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Caracterização molecular da comunidade bacteriana da pele de bacalhau

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palavras-chave PCR-DGGE; pirosequenciação, código de barras, peixe salgado, comunidade bacteriana

resumo Bacalhau salgado seco é um produto alimentar comercialmente importante e com uma vida de prateleira relativamente longa. Embora a análise microbiológica destes produtos seja crucial para a segurança alimentar, a maioria das abordagens empregadas envolvem apenas métodos moleculares clássicos e de cultivo. No presente trabalho, uma ampla gama de análises moleculares, como técnicas de PCR, electroforese em gel de gradiente desnaturante (DGGE) e pirosequenciação foram realizados a fim de caracterizar a composição da comunidade bacteriana da pele de três espécies estreitamente relacionadas da família Gadidae: O bacalhau do Pacífico *Gadus macrocephalus*, o bacalhau do Atlântico *G. morhua* e o Allaska pollock *Theragra chalcogramma*. Embora o processamento destes produtos tenha sido efetuado, na mesma fábrica, foram observadas diferenças significativas na composição bacteriana entre as espécies. Em geral, a diversidade bacteriana observada foi dominada por espécies Gram-negativas pertencentes à classe Gammaproteobacteria. Treze géneros diferentes representados por 19 OTU, incluindo OTU atribuído a espécies desconhecidas, foram detectados neste estudo. Os géneros mais frequentemente detectados foram Pseudomonas, Salinisphaera, Chryseobacterium, Psychrobacter, e Serratia. A ocorrência de novos grupos de bactérias associadas com o bacalhau salgado seco é relatada pela primeira vez (por exemplo, *Arthrobacter* sp., *Salinisphaera* sp., *Serratia marcescens*, *Rothia mucilaginosa*). A relevância destas descobertas é discutida a partir da perspectiva da segurança alimentar.

keywords PCR-DGGE; bar-coded pyrosequencing; salted fish; bacterial community

abstract

Dry salted codfish are commercially important food products with a relatively long shelf-life. Although the microbiological analysis of these products is of paramount importance for food safety, most approaches have only employed classical molecular and cultivation methods. In the present work a broad-range molecular analysis using PCR - denaturing gradient gel electrophoresis (DGGE) and pyrosequencing was performed in order to characterize the composition of bacterial assemblages in the skin of three closely related species (family Gadidae): the Pacific codfish *Gadus macrocephalus*, the Atlantic codfish *G. morhua* and the Allaska pollock *Theragra chalcogramma*. Despite the fact that all, previously salted, specimens were processed in the same factory, we observed significant differences in the bacterial composition among species. In general, the bacterial diversity observed was dominated by Gram-negative species belonging to the Gammaproteobacteria class. Thirteen different genera represented by 19 OTU's, including unknown OTU's assigned to unknown species, were detected in this study. The most frequently detected genera were Pseudomonas, Salinisphaera, Chryseobacterium, Psychrobacter, and Serratia. The occurrence of new bacterial groups associated with dry-salted codfish is reported for the first time (e.g., *Arthrobacter* sp., *Salinisphaera* sp., *Serratia marcescens*, *Rothia mucilaginosa*). The relevance of these findings is discussed from a food safety perspective.

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Introdução Geral

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1. Introdução geral

Neste trabalho, procuramos compreender melhor o produto gastronómico que mundialmente identifica Portugal, o bacalhau salgado seco. O lugar de privilégio que este ícone conquistou na mesa portuguesa representa mais de 40% do consumo interno de pescado e coloca o país no ranking dos três maiores consumidores de peixe do mundo (DGPA, 2007).

É no sentido de contribuir para uma fiável credibilidade do processo de transformação do bacalhau salgado seco que se iniciou o presente estudo. Nas próximas secções é apresentada uma pesquisa bibliográfica que serve como apoio documental para o enquadramento do presente trabalho.

1.1. Enquadramento histórico

A forma de consumo predominante do bacalhau em Portugal e outros países, como a Espanha, a França, o Brasil e Angola é o salgado seco (Dias et al., 2001). Países onde, sem surpresa, se verifica haver forte emigração portuguesa. Esta resistente afinidade que existe entre os portugueses e o bacalhau, deve-se a uma tradição profundamente enraizada na memória da população, (com destaque para a região central de Portugal, especificamente Aveiro/Ílhavo), que tiveram uma longa história de captura, processamento e secagem deste peixe (Duarte, 2002). Como consequência, o bacalhau é uma componente fundamental na dieta dos portugueses, quer estejam em território nacional ou em outros países.

Atualmente, existem diversas formas de consumo de bacalhau (ultracongelado, em refeições pré-cozinhadas, fresco), no entanto, de uma maneira geral, as

indústrias portuguesas importam o bacalhau sob a forma de salgado verde e/ou congelado para a sua transformação e posterior comercialização. Trata-se de uma indústria que persiste quase isolada das grandes tendências de globalização e de consumo, apoiando-se firmemente na fidelidade quase ancestral do consumidor português, consequência não só do sabor, odor e textura peculiares do bacalhau, mas também devido à sua alta estabilidade de armazenamento e valor nutricional (Lauritzsen, 2004).

1.2. Caracterização genérica dos gadídeos

O atual regime aplicável à comercialização do bacalhau salgado seco e espécies afins salgadas e secas é compreendido por: "produto que tenha sido sangrado, eviscerado, descabeçado, escalado e lavado e que, após maturação físico-química pelo sal, apresenta um teor de sal igual ou superior a 16%, expresso em cloreto de sódio; e que, após lavagem e posterior secagem por evaporação natural ou artificial, possui um teor de humidade inferior ou igual a 47% (Decreto-Lei n.º 25/2005).

No entanto, o bacalhau, na realidade, não é um peixe, mas sim o resultado de um processo de salga e secagem de uma família de peixes, a família Gadidae (Bacalhau da Noruega, 2012). Embora seja um nome vulgarmente dado a várias espécies de peixes, classificadas em vários géneros, de acordo com o Decreto-Lei n.º 25/2005, de 28 de Janeiro, para efeitos comerciais, são permitidas unicamente as seguintes denominações de bacalhau salgado seco, correspondentes a três espécies distintas:

- Bacalhau ou Bacalhau do Atlântico (*Gadus morhua*);

- Bacalhau da Gronelândia (*Gadus ogac*);

- Bacalhau do Pacífico (*Gadus macrocephalus*);

A denominação comercial permitida no que se refere as espécies afins, são as seguintes:

- Arinca ou alecrim (*Melanogrammus aeglefinus*);

- Bacalhau do Ártico (*Eleginus navaga*);

- Bacalhau polar (*Boreogadus saida*);

- Escamudo (*Pollachius virens*);

- Paloco ou juliana (*Pollachius pollachius*);

- Paloco do Pacífico ou escamudo do Alasca (*Theragra chalcogramma*);

- Abrótea ou abrótea do alto (*Phycis blennoides*);

- Lingue (*Molva molva*);

- Zarbo ou bolota (*Brosme brosme*).

Sendo os três últimos pertencentes a família Phycidae (subfamília da família Gadidae), e a família Lotidae (Lingue e Zarbo), da ordem dos gadiformes respectivamente.

Neste trabalho, foram estudadas três importantes espécies da família Gadidae. A espécie denominada escamudo do Alasca (*Theragra chalcogramma*), apesar de não pertencer ao género *Gadus*, Byrkjedal e co-autores (2008) sugerem que esta espécie é mais estreitamente relacionada com o bacalhau do Atlântico (*Gadus morhua*), e que *Theragra chalcogramma* deveria ser transferida de volta para o género *Gadus*, em que foi originalmente descrito como *Gadus chalcogrammus* (Carr & Marshal, 2008). Outra espécie igualmente popular, é o *Gadus macrocephalus*, que habita o oceano Pacífico, na região do Alasca. Já no Atlântico, a espécie mais conhecida e de maior relevância comercial, é, sem dúvida, o *Gadus morhua*, considerado como o verdadeiro e genuíno bacalhau (Nacional Oceanic and Atmospheric Administration, 2012).

As amostras por nós trabalhadas dizem respeito às três espécies acima referidas e foram gentilmente cedidas por um industrial da Gafanha da Nazaré/Ílhavo, nas quais as operações de fabrico, tratamento e manuseamento do pescado foram efetuadas nas instalações da empresa, cumprindo na íntegra as normas comunitárias de higiene e qualidade previstas pelo sistema HACCP (Análise de Perigos e Pontos Críticos de Controle).

1.3. O processamento do bacalhau (salga e secagem)

A água é a constituinte mais abundante dos animais aquáticos, com um teor que pode variar entre os 66 e 84% da sua composição. (Moreira et al., 2001). Quando o peixe é desidratado perde parte desse líquido e o grau de secagem representa justamente a quantidade de líquido que é eliminado do peixe, para ser transformado em bacalhau. Assim sendo, as fases principais do processo de transformação, consistem no aumento do teor de sal (max. de 20%) e na redução do grau de humidade para valores abaixo dos 47% (IPCP, 1991).

A salga (figura 1) é um método de preservação e uma operação preliminar para o processo de secagem. Atualmente, o pescado pode ser conservado de várias formas. Entre elas, as mais usuais de salga de peixe são: a salga húmida (picke salting), a salga seca (dry salting), a injeção de salmoura (injection salting), a salga em salmoura (brine salting) e a salga em vácuo (vacum salting). De todas as formas de salga referidas, a mais comum em Portugal (e concretamente para o processamento de bacalhau), é a salga seca; este processo consiste na utilização de NaCl sólido diretamente sob a superfície do peixe (o sal é trocado várias vezes conforme o seu tamanho e espessura). Esta técnica permite que o sal penetre no peixe e a água do pescado difunda para o meio exterior. Posteriormente, a água é drenada, promovendo a sua diminuição e também a do pH, de forma a obter um alimento estável microbiológica e bioquimicamente (Lauritzsen, 2004) durante muitos meses ou até anos, se bem conservado (Fernández-Segovia, 2006).

Figura 1. Salga do bacalhau em fardos.
http://www.grupeixe.pt/processo.html

No que se refere à secagem, existem duas formas de processamento industrial: a secagem natural e a artificial, cujo objetivo é extrair água da constituição dos tecidos, usando para isso a ajuda do calor e da circulação do ar.

- A secagem natural (já praticamente inexistente a nível industrial) consiste na exposição do peixe ao ar livre, colocado sobre solo pedregoso, sobre tabuleiros ou sobre estacaria feita de madeira (figura 2). Embora seja um processo económico no que se refere à instalação e ao tipo de energia utilizada, é também altamente dependente das condições climatéricas, apresentado ainda um risco elevado de contaminação ambiental, tempos de secagem elevados, e uma grande necessidade de mão-de-obra (Duarte, 2002).

Figura 2. Secagem natural do bacalhau.
http://www.grupeixe.pt/processo.html

- Já a secagem artificial, necessária para suprir a forte demanda por parte dos consumidores, é processada em estufas, onde a temperatura, a humidade e a velocidade do ar são rigorosamente controladas, apresentando um menor tempo de secagem (cerca de 70-75 h). No entanto, a construção de estufas de secagem exige um elevado investimento inicial, somado ao elevado custo energético associado à sua operação (figura 3). (Caderno de especificações e obrigações do produto bacalhau de cura tradicional portuguesa, 2010).

Figura 3. Secagem artificial do bacalhau.
http://www.grupeixe.pt/processo.html

1.4. Microbiologia e antecedentes do bacalhau salgado e seco

O bacalhau passa por três processos importantes antes de chegar a cozinha dos consumidores: a salga, a secagem e a demolha (Figura 5). Ao longo destes três processos ocorrem diversas alterações sensoriais e organoléticas que conferem ao produto as características tão apreciadas pelos consumidores (Barat et al., 2006).

Embora a salga seja um dos vários métodos de preservação para evitar a deterioração e o crescimento de micro-organismos patogénicos em peixes (Huss,

1994), eles não estão livres de sofrer deterioração (Aiura et al., 2008), visto que existem micro-organismos halotolerantes ou halofílicos que não são afetados pelo sal (Yeannes et al., 2011). Os constituintes do peixe (por exemplo, hidratos de carbono, proteínas e lípidos) servem também como substrato para a proliferação destes microrganismos, que juntamente com enzimas endógenas, produzem compostos de sabor desagradável, provocando a deterioração da textura, descoloração e outras alterações adversas que podem ocorrer no músculo do peixe (Zare, 2004).

Atualmente existem poucos trabalhos publicados na literatura científica que tenham abordado a caracterização da comunidade microbiológica existente no bacalhau salgado seco. Os estudos mais relevantes até à data da realização da presente dissertação estão resumidos na Tabela 1.

Tabela 1. Sumário da revisão de literatura das comunidades microbianas encontradas no bacalhau salgado seco.

Género e/ou espécie	Tratamento	Análise microbiana	Referência
Staphylococcus arlettae	[a]NM	[b]CMD	(Vilhelmsson et al., 1997)
Psychrobacter sp.	[c]GM	CMD	(Bjorkevoll et al., 2003)
Aeromonas hydrophila	NM	CMD	(Rodrigues et al., 2003)
Actinobacillus urea			
Aeromonas caviae			
Pantoae agglomerans			
Flavimonas oryzihabitans			
Staphylococcus auricularis			
Aerococcus			
Staphylococcus capitis			

[a]NM- não mencionado • [b]CMD – cultura de método dependente • [c]GM – *Gadus morhua*

1.5. Metodologia de estudo

Tradicionalmente, a caracterização das comunidades microbianas existente nos alimentos, baseia-se em técnicas de cultura, isolamento e identificação das amostras através da análise das características morfológicas, fisiológicas e metabólicas (métodos *ex situ* ou métodos dependentes de cultura). No entanto, este conjunto de metodologias, além de ser morosas e não permitirem determinar exaustivamente a comunidade microbiana, favorece também a uma disparidade entre as amostras cultiváveis e a diversidade *in situ* (Nocker et al, 2007), aumentando por isso a importância destes métodos serem complementados com ferramentas moleculares.

A aplicação de técnicas moleculares (métodos independentes de cultura), permite um census mais exato e completo das comunidades microbianas, revolucionando as metodologias tradicionais que têm sido utilizadas para caracterizar e determinar as populações microbianas envolvidas não só em alimentos, mas também em outros variados tipos de pesquisa.

Para este trabalho, utilizamos três métodos independentes de cultura (Nested-PCR, DGGE e pirosequênciação) cujos resultados permitiu-nos identificar em profundidade, a comunidade bacteriana da pele do bacalhau salgado e seco.

- Em “nested PCR” (Puig et al., 1994), efetua-se inicialmente uma reação de amplificação de um alvo genómico (e.g. com “primers” universais), seguida da reamplificação de uma região interna do genoma com “primers” mais específicos (e.g. exclusivos de uma espécie em particular), aumentando o nível de especificidade e a eficiência da amplificação;

- O método com electroforese em gel com gradiente desnaturante (DGGE “denaturing gradient gel electrophoresis”) é uma forma de comparar diferentes comunidades em simultâneo, sendo o perfil DGGE de uma amostra, a impressão digital (“fingerprint”) da sua comunidade. Os fragmentos de cadeia dupla de DNA obtidos por amplificação do PCR são separados por electroforese em condições

desnaturantes providenciadas por ureia e formamida, cujos produtos PCR de espécies microbianas diferentes com tamanho idêntico mas sequências de pares de bases diferentes (Doaré-Lebrun et al., 2006) são separados, durante a migração electroforética, porque atingem os respectivos pontos de fusão em locais diferentes do gradiente do gel (Moeseneder et al., 1999; Zhang et al., 2000). Com este método, é ainda possível realizar uma estimativa semi-quantitativa da abundância dos filótipos por comparação das intensidades das bandas do gel DGGE (Marzorati et al., 2008; Nocker et al., 2007).

- O método “454 pyrosequencing” é uma técnica de sequenciação de DNA, que permite a geração de curtas leituras rapidamente e com precisão, evitando clonagens (Ronaghi e Elahi 2002; (Dinsdale et al., 2008).

Uma análise comparativa entre os métodos por nós utilizados é mostrada na Tabela 2.

Tabela 2. Análise qualitativa do custo-benefício para os dois métodos estudados (DGGE e pirosequenciação).

	DGGE	pirosequenciação 454
Custo		
Tempo consumido	Alto	Médio
Equipamento	Médio	Alto
Reagentes	Alto	Alto
Benefícios		
Precisão taxonómica	Baixo	Alto
Facilidade de desempenho e interpretação	Médio	Baixo
Cobertura da diversidade microbiana	Médio	Alto

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Capítulo 2

Molecular Analysis of Skin Bacterial Assemblages from Dry-Salted Codfish and Pollock

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Molecular Analysis of Skin Bacterial Assemblages from Dry-Salted Codfish and Pollock

Rodrigues, NP, Calado, R, Duarte, LN, Manco, SC, Fernandes, JF, Cleary, DFR &

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Abstract

Dry salted codfish and pollock are commercially important food products with a relatively long shelf-life, although they may spoil due to the growth of halophilic bacteria. Although the microbiological analysis of these products is of paramount importance for food safety, most approaches have only employed classical molecular and cultivation methods. In the present work a broad-range molecular analysis using PCR - denaturing gradient gel electrophoresis (DGGE) and pyrosequencing was performed in order to characterize the composition of bacterial assemblages in the skin of three closely related species of the Gadidae family: the Pacific codfish *Gadus macrocephalus*, the Atlantic codfish *G. morhua* and the Allaska pollock (*Theragra chalcogramma*). Despite the fact that all, previously salted, specimens were processed in the same factory, we observed significant differences in the bacterial composition among species. In general, the bacterial diversity observed was dominated by Gram-negative species belonging to the Gammaproteobacteria class. Fifteen different genera represented by 19 OTU's, including unknown OTU's assigned to unknown species, were detected in this study. The most frequently detected genera were *Pseudomonas, Salinisphaera, Chryseobacterium, Psychrobacter*, and *Serratia*. The occurrence of new bacterial groups associated with dry-salted codfish and pollock is reported for the first time (e.g., *Arthrobacter* sp., *Salinisphaera* sp., *Serratia marcescens*, *Rothia mucilaginosa*). The relevance of these findings is discussed from a food safety perspective.

***Keywords**:* PCR-DGGE, bar-coded pyrosequencing, salted fish, bacterial community

1. Introduction

The particular flavor and texture of dry-salted gadoid fish is highly appreciated in Portuguese speaking countries; their trade and consumption play an important economic and cultural role (Dias et al., 2001). Wet salted fish is used as raw material in Portuguese drying factories for the production of dry-salted codfish and pollock. The salt content and moisture level marketed in these products is regulated and must be between 16-20% and lower than 47%, respectively (IPCP, 1991). Due to its salt content and moisture level, dry-salted fish generally have a relatively long shelf life (Bjorkevoll et al., 2003).

Dry-salted products are rarely (if ever) sterile, and display distinctive microbial associations, whose composition is determined by the raw materials used, food processing parameters and subsequent storage conditions (Gram & Huss, 1996). Furthermore, previous authors (e.g., Bjorkevoll et al., 2003; Rodrigues et al., 2003) have already reported that certain microorganisms are able to remain active under high salt concentrations (15-20%). The correct characterization of the bacterial microflora of dry salted codfish and pollock is crucial to control food production and quality, particularly when hazard analysis of critical control points (HACCP) is employed.

One of the main tasks of seafood inspection is to perform an accurate bacterial species differentiation in order to achieve an early detection and identification of pathogenic microorganisms. The presence of pathogenic bacteria in finfish for human consumption can be the result of microorganisms naturally present in the marine environment, environmental contamination by animal and/or anthropic sources (e.g., feces), as well as post-harvest handling and/or processing of the fish (Gram & Huss, 1996; Huss, 1997). Several pathogenic bacteria are also able to survive as non-growing cells during the dry-salting process and recover during desalting (Bjorkevoll et al., 2003; Pedro et al., 2002).

Classical microbiology analyses are widely used to characterize seafood quality, but commonly overlook important microorganisms, which may not grow in artificial

media or that belong to less abundant microbial groups (Broekaert et al., 2011). Polymerase chain reaction (PCR) based methods, cloning and sequencing of phylogenetic marker genes are currently the most common molecular techniques used to rapidly detect pathogenic microorganisms in the food industry (Justé et al., 2008). PCR-DGGE has already been used in several fields of food microbiology for the detection of microorganisms, the evaluation of community structure and food quality assessment (Ercolini, 2004). However, none of these technologies provide a single and thorough in depth characterization of the microbiological composition. Recent developments in sequencing technologies, such as bar-coded pyrosequencing (Hamady et al., 2008) and microarray technologies (Bae et al., 2005), have enabled researchers to perform large-scale and in-depth characterizations of complex microbial communities. Although both technologies described above can be used to asses microbial composition, only bar-coded pyrosequencing can detect unknown microbes (novel sequences) (Rothberg & Leamon, 2008).

In the present study we use a classical molecular technique (PCR-DGGE) and state of the art bar-coded pyrosequencing to compare the composition and diversity of bacteria in the skins of three closely related (Teletchea et al., 2006) dry-salted gadoid species: the Pacific codfish *Gadus macrocephalus*, the Atlantic codfish *G. morhua* and the Allaska pollock (*Theragra chalcogramma*).

2. Material and Methods

2.1. Dry salted codfish sampling

Samples of dry-salted *G. macrocephalus*, *G. morhua* and *T. chalcogramma* were directly supplied by the Quality Department of a commercial enterprise which processes and trades dry-salted Gadoid fish in Portugal.

The samples ceded were in the final stage of production (dry-salted) and ready to be delivered for retail selling, kept in refrigerated chambers (between 2 and 5 °C). Its shelf life, if properly stored, is 12 months, according to national specification. These samples were sealed in plastic bags, in the factory and transported immediately to the laboratory where they were kept at -20 ºC until processing.

2.2. DNA extraction and polymerase chain reaction (PCR) amplification of 16S rRNA gene fragments

Four fishes were sampled and analyzed for each gadoid species studied. Each fish sample consisted of three haphazardly collected pieces of fish skin (~200 mg each), processed separately and combined into a single tube (1.5 ml microcentrifuge tube) before the total community DNA extraction. Approximately 200 mg of fish skin were removed aseptically and placed separately into 1.5 ml microcentrifuge tubes containing 0.5 g of DNA free glass beads. Total community DNA was extracted directly from skin samples using a commercial kit (OMEGA E.Z.N.A. Soil DNA Kit, Bio-Tek, USA) following the manufacturer's instructions. Amplified 16S rRNA gene fragments suitable for bacterial DGGE fingerprints of total microbial community DNA samples were obtained after a nested approach as described by Gomes et al. (2008).

2.3. Nested PCR condition

In the first PCR, the universal bacterial primers F-27 and R-1492 were used to amplify c. 1450 bp of the 16S rRNA gene (Weisburg et al.,1991). The PCR reaction mixtures (25 µL) consisted of: 1 mL template DNA (c. 20 ng), 1_ Stoffel buffer (Applied Biosystems), 0.2mM dNTPs, 3.75mM $MgCl_2$, 2.5 µg bovine serum albumin (BSA), 0.1 mM primers and 2.5U Taq DNA polymerase (Stoffel fragment, Applied Biosystems). After 5 min of denaturation at 94°C, 30 thermal cycles of 1 min at 94°C, 1 min at 56°C and 2 min at 72°C, the PCR was finished by an

extension step at 72°C for 10 min. The amplicons obtained from the first PCR were used as a template, whereas for *Theragra chalcogramma* was used 1:5 diluted, both, for a second PCR with bacterial DGGE primers F984-GC and R1378 (c. 473 bp) according to Heuer et al. (1997), and 30 cycles were done.

2.4. Denaturing gradient gel electrophoresis (DGGE)

Bacterial PCR amplicons (16S rRNA gene fragments) were analyzed by DGGE using a 40% to 58% chemical denaturing gradient. Amplicon concentration was determined visually after elecrophoresis in an agarose gel. Subsequently, the volume of sample to be used in each DGGE lane was adjusted according to amplicon concentration. Amplicon separation was achieved by DGGE in 1x TAE buffer (40 mM Tris–acetate, 1mM EDTA, pH 8.0) for 16 hours at a constant voltage of 160 V and a temperature of 58°C (Biorad DCode system). The gel was silver-stained according to Heuer et al. (1997).

2.4.1. Statistical analysis of DGGE fingerprints

The gel was transmissively scanned and the digitalized profiles were analysed using the software package GelCompar 4.0 (Applied Maths) following Gomes et al. (2010). The DGGE band surface was converted to relative intensity by dividing its surface by the sum of all band surfaces in a lane. This was subsequently log10 (x + 1) transformed and a distance matrix constructed using the Bray-Curtis index in PRIMER 5 (Clarke & Gorley, 2001). The Bray-Curtis index is one of the most frequently applied (dis)similarity indices used in ecology (Legendre and Gallagher, 2001; Cleary, 2003; Cleary et al., 2004; de Voogd et al., 2009). Variation in bacterial composition among species was assessed with Multidimensional Scaning (MDS) Analysis in PRIMER. We tested for significant differences in the

skin bacterial composition among fish species using an ANOSIM analysis in PRIMER with 999 permutations.

2.5. Pyrosequencing analysis

A bar-coded pyrosequencing approach was used for a more in-depth analysis of bacterial composition. Prior to pyrosequencing, DNA from the four pooled replicates of each codfish species were combined in order to obtain one DNA library per fish species (named GM for *G. morhua*, GMC for *G. macrocephalus* and TC for *T. chalcogramma*). Fragments of the 16S ribosomal RNA (rRNA) gene were sequenced with primers V3 Forward (5'-ACTCCTACGGGAGGCAG-3') and V4 Reverse (5'-TACNVRRGTHTCTAATYC-3') using the 454 Genome Sequencer FLX Titanium (Life Sciences Roche Diagnostics Ltd, West Sussex, UK). Only sequences containing exact matches to primer sequences and barcode tags were used for further analysis. Raw sequencing reads were quality trimmed according to published recommendations (Huse et al., 2007); The Qiime software package (Caporaso et al., 2010) following Cleary et al. (2012) was used.

2.5.1. Assignment of 16S rRNA gene sequences

The 'pick_otus.py' function in Qiime (QIIME 1.5.0 (release) AMI: ami-e4bf1b8d, latest version) with the uclust method and the default sequence similarity threshold of 0.97 were used to assign sequences to operational taxonomic units (OTUs) (Díez et al., 2004; Pommier et al., 2010). OTU richness was assessed using rarefaction; a rarefaction curve for each sample was computed using a self-written function in R (Gomes et al., 2010). A representative OTU set was selected with the 'pick_rep_set.py' function in Qiime using the 'most abundant' method. Sequences belonging to the representative set were classified taxonomically with the 'assign_taxonomy.py' function in Qiime with the Naive Bayesian rRNA RDP

Classifier method and 80% minimum confidence score. Chimeric sequences or sequences not classified as Bacteria were removed using the parallel_identify chimeric_seqs function in Qiime. In addition to this, 16S rRNA gene fragments representing dominant OTUs were mapped back to reference genomes by sequence alignment using the NCBI (National Centre of Biotechnology Information) tool 'Basic Local Alignment Search Tool' (BLAST) (Altschul et al., 1990). Reads with hits were assigned to the genome corresponding to their top BLAST hit only if the top hit had sequence identity higher than 95% (Morgan et al., 2010b).

3. Results and Discussion

In the present study, interferences in the PCR-DGGE analyses were detected (PCR inhibition and faint bands, data not shown) following total community DNA extraction. This may be due to the high salt content (a well-known inhibitor of PCR) of surveyed samples. To overcome this problem, a nested PCR approach was employed to improve the yield and sensitivity of the reaction (Dar et al., 2005; Loffler et al., 2000). The DGGE profiles of bacterial assemblages of the three gadoid species indicated the dominance of a few bacterial populations (bands) (Fig. 1.). Some DGGE-ribotypes were, furthermore, present in both *G. macrocephalus* and *G. morhua* samples (indicated with arrows in Fig. 1.) but not in *T. charlcogramma* samples.

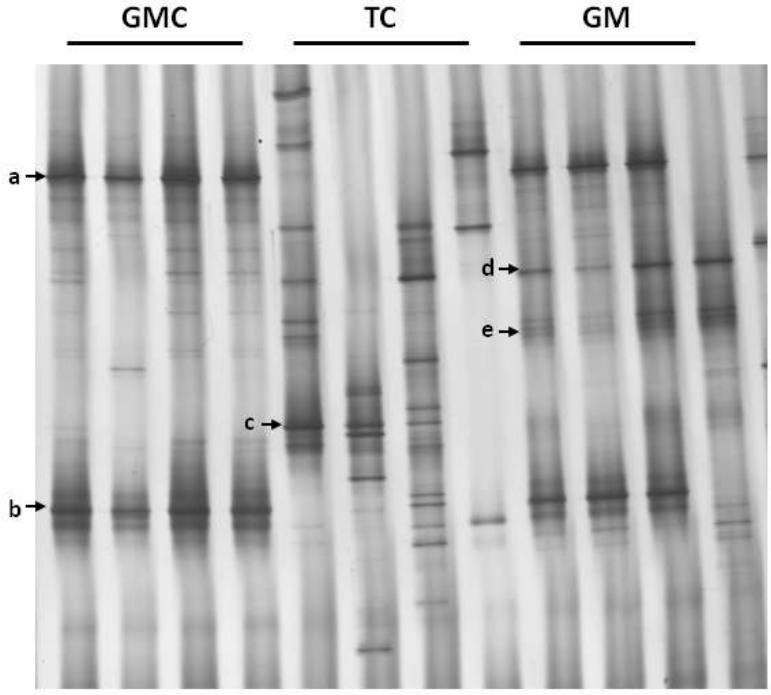

Figure 1. Denaturing gradient gel electrophoresis (DGGE) fingerprint of 16S rRNA gene fragments amplified from four replicates of three different gadoid species: *G. macrocephalus* (GMC); *T. charlcogramma* (TC); *G. morhua* (GM) DNA templates are shown. Arrows indicate differentiating DGGE ribotypes.

In line with these results, the ANOSIM analysis revealed differences (*G. macrocephalus* vs *T. charlcogramma* – R = 0.62, *G. macrocephalus* vs. *G. morhua* - R = 0.48 and *T. charlcogramma* vs. *G. morhua* - R = 0.39) in bacterial composition between different fish species. The R statistic in ANOSIM ranges from 0 to 1. In general values of R > 0.75 indicate strong separation, values > 0.5 but < 0.75 moderate separation and values < 0.25 poor separation (Clarke, 1993). This was also apparent in the MDS ordination (Fig. 2.); samples of *G. macrocephalus* and *G. morhua* formed distinct clusters while there was pronounced variation in the composition of *T. charlcogramma* samples.

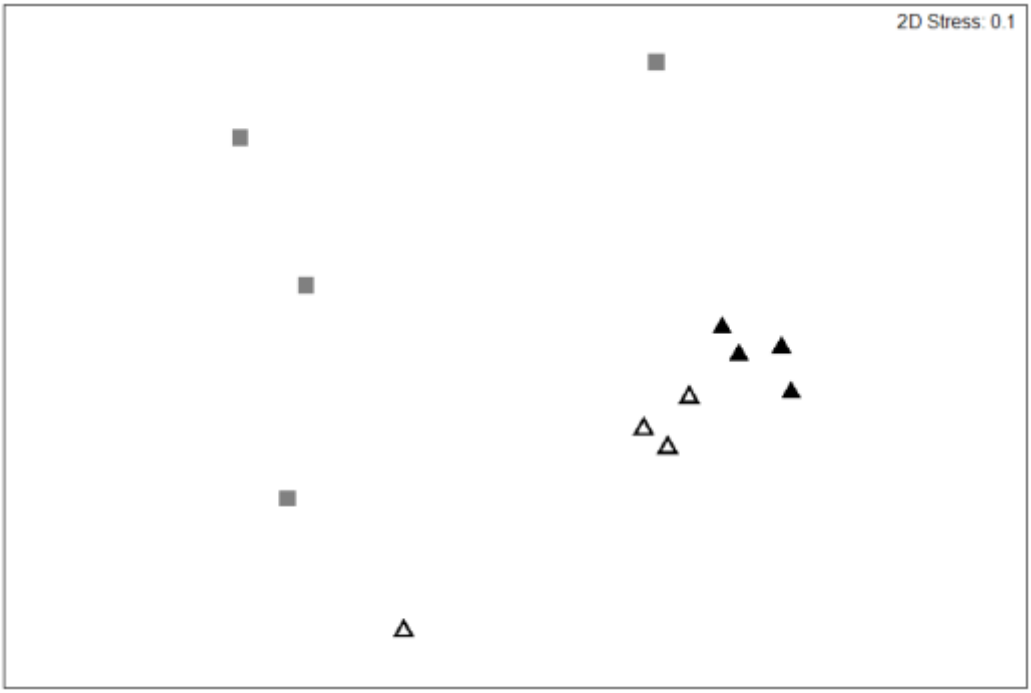

Figure 1. MDS analysis of the bacterial community structure based on DGGE profiles comparing similarities between bacterial skin assemblages of *G. morhua* (∆); *G. macrocephalus* (▲); *T. charlcogramma* (■).

The surface of fish skin provides an ideal surface for bacteria to attach to in their natural environment (Cahill, 1990). It is, therefore, likely that fish skin is also exposed to sources of microbial contamination during industrial processing.

Cleary et al. (2012) previously showed that DGGE fingerprinting data yielded results that were significantly congruent with bar-coded pyrosequencing data and highlighted the advantages of combining these two molecular approaches for a fast and cost-effective characterization of microbial communities. In the present study, we used this approach for a more in depth assessment of bacteria than would be possible using DGGE fingerprinting alone. The pyrosequencing analysis of bacterial populations associated to the skin of the three dry-salted gadoid species generated a total of 665 sequences (after quality control), 239 were found in *G. macrocephalus*, 234 in *G. morhua* and 192 in *T. chalcogramma*. The number of sequences yielded for each sample, when compared to our previous studies,

was about an order of magnitude lower (Gomes et al., 2010a). In line with PCR-DGGE analysis, these results can be an indication of interferences in the pyrosequencing reaction due to the presence of inhibitors in the total community DNA extracted from dry-salted fish samples. However, the bacterial abundance in the samples was not measured in this study and therefore the effect of a low amount of target bacterial DNA should not be ruled out. For future studies employing pyrosequencing analysis of salted dry fish products we suggest the use of an indirect approach for total community DNA extraction. This strategy can be more appropriate for molecular microbial community analyses, when samples containing PCR inhibitors are analyzed (Milling et al., 2005). Nevertheless, despite the low yield of sequence reads, cumulative bacterial richness analysis (Fig. 3.) showed a relatively high richness of the samples studied. OTU richness was highest in *G. morhua*, intermediate in *G. macrocephalus*, and lowest in *T. charlcogramma.*

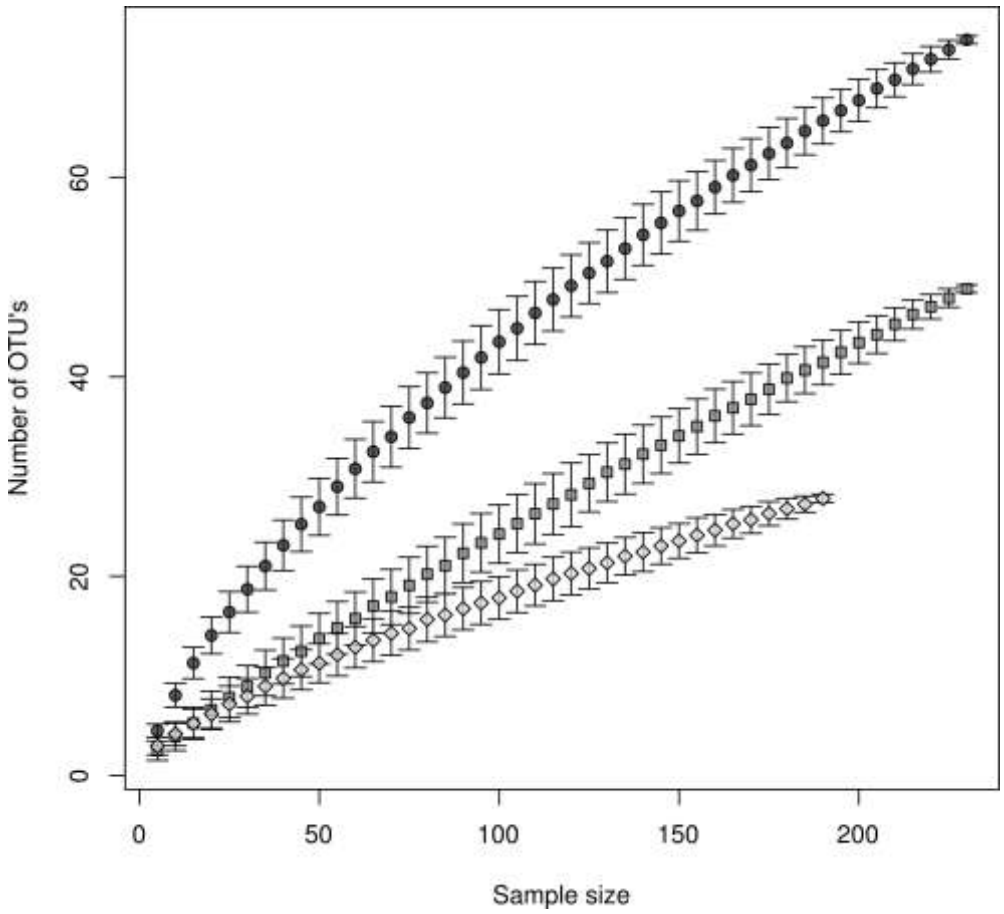

Figure 3. Species accumulation curves as a function of the number of sequences, by using resampling of 16S rRNA gene sequences from *G. morhua* (●), *G. macrocephalus* (■), *T. charlcogramma* (◊).

In addition to this, there was no evidence of an asymptote for any skin bacterial assemblage studied, suggesting that true richness is higher than what was observed in this study.

The 'uclust' method in the pick_otus.py function of QIIME yielded a total of 74, 49 and 28 OTU's associated to *G. morhua*, *G. macrocephalus* and *T. chalcogramma*, respectively. In line with the DGGE profiles only a few OTU's were dominant in the skin of dry-salted gadoid fish species (Table 1). Different dry-salted fish species processed in the same factory showed a dominance of different bacterial groups. While *Chryseobacterium* sp. was the most abundant bacteria in *G. morhua* (16%), *Pseudomonas* sp. and an OTU related to *Salinisphaera* sp. were the most

dominant in *G. macrocephalus* (66%) and *T. chalcogramma* (48%), respectively. Microbial species within the genus *Chryseobacterium* have been considered as potential emergent pathogens under various fish farming conditions and over different geographical areas (Gonzalez et al., 2000). Furthermore, members of this genus are known for their high proteolytic activity (Yamaguchi & Yokoe., 2000). Despite the ubiquitous nature of *Chryseobacterium* spp., which is often found in aquatic and terrestrial environments (Kampfer et al., 2003) or food products (Jooste & Hugo, 1999; Vandamme et al., 1994), this genus has not been previously detected in dry-salted *G. morhua*. *Pseudomonas* spp. is also widely found in nature and has been previously found in association with codfish (Wilson et al., 2008). Earlier studies have shown that *Pseudomonas* spp. can be considered a "specific spoilage organism" in food products (Dalgaard, 1995). This group of microorganisms is able to reduce the shelf life of fish and affects the flavor and taste (Dalgaard, 1995; Koutsoumanis & Nychas, 1999; Vogel et al., 2005). In contrast to *Chryseobacterium* sp., sequence reads assigned to Pseudomonas sp. were also abundant in *G. morhua* (9%) and *T. chalcogramma* (28%). Sequence reads related to the genus *Salinisphaera* were more abundant in *T. chalcogramma* (48%). *Salinisphaera hydrothermalis* (mesophilic, halotolerant, gammaproteobacterium) was the closest relative detected in GeneBank (Table 1) and was recently isolated from hydrothermal fluids from diffuse-flow vents on the East Pacific (Crespo-Medina et al., 2009). Due to its ability to grow at environmental temperatures and tolerate high salt concentrations, members of this group of bacteria may contribute to the spoilage of salted codfish and pollock. However, to the best of our knowledge, there have been no previous studies on the occurrence of members of this genus in dry-salted fish products and their effect on sea food quality and safety.

Psychrobacter (*Psychrobacter namhaensis*) and *Serratia marcescens* were also associated with dominant taxa detected in *G. morhua* (12%), *G. macrocephalus* (8%), respectively, and were detected at lower levels in all fish species studied (Table 1). Members of the genus *Psychrobacter* are psychrotolerant, and are commonly found in the skin of raw fish and processed fish products. Bjorkevoll et al. (2003) have shown that a dominant *Psychrobacter* bacterium was present in

the skin of *G. morhua* immediately after its capture and remained dominant in salt-cured and dry-salted codfish products. The same authors also showed that inoculation with *Psychrobacter immobilis* can accelerate spoilage of dry-salted codfish samples.

Serratia spp., have been often reported as part of the fish microflora (Gonzalez et al., 2000; Olsson et al., 2004) and in some cases a potential fish pathogen (Baya et al., 1992). *Serratia marcescens* is well known due to its ubiquitous distribution, but can also be a human pathogen and is commonly associated with opportunistic infections (Curtis et al., 2005; Takahashi et al., 2004). To the best of our knowledge nothing has been previously described about the colonization of gadoid fish (either fresh or processed) by *S. marcescens* and its relevance for food safety. Interestingly, Son et al. (2008) published a case report on the occurrence of a deep cutaneous ulcer caused by *S. marcescens* in an immune-compromised patient after her thumb had been pricked by a thorn while processing a codfish. However, Son et al. (2008) suggested that water, rather than the codfish, was the vector for *S. marcescens* infection.

Bacterial composition analysis also revealed the presence of a more diverse range of bacterial taxa associated with the skin of *G. morhua* [*Rothia mucilaginosa* (6%), *Arthrobacter* sp. (5%), *Acinetobacter* sp. (5%) and *Pseudoalteromonas* sp. (5%)]. *Acinetobacter* spp. and *Pseudoalteromonas* spp. are often found in the natural microbial community of G. morhua (Wilson et al., 2008) and are most likely also involved in the process of fish spoilage. *Arthrobacter* species, in contrast, are commonly associated with soil microbes, while *R. mucilaginosa* is often found in the human oral and respiratory tract, and has been related to infections in immunocompromised patients (Collins et al., 2000; Morgan et al., 2010a). It is unclear at present what effect *Arthrobacter* spp. and *R. mucilaginosa* have on fish product quality and/or safety.

Table 1. Overview of the microbial community composition in dry salted codfish determined by bar-coded pyrosequencing analysis of the 16S rRNA gene sequence based on the Naive Bayesian rRNA RDP Classifier method and similarities to closest relatives in the GenBank database.

Taxonomic position of dominant OTUs (Class and Family)	Closest relative	16S rRNA gene Fragment reads (%)			Similarit[a] (%)	GenBank[b]
		GM[c]	GMC[d]	TC[e]		
Gammaproteobacteria\Pseudomonadace	*Pseudomonas* sp	9	66	28	99	JN609540
Gammaproteobacteria\Salinisphaeraceae	*Salinisphaera hydrothermalis*	_	0.4	48	96	EU740416
Gammaproteobacteria\Moraxellaceae	*Acinetobacter* sp.	5	1	0.5	100	FR749840
	Psychrobacter namhaensis	12	1	3	100	JF711000
	Psychrobacter psychrophilus	2	_	_	99	AJ748268
Gammaproteobacteria\Pseudoalteromonadaceae	*Pseudoalteromonas* sp	5	0	0	98	JQ072069
Gammaproteobacteria\Enterobacteriaceae	*Serratia marcescens*	2	8	5	99	JF494817
Gammaproteobacteria\ Halomonadaceae	*Halomonas jeotgali*	1	_	2	100	EU909458
	Halomonas salina	_	_	1	97	AM945688
Gammaproteobacteria\Idiomarinaceae	*Idiomarina loihiensis*	1	_	_	98	AE017340.1
Alphaproteobacteria\Rhodobacteraceae	*Paracoccus* sp.	2	_	1	99	HM854519
Alphaproteobacteria\Sphingomonadaceae	*Caulobacter leidyia*	_	2	0.5	100	AF331660
Epsilonproteobacteria\Campylobacteraceae	*Arcobacter butzleri*	_	_	2	98	AP012047
Flavobacteria\Flavobacteriaceae	Chryseobacterium sp	16	_	_	100	JF710966
Bacilli\Staphylococcaceae	*Macrococcus carouselicus*	1	0.4	_	100	NR044927
	Staphylococcus equorum	1	_	_	100	FR691468
Actinobacteria\ Micrococcaceae	*Arthrobacter* sp	5	_	0.5	100	JQ691547
	Rothia mucilaginosa	6	_	_	100	DQ870701
	Pseudoclavibacter helvolus	2	_	0.5	100	FJ795667

[a]Sequences obtained from partial 16S rRNA gene and were aligned to the closest relative (Genus-Species) based upon BLAST search in the GenBank database. Closely related • [b] Reference accession number • [c]GM – Gadus Mohrua • [d]GMC – G. macrocephalus • [e]TC – T. charlcogramma • − Not Detected

4. Conclusions

The rapid and accurate identification of microbes in food products is crucial for the timely identification of food spoilage, hazard analyses and monitoring of critical control points during food production. Traditionally, bacterial species identification in food has been performed by culture-dependent methods, biochemical characterization and traditional molecular tools (e.g. PCR and Quantitative PCR). In this study, a combined PCR DGGE and bar-coded pyrosequencing approach revealed significant differences in the structure and composition of skin bacterial assemblages of three commercially important dry-salted gadoid species. Furthermore, we have shown, for the first time, new bacterial groups associated with dry-salted codfish and pollock. However, the impact of these bacterial groups on fish quality and safety remain unknown. Further studies are needed to clarify the dynamics of fish skin bacterial flora after capture and during the dry-salting process and the importance for human consumption.

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Capítulo 3

Considerações Finais

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Os resultados apresentados nesta tese de mestrado mostram um trabalho de investigação feito a partir da comunidade microbiológica presente na pele de três importantes espécies vendidas vulgarmente como bacalhau salgado seco em Portugal. O objetivo foi o de combinar diferentes abordagens moleculares e conhecer, em pormenor, o que as análises clássicas feitas com métodos dependentes de cultura não mostram.

Para este efeito, a pirosequenciação revelou que embora mais dispendiosa, apresentou uma cobertura elevada riqueza bacteriana e ofereceu uma perspectiva geral sobre diversidade de espécies bacterianas no bacalhau salgado seco. O DGGE também contribuiu para comparar as comunidades microbianas e foi preliminar para posterior pirosequenciação. Adicionalmente, espécies de bactérias, encontradas na pele *G. morhua*, *G. macrocephalus* e *T. chalcogramma* (salgados e secos) ainda desconhecidas e negligenciadas pelos métodos tradicionais de cultivo foram descobertas. No entanto, mais pesquisas são necessárias para discriminar as diferentes hipóteses que se colocam a respeito da proveniência destes microrganismos e qual a sua relevância para a saúde pública. Idealmente, um estudo comparativo entre a microflora da pele após a captura do peixe e durante o seu processamento, adicionado a uma análise de cultura dependente, complementar ao DGGE e a pirosequenciação poderia ser feito, de modo a construir uma ponte entre o que já foi descrito e o que pode ser adicionado para segurança do risco microbiológico desses alimentos.