had a unique profile (Fig. 2). Using sequence analysis to determine the bacteria present, a clear shift in the bacterial composition in the various sections of the intestine was observed (Table 1). The fore-gut was dominated by *Janthinobacterium* sp., *Pseudomonas* sp., *Acinetobacter* sp. and *Vibrio* sp., and the mid-gut by *P. phosphoreum* and *Pseudomonas* sp. In the hind-gut *Vibrio* sp. (sea cage I), and *P. phosphoreum* (sea cage II) were detected. Overall, the DGGE profiles and sequence analyses of excised bands revealed different bacterial composition in samples from direct DNA extraction compared to bacterial DNA obtained from TSA cultivation.

4. Discussion

One reported constrain in the analysis of the fish intestine microbiota is the individual variations (Spanggaard et al., 2000). To minimize the influence of these variations, the gut content 5 fish were pooled prior to analyses. Cultivation on TSA has previously been reported by several authors to be suitable for the study of the bacterial composition of fish intestine (Spanggaard et al., 2000; Huber et al., 2004; Korsnes et al., 2006; Ringø et al., 2006b). This agar was, therefore, included in the present study, in order compare this work and previous results. According to Spanggaard et al. (2000) and Ringø et al. (1995), most of the bacteria in the fish intestine are found to be aerobic and cultivable on TSA. This is in contrast to the findings of Huber et al. (2004), who found that 11-50 % was cultivable, and for one particular sample only 2 % of the intestinal bacteria could be cultured. In the present study, the bacterial counts on TSA increased from the fore-gut to the hind-gut. Compared to Holben et al. (2002), who found the distal intestinal content of farmed Norwegian and Scottish salmon to be log 7.2 and 7.8 cfu/g digesta, we reports lower bacterial counts of log 3.7-6.4 cfu/g digesta. Recent

232 analyses of Atlantic cod reported a variable bacterial content in the different parts of the gut,
233 ranging between log 3.9-5.6 cfu/g wet weight (Ringø et al., 2006b). In this study, Ringø et al.
234 (2006b) found that the adherent bacteria in the hind-gut gave the highest bacterial numbers of
235 log 4.7 to log 5.6 cfu/g wet weight, depending on diet. Other studies report a bacterial number
236 between log 2.3-6.3 cfu/g (Pond et al., 2006; Ringø et al., 2006a; Seppola et al., 2006). It is,
237 however, difficult to compare all the available results as different studies examine different
238 parts of the gut, different fish species, and furthermore, the fish sampling locations and times
239 between feeding and analyses may vary.
240
241 In the present study, traditional morphological, and biochemical criteria for identification of
242 representatives from the intestinal microbiota failed to differentiate between the isolates.
243 Ringø and Olsen (1999) used an extended and adjusted procedure to identify such isolates.
244 The insufficiency of the API system, when testing environmental samples, has been discussed
245 by several authors, and is reviewed by Popovic et al. (2007). Hansen and Sørheim (1991)
246 developed a multidish system as an alternative to API for characterising marine bacteria. For
247 further analyses one should consider using these modified procedures.
248
249 To identify the isolates we used 16S rDNA sequencing. A total of 18 hind-gut colonies from
250 Iron Agar were sequenced. Out of these, 10 appeared as black H_2S-producing bacteria on the
251 agar. From previous analyses of ice stored fish fillets the majority of black colony forming
252 bacteria on Iron Agar were found to be *Shewanella putrefaciens* (Gram et al., 1987; Dalgaard
253 et al., 1993), although formation of H_2S can occur among members of the family
254 *Vibrionaceae* (Gram et al., 1987; Lund et al., 2000). Sequence analyses of the 16S rDNA
255 assigned the black colonies as *Vibrio* sp. V170 (DQ146979) and *Vibrio* sp. DAI (AM159569)
256 with a sequence similarity of 97-99 %. None of the selected Iron Agar pure colonies were

identified as *Shewanella* species, only *Vibrio* sp. and *P. phosphoreum* were found in the hind-gut samples.

A total of 50 colonies isolated from TSA were sequenced, with *Vibrio* spp. (50 %) and *P. phosphoreum* (46 %) as the dominating taxa. *Vibrio* species have also previously been detected in gut of halibut larvae (Verner-Jeffreys et al., 2003; Jensen et al., 2004) and Atlantic cod (Korsnes et al., 2006; Ringø et al., 2006b). Several papers discuss the importance of the genera *Vibrio* in aquaculture, and the presence in the intestinal microbiota of fish (Vandenberghe et al., 2003; Thompson et al., 2004; Austin, 2006). *P. phosphoreum* is known as a marine bacterium, found in fish intestine, and it is lately reported in farmed Norwegian salmon (Holben et al., 2002) and UK halibut (Verner-Jeffreys et al., 2003). The bacterium is also known for its spoilage potential of fish fillets, stored in air or modified atmosphere with high CO_2 concentrations (Gram and Huss, 1996). Identifying this bacterium as part of the intestinal microbiota can be of interest to determine contamination routes, from the gut to the fish flesh. *Psychrobacter glacincola*, reported as 2 % of the sequenced bacteria, and *Brevundimonas* sp. (2 %) were also identified using 16S sequencing. Ringø et al. found *Psychrobacter glacincola* in the hind-gut of Arctic charr (2006a) and Atlantic cod (2006b), whilst *Brevundimonas* has previously been found on gills and in the spleen from Bluefin tuna (Kapetanovic et al., 2006).

Sequencing DGGE bands from TSA cultivated bacteria identified *Acinetobacter* sp. and *Pseudomonas* sp. These taxa are known to be members of the intestinal microbiota of salmonids (Ringø et al., 1995). The DGGE approach confirmed the results from 16S rDNA sequencing of the hind-gut isolates, finding *Vibrio* spp. and *P. phosphoreum.*

As plate counts only detected the cultivable aerobic bacteria, a cultivation independent method was used to visualize the total bacterial flora in the intestine. We describe the recovery and subsequent 16S rDNA analysis of the total bacterial community of salmon intestine, using universal PCR primers and DGGE profiling. By extracting DNA directly from the intestine without prior cultivation, it is possible to detect uncultivable anaerobic bacteria and other bacteria requiring special growth conditions, not obtained on conventional media.

It is well documented, during several investigations, that lactic acid bacteria (LAB) constitute a part of the native microbiota of aquatic animals (for a review (Ringø, 2004)). Generally, LAB from fish is known to be slow growing, and the recommended growth conditions on agar-media at low temperatures is up to 4 weeks (Ringø and Gatesoupe, 1998). This may explain why *Lactobacillus* spp. and *Lactococcus* sp. were only detected from direct extraction of bacterial DNA and not from TSA, after incubation at 15 ^{o}C for one week. Moreover, the detection on growth media can be lower than the actual bacterial number.

The unidentified bacterium detected in our study has earlier been found in chicken intestine, with 85 % sequence similarity (Gong et al., 2007). It has been demonstrated that 18S rDNA sequences may be amplified using the present primer set (Hovda et al., 2007b), hence the angiosperm environmental sample and the eukaryotic 18S rDNA can be traces of plants and other eukaryotes eaten by the fish. Analysing the bacteria from direct DNA extraction did not show any differences in the microbiota of the different parts of the gut. This is contrary to the results obtained during cultivation, where the DGGE profiles differed between the fore-, mid- and hind-gut. Furthermore, no differences were found between the squeezed and washed hind-gut fraction. The cultivation based method showed a decreasing bacterial diversity from the fore- to the hind-gut (Table 1).

The direct extraction of intestinal bacterial DNA gave a different microbiota compared to the results obtained by cultivation. As PCR-DGGE monitors the predominant bacteria in a sample, differences in the bacterial composition based on cultivation can be biases due to media selectivity (Amann et al., 1995). Our results indicate that *Acinetobacter* sp., *Pseudomonas* sp. and *Vibrio* sp. might not be part of the predominant bacteria in the gut. During cultivation these bacteria can dominate over bacteria as LAB, and thus explain the differences in the microbial composition obtained. The cultivation-based method detected two bacteria in the hind-gut, whereas the direct extraction detected four bacteria species. The universal PCR primers targeting the highly conserved hypervariable 16S rDNA V3-region employed here were appropriate for surveying the microbial community and possible community changes throughout the salmon intestine.

Further studies could benefit from applying the molecular approach described, for additional analyses of the differences between microbiota of cultivated and non cultivated samples. Such studies will be helpful to determine whether the observed results are due to sample variations, or occur as a result of cultivation biases.

Conclusion

Population analysis based on DGGE and sequencing of DNA obtained directly from samples of salmon intestine was shown to be different from those obtained after prior cultivation. Whereas cultivation-based methods detected *Vibrio* sp., *Pseudomonas* sp., *Janthinobacterium* sp., *Acinetobacter* sp. and *P. phosphoreum*, the direct DNA extraction approach found *Lactobacillus fermentum*, *Lactococcus* sp., *P. phosphoreum* and *Bacillus* sp. as the predominant bacteria in the intestine of Atlantic salmon.

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Table

Table 1. The bacterial growth on TSA in the different parts of the Atlantic salmon intestine, and identification of the bacteria present in the intestinal microbiota. The gut microbiota is detected by sequencing DGGE bands from both direct DNA extraction, and from cultivated samples.

Bacterial number (log cfu/g) / Bacterial groups	Direct extraction			Cultivated bacteria		
	Fore	Mid	Hind	Fore	Mid	Hind
				3.9	3.7	6.4[1] and 4.8[2]
Lactobacillus spp.	x	x	x			
P. phosphoreum		x	x		x	x
Lactococcus sp.	x	x	x			
Bacillus sp.	x	x	x			
Unidentified bacterium	x	x	x			
18S rDNA	x		x			
Angiosperm environmental sample	x	x	x			
Pseudomonas sp.				x	x	
Janthinobacterium sp.				x		
Acinetobacter sp.				x		
Vibrio sp.				x		x

[1]Bacterial number in the squeezed hind-gut sample.

[2]Bacterial number in the washed hind-gut sample.

458 **Figure captions**

459 **Figure 1.**

460 DGGE bacterial profiles of gut microflora obtained after direct DNA extraction. Lane 1
461 represents the fore-gut, lane 2; the mid-gut, lane 3; hind-gut squeezed and lane 4; hind-gut
462 washed. The indicated bands (1-16) represent the sequenced bands with a sequence similarity
463 of ≥ 97 %, if nothing else is mentioned. Band #1 identified *Lactobacillus* sp., #2 and 3
464 *Lactobacillus fermentum*, #4 and 15 18S rDNA, #5 unidentified bacterium clone (AY654985)
465 with 85 % identity, #6 and 13 *Bacillus* sp., #7 Angiosperm environmental, #8-10 *P.*
466 *phosphoreum*, #11 *Lactococcus* sp., #12 and 14 *Photobacterium* sp. and #16 *Lactobacillus*
467 *fermentum* with 96 % similarity.

468

469 **Figure 2.**

470 DGGE profiles of DNA from gut microflora obtained after cultivation of bacteria on TSA.
471 The fore-gut (lane 1 and 5), mid-gut (lane 2 and 6) and hind-gut squeezed (lane 3 and 7) and
472 washed (lane 4 and 8), which represents samples from the two cages included in the
473 experiment. The indicated bands (1-18) represent the sequenced bands with a sequence
474 similarity of ≥ 97 %, if nothing else is mentioned. Bands #1, 4 and 5 identified *Pseudomonas*
475 sp., #2 *Janthinobacterium* sp. (EF422171), #3, 9 and 10 *Acinetobacter* sp., #6, 11 and 12
476 *Vibrio* sp., #7 and 8 *Acinetobacter* sp. (95-96 % similarity), #13, 14, 16 and 18 *P.*
477 *phosphoreum* (95-96 % similarity), #15 and 17 *P. phosphoreum*.

Figure 1

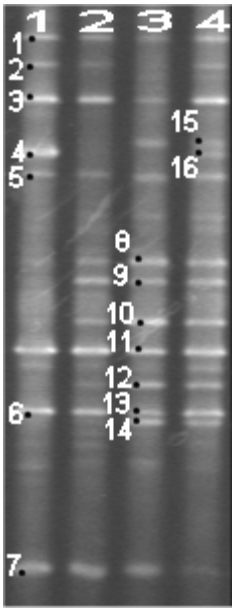

Figure 2