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Impacts of high pressure processing conditions on the microbiome of sea bass fillets

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Statement of authorship

I hereby declare that I am the author of this work, which is, to the best of my knowledge and belief, original, except as acknowledged in the text. Authors and work consulted are properly cited in the text and listed in the references in the required format. The material has not been previously submitted, in whole or in part, for a degree at this or any other university.

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As detailed in section 2. Methodological Approach, the samples here analysed were produced in an experiment carried out at the National Technical University of Athens (NTUA) and then shipped to the CCMAR for this metagenomics analysis, under the frame of these projects.

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Abstract

Fish and seafood products are some of the most important protein sources in human nutrition and more than 45% of the fish used for human consumption comes from aquaculture. However, fish products are easily perishable and often associated with human diseases, leading to development of different preservation methods. Recently, novel processing techniques like High Pressure Processing (HPP), that uses pressure to inactivate microbes without heat, are being optimized to extend shelf-life without affecting food quality.

We used metagenomics (the global study of genetic material from environmental or organism samples) to characterize in a global way the microbes present in sea bass fillets treated with different pressures (300-600 MPa) and processing times (2-5 min) and after refrigerated storage for 1 or 11 days, contributing to optimize HPP of these products.

We extracted and selected 42 DNA samples (of 14 experimental groups) based on their quantity and quality and sent them to a sequencing company to construct microbiome libraries based on the 16S rRNA gene sequencing. With the sequencing and bioinformatics analysis using QIIME it was possible to evaluate the impacts of HPP treatments on the bacterial microbiome of the samples. There was a decrease in bacterial load in the treated samples when compared to the control (confirmed by quantitative PCR of 16S), which was especially evident when we compared the control at 11 days with the treated samples at 11 days, notably in the 450MPa and 600MPa treatments. The most abundant genera of bacteria in the control fillets significantly changed after storage for 11 days, leading to a different composition to 300 MPa fillets and separated by PCoA from 450-600 MPa fillets. These had a microbiome more similar to the initial control fillets. These results will help optimizing HPP of fish fillets and identifying the main genera of deterioration bacteria and HPP effects.

Keywords: High pressure processing, Metagenomics, Spoilage, Microorganisms, Shelf life

Resumo

Peixes e mariscos são importantes fontes de proteína na nutrição humana. A produção de peixe aumentou ao longo dos anos, muito devido ao crescimento da aquacultura e o interesse por uma alimentação mais saudável. Atualmente mais de 45% dos peixes para consumo humano são provenientes da aquacultura e Portugal é o país mais relevante da UE em consumo *per capita*. No entanto os produtos alimentares à base de peixe (ricos em nutrientes e água) são facilmente deteoráveis e podem estar associados a doenças (difilobotríase), especialmente quando consumidos crus ou pouco cozinhados.

Diversas técnicas de conservação têm sido utilizadas (ex. congelamento, defumação, salga). Atualmente a procura por produtos mais frescos e aumento de produção por aquacultura requer otimização de novas tecnologias de processamento para diminuir a deterioração alimentar (ex. alterações de cor, textura, cheiro ou sabor, frequentemente causada por microrganismos), estender o prazo de validade e a segurança (ex. intoxicações causadas pelos microrganismos e seus metabolitos) e diminuir o desperdício alimentar e perdas económicas (cerca de um terço da produção alimentar mundial é perdida todos os anos).

Nesta tese, focámos na otimização do Processamento por Alta pressão (HPP) para preservação de filetes de robalo de aquacultura, pois é uma técnica promissora e em expansão de processamento alimentar não térmico, que aplica pressões entre 300MPa e 1000MPa durante vários minutos à temperatura ambiente para inativar microrganismos em alimentos sólidos ou líquidos.

HPP tem sido aplicada a mariscos, frutas, molhos, carnes e vegetais, preservando a qualidade e sabor dos alimentos, mas aumentando a sua segurança e tempo de vida. Os parâmetros críticos são a pressão; os tempos para alcançar a pressão, de tratamento e de descompressão; as temperaturas iniciais do produto e durante tratamento e as características iniciais do produto (pH, composição nutricional e microbiológica, humidade) e o embalamento, pelo que a aplicação a novos produtos requer cuidada otimização para ser mais efetiva e ter menor custos de operação.

Esta tese vem na continuação de trabalhos anteriores em que HPP melhorou a qualidade dos filetes de robalo, aumentou a sua vida útil e reduziu a carga microbiológica e proporção de géneros bacterianos de deterioração alimentar. No estudo atual, a metagenómica foi usada para estudar o microbioma presente nos filetes de robalo depois

de aplicados diferentes tratamentos com o HPP (pressões de 300-600 MPa e tempos de 2-5 minutos) e tempos de armazenamento refrigerado (1-11 dias a 2ºC), para caracterizar globalmente os microrganismos nas amostras e o efeito de HPP e contribuir para a sua otimização.

Os filetes sujeitos a estas condições experimentais foram fornecido por colaboradores na Grécia e no CCMAR procedeu-se à otimização e extração de DNA de amostras da sua superfície; controlo de qualidade por quantificação em espectrofotómetro Nanodrop e géis de eletroforese; seleção de amostras representativas (triplicados para 14 grupos experimentais) e envio para empresa de sequenciação (Laragen, USA) para produção de bibliotecas de microbioma, em que se usou o sequenciamento direcionado por PCR com o gene 16S rRNA como alvo. Este é o marcador taxonómico mais usado para bactérias, com zonas variáveis alternadas com conservadas, cobrindo a maioria das bactérias e boa disponibilidade em bases de dados.

As bibliotecas foram analisadas usando o Qiime (com a base de dados SILVA e normalmente usado em metagenómica), em colaboração com parceiros no CCMAR. Às OTU (unidades taxonómicas operacionais, grupos de sequências identificados a um dado nível taxonómico) obtidas a partir da análise com conjunto de aplicações Qiime, foram retiradas as sequências identificadas como cloroplastos, sequencias não identificadas, singletons e doubletons (que apareceram menos de 3 vezes nas amostras) e os dados foram rarefeitos para o número mínimo de sequências obtidas nas bibliotecas. Produziram-se tabelas dos diferentes níveis taxonómicos e os cálculos da alfa diversidade (como Shannon Index e número de espécies identificadas). Identificaram-se assim os microrganismos mais abundantes ao nível do género e que poderão ser usados como potenciais marcadores para tratamentos específicos: *Massilia, Carnobacterium, Shewanella* e *Janthinobacterium*. O desenho de primers e testes para estes microrganismos mais abundantes estão a ser ainda desenvolvidos pelo que não serão inseridos nesta tese.

Os resultados de culturas microbiológicas das amostras e testes sensoriais feitos pelos nossos colaboradores na Grécia (não incluídos nesta tese) apontam para uma diminuição da carga bacteriana das amostras sujeitas a pressões altas, mas pouca perda de qualidade, valores que no geral confirmam os obtidos no projeto piloto anterior (Tsironi et al. 2019).

A aplicação de diferentes pressões levou a resultados muito semelhantes entre os diferentes grupos, com maior divergência no grupo 300MPa 11 dias que teve microbioma bastante diferente dos restantes a 11 dias (com o grande aumento de *Carnobacterium*). Nos outros grupos 450/600Mpa 11 dias houve uma diminuição de *Massilia* e um aumento de *Shewanella,* como no controlo, mas o microbioma a estas altas pressões foi mais semelhante ao do grupo controlo inicial.

Resultados da diversidade com os valores de Shannon, observed_species e pelas curvas de rarefação (obtidas usando os ficheiros do Qiime com as OTUs após limpeza de dados e rarefação) confirmaram a diversidade dentro das amostras, que no global aumentou entre os 1 e 11 dias de armazenamento.

O qPCR do 16S feito a maior número de amostras (n=5-7 por grupo) do que usado na sequenciação, pode confirmar que o controlo a 11 dias continha muita carga bacteriana, enquanto os tratamentos com 450MPa e 600MPa foram eficazes na redução do número de bactérias, contendo valores muito perto daqueles obtidos nas amostras com armazenamento de apenas 1 dia, e a pressão 300 MPa revelou valores intermédios.

Os tratamentos com HPP com pressões de 450/600 MPa tanto em 2 mins como em 5 mins poderão ser eficazes para tratar este tipo de amostras, obtendo cargas e composições bacterianas aos 11 dias perto das do armazenamento inicial, e poderão contribuir para aumentar o seu tempo de vida útil. A pressão a 450 MPa poderá ser a melhor neste tipo de processamento de filetes por ser provável que cause menos danos físicos e sensoriais ao produto (ainda em estudo), tendo uma eficácia muito semelhante ao tratamento com 600Mpa, com menores gastos de energia.

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1. Introduction

1.1. Aquaculture production and food preservation

Fish and seafood products are important protein sources in human nutrition. The production of fish has increased over the years, mainly due to the growth of the aquaculture sectors, and more than 45% of the fish used for human consumption come from aquaculture (FAO 2019). According to EUMOFA (2019), Portugal has the highest *per capita* consumption of fish in the EU. In 2017, the estimated level of fish consumption was 56.8 kg *per capita*, which was more than twice the EU level. However, fish products are highly perishable, and, if left unpreserved, they spoil rapidly. Fish and fish products are often associated with human diseases, especially when consumed raw or undercooked (Novoslavskij et al. 2015). Commonly used conservation techniques, include curing (dried and salted) and make fish and derivatives shelf stable at ambient temperature. A high number of fish products are also preserved using low levels of salt, cooling, packaging in a modified atmosphere and by addition of low levels of preservatives. The microbiota of these products is complex, and spoilage is mostly caused by microbial action (Gram 2009).

While the most common preservation methods are drying, salting, pickling, smoking, freezing, or canning (as detailed below), the growth in aquaculture has caused a change in the way that fish are preserved. Fish is still mainly consumed "fresh", and canned and cured products have become less popular (Pedro et al. 2007). In addition, the intensive growth of the industry and agriculture sectors in the last decades may have caused contamination of aquatic environments with chemicals and may affect not only the health of fish and their defences, but also raise safety concerns regarding fish used for human consumption (Carvalho 2017).

It is well known that fish and fish products may lead to human diseases if they are contaminated when consumed. Thus, it is necessary to study the prevalence of pathogens in fish to ensure the safety of fish products and the processing plants. Microbial assessment of fish also gives some additional information about the hygienic status of aquatic environments including lakes, rivers, ponds, and fish farms. Detection of

pathogenic microorganisms and analyses of changes in the natural microbiota in the aquatic environments could be important indicators of possible contamination, with pollutants or with additional microorganisms (Novoslavskij et al. 2015).

For example, the European bass (*Dicentrarchus labrax*), the species studied in this thesis, was one of the first species of fish to be commercially farmed in Europe; one of the largest success stories in European aquaculture has been the Mediterranean seabass aquaculture industry that produces around 191,003 tonnes of this species yearly, and therefore, there is a particular need to "protect" it from spoilage (FAO 2017).

1.2. Food spoilage, deterioration, and safety

Food spoilage is a process where the food products (meat, fish, vegetables, etc.) become unsuitable for ingestion by the consumer and may be unacceptable from a sensory point of view. Signs of food spoilage may include an appearance different from the food in its fresh form, such as a change in colour, a change in texture, an unpleasant odour, or an undesirable taste (Magoulas 2016).

Food spoilage is a big problem worldwide with roughly one-third of the world's produced food for human consumption being lost every year (FAO 2011). There is, thus, a need to discover and optimize novel storage or processing techniques, to improve food safety but also to reduce the amount of food waste worldwide. This is particularly important for meat and fish products, which are highly perishable as these aliments are very rich in nutrients and water; so, they are optimal for the growth of many bacteria species and therefore they result, very easily, in spoiled food if not handled or conserved with care (Rawat 2015).

Lipid hydrolysis and oxidation are two very common causes of spoilage in many fatty fish species such as anchovies, that can cause "belly burst" in which the enzymes and microorganisms of the digestive tract cause massive gas development (Careche et al. 2002). Protein denaturation and development of odd flavour due to changes in the protein and lipid fraction are common causes of spoilage of frozen fish products. However, microbial growth and their metabolism are the major causes of spoilage of fresh, semi-preserved fish, and lightly preserved fish products. Microbial spoilage involves growth

of bacteria, yeast or fungi in high levels and the products of their metabolism can give rise to the sensory impressions perceived as spoilage (Gram 2009).

The diverse factors that can cause food spoilage making items unsuitable for consumption (such as light, oxygen, humidity, temperature, heat and spoilage bacteria, fungi, or yeast) can affect both quality and safety of easily perishable foods and when subject to these factors' food will gradually degrade. The spoilage occurs not only because of the storage conditions but also due to the food-specific microbiota, bacteria, or fungi to which it may be exposed, which are the main causes of spoilage and can have severe consequences for the consumers (Gram et al. 2002).

1.3. Microorganism food spoilage

Microorganisms are the most important factor in food spoilage originating chemical reactions that cause offensive sensory changes in foods and are produced by a variety of microbes that use food as a carbon and energy source. Since microorganisms are ubiquitous in the environment there is always a risk of spoilage, and so special techniques for processing or safe storage conditions are essential (Sperber 2009).

Microbial spoilage results from bacteria, yeast and moulds that normally manifest themselves as slime and strong off-odours and off-flavours (Gram et al. 2002). Spoilage microbes are often common inhabitants of water, soil, or are present in the intestinal tracts of animals and may be dispersed through the water and air and by the activities of small animals, particularly insects (Rawat 2015).

Some of the most common bacteria that can cause microbial spoilages belong to the genera *Clostridium*, *Bacillus*, *Geobacillus* or *Vibrio*. Spoilage can also be caused by pathogenic bacteria such as *Bacillus cereus*, known for the spoilage of milk and cream, or *Listeria monocytogenes* that infects mainly meat, poultry, and seafood and these bacteria in addition to causing food deterioration, can also have serious health consequences (Magoulas 2016; Farber et al. 1991).

Food spoiling can be very problematic for human health with the possibilities of food poisoning or foodborne illnesses, caused by the microorganism growth and the products of their metabolism, including toxins present in deteriorated food. As a safety measure spoiled food is removed from sale and discarded and this is resource wasteful

and also causes big economic losses (Chaillou et al. 2015). There is thus a need to improve the existing processing and storage methods to extend the shelf-life duration of food products, particularly highly perishable seafood, and fresh fish products that rapidly spoil compared to other types of food, due to their microbiological complexity (Macé et al. 2013).

1.4. Classical and novel fish preservation techniques

Many food products are perishable and require protection from spoilage during their preparation, storage, and distribution to give them the desired shelf-life, while preserving quality and safety. Because food products are now often sold in areas of the world distant from their production sites, there is also a need to extend their shelf-life while maintaining the safety of these products (Rawat 2015). To combat this problem, food preservation techniques have been used since the beginning of mankind and they were very important for survival. The development of food preservation processes has been driven by the need to extend their shelf-life. Food preservation is a continuous fight against microorganism spoiling food or making it unsafe for consumption (Ramesh 2008; Sancho-Madriz 2003).

Several food preservation systems such as smoking, freezing, canning, heating, refrigeration, and addition of antimicrobial compounds can be used to reduce the risk of outbreaks of food poisoning. However, these techniques are frequently associated with adverse changes in organoleptic characteristics and loss of nutrients (Rawat 2015). Some older preservation techniques such as drying, salting or fermentation are still being used but with improved and adapted new techniques. Nowadays the need to prevent food spoilage has led to the development of different types of food storage techniques, directed at preventing economic losses (Hammond et al. 2015).

Within the preservation techniques already known, the food industry is seeking to replace traditional food preservation techniques with new preservation techniques such as different storage temperatures, modified-atmosphere packaging, dehydration, curing protocols, vacuum packaging, and pasteurization (Niakousari et al. 2018). There is an increased consumer demand for tasty, nutritious, natural, and easy-to-handle food products. Improvements in the cold distribution chain have made international trade of

perishable foods possible, but, unfortunately, refrigeration alone cannot assure the quality and safety of all perishable foods or significantly extend the shelf-life (Rawat 2015).

Novel techniques such as High Pressure Processing (HPP), which is the focus of this thesis (detailed in topic 1.5), and the utilization of novel chemical preservatives or food additives have started to grow in the recent decades. Chemical preservatives are substances that are added to food to retard or inhibit the activity of microorganisms such as putrefaction fermentation and decomposition. Food additives are substances or mixtures of substances, which are present or added to foods during production, processing, storage, or packaging. Sugar, salt, acids, and spices are some examples of chemical preservatives/food additives (Rawat 2015).

1.5. High pressure processing

High pressure processing (HPP), also known as ultra-high-pressure processing (UHP) and high hydrostatic pressure processing (HHP), has a great potential in food processing. Contrary to common thermal processing methods such as pasteurization and sterilization that use heating and may affect food quality, HPP uses pressure to inactivate microbes. The pressures applied are in the range of 300MPa to 1000 MPa for several minutes at ambient temperatures and can be used both on solid and liquid foods, with or without the addition of heat (Farr 1990; Balasubramaniam et al. 2008). HPP does not break covalent bonds in nutrients and so this contributes to retain food quality and its natural freshness while extending the shelf-life (Balasubramaniam et al. 2008).

The studies on this type of processing began in 1899 with studies on milk and meats (Hite 1899). Nowadays this technology is increasingly being used by the food industry as an intervention technology to kill pathogens in food products (such as *Escherichia coli, Salmonella, Pseudomonas, Listeria* and *Vibrio*), without additional heat processing. HPP can be effective in a wide variety of foods such as, seafood, meat, fruits, sauces, and vegetables, preserving the natural flavour of the food, enzyme regulation and the starch digestibility (Balasubramaniam et al. 2008). However, there is not yet enough knowledge about the type and range of microorganisms that are affected by HPP or those that are resistant, and metagenomics (topic 1.8) can contribute to this global analysis.

Traditional thermal pasteurization technology negatively affects the characteristics of the food, such as flavours and nutritional content. So, there is a need for a different treatment method, such as HPP that ensures the microbial safety of the food, without adding preservatives thus allowing processed food to maintain its natural flavour and nutritional value. The effect of high hydrostatic pressure, unlike that of thermal processes and other conventional conservation technologies, is almost instantaneous and uniform, HPP effects are also relatively independent of the equipment and product geometry and size; therefore, scaling of laboratory and pilot plant findings to commercial production is both simple and safe, but for financial feasibility reasons, HPP treatments must be kept short (Mújica-Paz et al. 2011).

The critical process factors in HPP are: pressure, treatment time, time to achieve treatment pressure, decompression time, treatment temperature, product initial temperature, vessel temperature distribution at pressure, product pH, product composition, product water activity, packaging material integrity.

1.6. High pressure processing of fish fillets

HPP of fish fillets is the focus of the thesis work, as it can be a novel type of processing that extends the self-life of fish products and has been found in some studies to lower the microbes present in food that can cause spoilage. HPP uses pressures between 100 and 1000MPa, leading to changes in the morphology, biochemical reactions and cell membrane and wall of associated microorganisms rendering them inactive (Huang et al. 2014, Kiera et al. 2008). Fish gels obtained by HPP were reported to have a smoother and glassier appearance with improved texture relative to heat-induced gels (Truong et al. 2014). HPP inhibits endogenous enzyme activities due to denaturation of the protein fraction and in this context, proteomics may be an effective tool for evaluation of the efficiency of HPP for food applications. But HPP can also induce undesirable changes in the appearance, texture, and chemical structure of fish flesh, which need to be minimized by further investigation.

HPP has many advantages for products that cannot be thermally treated, such as fresh fish products that are highly perishable and require very low storage temperatures (0-2ºC) during their very short shelf life. HPP treatments may allow the use of higher

temperatures (5-7ºC) in the cold chain and can result in an extended shelf life (by at least 3-fold) expected to reduce energy and food waste (Tsironi et al. 2015).

Processing at pressures ranging from 300 to 800 MPa at ambient temperature has been reported to be sufficient to inactivate vegetative microorganisms due to unfolding and denaturation of important cell enzymes and proteins.

In a recent paper from the host group at CCMAR, HPP was shown to improve fish (sea bass) fillet quality during refrigerated storage, it extended shelf-life by reducing food spoilage genera and generally improved the microbial quality by reducing initial microbial loads and changing their microbial diversity, evaluated using a preliminary metagenomics approach (Tsironi et al. 2019).

1.7. Quality control of food and fish products

Food quality control (QC) is a process by which entities review the quality of most factors involved in production. The main objectives of fish product QC are to ensure they are unadulterated, have a high nutritional value and produce the desired properties. Product quality includes physical aspects such as freshness and appearance, and organoleptic properties as well as nutritional quality. QC is now very common in the fish industry and its use is increasing steadily due to the demand for high-quality products. Some of the QC points taken into consideration for the fish product supply chain are hygiene, fat stability, raw material freshness, safety, and purity. Some of the more important factors that determine quality from the customer's point of view are appearance, odour, flavour, freshness, condition, packaging, and composition (Tahsin et al. 2017).

QC assists the maintenance or improvement of profitability by minimizing customer complaints about quality/safety and contributes to avoid losses and Hazard Analysis Critical Control Point (HACCP) is one type of quality control very common nowadays. The HACCP approach is a systematic preventive approach for food safety and prevention from a biological, chemical, and physical hazards (and more recently radiological hazards) perspective in the context of production processes that can cause the finished product to be unsafe and it designs measures to reduce these risks to a safe level. In this manner HACCP attempts to avoid hazards rather than attempting to inspect finished products for the effects of those hazards. The HACCP system can be used at all

stages of the food supply chain, from food production and preparation processes including packaging and distribution (IFFO 2017; FAO 2000; Tahsin et al 2017).

1.8. Metagenomics as a tool to improve and monitor food safety and quality

Metagenomics is the study of genetic material of a mixture of organisms recovered directly from environmental samples or from the interior or surface of organisms, it studies the community of microorganisms without the need for isolation of individual species (Thomas et al. 2012). Metagenomics can give a global characterization of all the microbes in a given sample (Ghosh et al. 2018). Using this approach microbial ecology can be investigated at a bigger scale and detail than previously, including both cultivable and non-cultivable species, as well as evaluating the effects of different conditions (biotic or abiotic) or treatments in the microbiomes of the samples. For example, in this study and in the previous study carried out in this group (Tsironi et al. 2019), the microbiome of sea bass fillets under different HPP treatments were studied using metagenomics.

The current metagenomics approaches allow us to understand the complex properties, their dynamics and function in the natural system, of the various bacteria present in the samples (Janda et al. 2007). Metagenomics approaches may answer very important questions such as which organisms are present (taxonomic diversity), and what roles they play (functional metagenomics). The two most used methods of microorganism identification using high throughput sequencing (metagenomics) are: amplicon-based methods (in which specific genes are amplified and their PCR products are sequenced) and whole metagenomic shotgun sequencing. In the amplicon-directed approaches, the most used targets include the 16S ribosomal RNA (16S rRNA) gene for the identification of bacteria; the internal transcribed spacer (ITS) region for fungi and the 18S rRNA gene for eukaryotes (Ghosh et al. 2018).

For the characterization of the bacteria present in a sample, the PCR-directed sequencing with the 16S rRNA gene as target is the most common method (Clarridge 2004), and the one chosen for use in this study as detailed below; this gene is present in all bacteria and has a good combination of variable and conserved regions (Janda et al. 2007), and therefore covers a substantial part of most bacteria in samples and allows their taxonomic

identification by comparing to 16S rRNA gene databases that are very complete. The general approach for metagenomics integrates genomic technologies and bioinformatics tools to directly assess the genetic content of entire communities of organisms, and this field of study is responsible for a considerable increase in the knowledge of microbiology ecology evolution and diversity in the past years, and many research labs are engaged in it now.

Some important steps in a metagenome project include sampling and processing, DNA extraction, DNA sequencing, assembly, binning, annotation, statistical analysis, and storage and sharing of data (Thomas et al. 2012). Aseptic sampling and processing are the most important step in metagenomics and consists in the extraction of the total DNA from the sample that should represent all the microbial content present, and it must have enough high-quality nucleic acids for library production and sequencing (Thomas et al. 2012).

1.9. Metagenomics advantages and applications

Metagenomics has a wide range of application from environmental samples to food safety, from industrial waste to medical fields because it can identify normal flora, deterioration microbes and pathogens. It has also been used to identify new enzymes, microorganisms capable of degrading toxic pollutants or pathogens present in food which is very important in food safety management (NRC CM 2007; Ghosh et al. 2018). Metagenomics can have an important contribution in diverse areas of science, such as biotechnology, bioremediation, agriculture, aquaculture, bioenergy and particularly food safety, authenticity, and processing (Techtmann et al. 2016; Chroneos 2010).

There are groups such as the Consortium for Sequencing the Food Supply Chain (CSFSC), founded by IBM and Mars Incorporated, that are putting efforts into the collection of genomic information on pathogenic bacteria across the food supply chain and characterizing and quantifying microbiomes before and after processing, to improve food safety, authenticity, and traceability (IBM 2015). With this information on different microbiomes, it is possible to identify pathogens, which are normally present in the food, and permits the efficacy of the food preservation / processing techniques to be established.

There is still very little understanding about the roles played by the large spectrum of species involved in food spoilage, but many studies have shown that bacteria are the most predominant microorganisms present in spoilage of meat and seafood (e.g., spoilage species belong to the orders *Lactobacillales, Vibrionales, Pseudomonadales and Enterobacteriales*). The effectiveness of storage methods can be investigated using metagenomics to help discover what type of microbes are present in food after the processing technique used, as metagenomics provides a way to study the different microbial communities in their own “habitat”. Complex ecological interactions including lateral gene transfer, phage-host dynamics, and metabolic complementation can also be studied using metagenomics (NRC CM 2007). The relationship between foods and their microbiome is fundamental to their quality and safety, as beneficial microbial communities can be responsible for rheological and organoleptic traits of fermented foods.

1.10. Objectives

The global aim of this thesis was to further investigate the effect of HPP on European sea bass (*Dicentrarchus labrax*) fillets using metagenomics and relating it with other measurements of microbiological analyses, inserted into a larger study in which physicochemical and sensory indices are also being evaluated by other collaborators. The effectiveness of HPP was evaluated using different treatment pressures, processing times and storage times with sea bass fillets (aiming for the optimization of its future use as preservation technique for fish products) and in this thesis the respective microbiome libraries were sequenced and analysed, with the long-term goal to identify novel markers for quality assessment and microbiological spoilage fingerprints. We hypothesised that higher pressure and exposure times will reduce the spoilage microorganisms of fish fillets.

2. Methodological approach

2.1. HPP processing experiment (NTUA):

Sea bass fillets were provided by a Greek aquaculture company and transported within 2 - 4h directly to the Laboratory of Food Chemistry and Technology at the National Technical University of Athens (NTUA, collaborators of the project), in polystyrene boxes with flaked ice (0°C). HP was applied using a laboratory pilot scale HP equipment with a maximum operating pressure of 1,000 MPa (food pressure unit FPU 1.01, Resato International BV, Roden, Holland). The sea bream fillets were processed with different treatment pressures (300MPa, 450MPa or 600MPa), pressure times (2 minutes or 5 minutes) and then stored for different times at 2ºC (1day or 11days)- Figure 1.

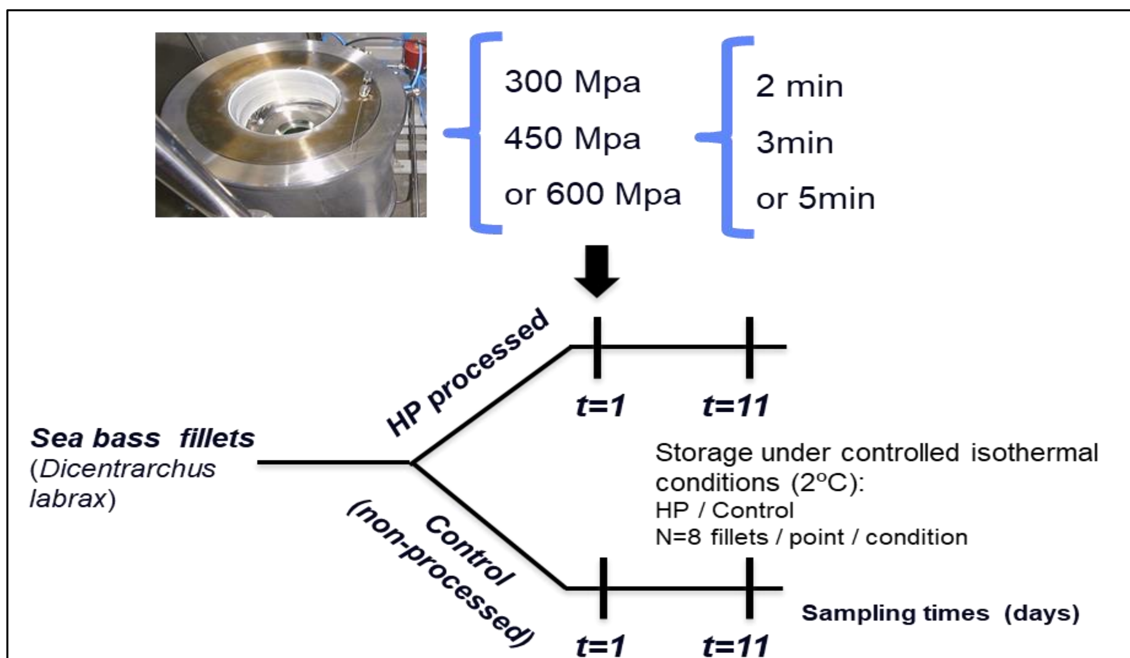

Figure 1- Experimental design of the high pressure processing (HPP) experiment. Three different pressures were used (300/450/600MPa) at three different processing times (2, 3 or 5 min) and two different storage times (1 day and 11 days) at 2ºC. Control groups were not processed but were also stored refrigerated at the same storage times. Classical microbiological analyses (using general and selective culture media), pH measurement, moisture content, sensory evaluation, and texture analysis tests were conducted by our collaborators (not included in this thesis) in these samples; the metagenomics analyses (of the two experimental groups, three pressures and two processing times 2 and 5 min) were run at CCMAR as part of the thesis work, followed by quantitative PCR validation.

2.2. Metagenomics experimental design (CCMAR)

Samples of the fish fillets processed by the NTUA collaborators were aseptically sampled for microbiome analysis (by excising approx. 100-300 mg slices from the fillet surface), immediately frozen and shipped on dry ice to CCMAR, where they were stored at -80ºC until extraction. The steps carried at CCMAR / University of Algarve during this thesis are represented in grey in Figure 2 (Methodological approach).

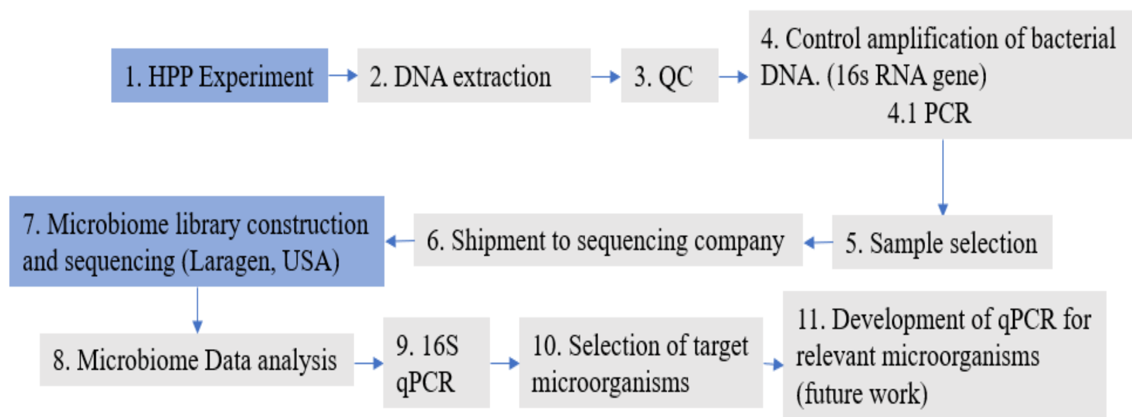

Figure 2- Methodological approach of the high pressure processing (HPP) experiment. The steps carried out during this thesis (grey) are described in the next section and in blue are the steps that were done by collaborators (1) or a company (7). QC – Quality Control, HPP – High pressure processing, PCR – polymerase chain reaction, qPCR – quantitative real time polymerase chain reaction.

3. Materials and methods

3.1 DNA extraction

Extraction of total DNA from fish fillet muscle samples (6-8 individuals per group) was performed using the DNeasy Blood & Tissue Kits (Qiagen), as previously described for this type of sample in Anjos et al. 2019. Inside of a laminar flow cabinet (to maintain sterile conditions) the sea bass fillet slices were weighed, cut into small pieces and 40-225 mg of tissue were placed into sterile 1.5 ml microcentrifuge tubes together with 200 µl of lysis buffer (20Mm Tris-HCl, pH 8; 2mM sodium EDTA; 1.2% Triton X-100; 40 mg/m Lysozyme) and 200 ml of AL buffer from the kit. Samples were then lysed

using a Qiagen Tissue Lyser with iron beads (2x30 secs) for the first rupture and glass beads (3x 5 mins) for the second rupture. Disrupted samples were incubated at 37º for 30 mins to complete enzymatic lysis and then proteinase K (for protein lysis) was added, followed by another incubation at 56º for 30mins. Tubes were centrifuged for 1 min at 6093 g and the lysed supernatant was recovered and incubated with RNAse A (10 µl of 10 mg/ml) for 10 min at room temperature. The supernatant of the samples was then transferred to microcentrifuge tubes and ½ volume of 100% ethanol was added. The samples were then loaded onto a DNeasy Mini spin column and then centrifuged, so that the DNA was selectively bound to the DNeasy membrane. Contaminants and enzyme inhibitors were removed in two wash steps (Washing buffer 1 and Washing buffer 2 from the kit) and DNA was then eluted with a buffer (EB buffer 10Mm Tris-HCl, ph8). DNA was eluted in two consecutive steps using 50 µl of elution buffer each (to guarantee the maximum recovery of DNA with not too much dilution), and both eluted fractions were quantified.

3.2 Quality control (QC) of the extracted DNA

The quality and integrity of the extracted DNA was verified using a Nanodrop spectrophotometer and by agarose gel electrophoresis. The nanodrop gives the concentration of the DNA in solution and its purity ratios (260/280 ratio and 260/230 ratio). A 260/280 ratio of approximately 1.8 is generally accepted as "pure" enough for DNA. The 260/230 ratio is used as a secondary measure of nucleic acid purity and the values of this ratio are often higher than the 260/280 ratio values. Expected 260/230 values are in the range of 2.0-2.2. If the ratio is lower than expected this may indicate the presence of contaminants (Thermo scientific 2009; Thermo scientific 2012). Agarose gel electrophoresis indicates the DNA integrity, which is very important when selecting the DNA samples for sequencing. Nanodrop values of the muscle DNA extracts varied from A260/280 between 0.951 - 1.898 with a mean of 1.378 which are good concentrations and close to the concentration of 1.8 that is accepted as pure for DNA; the obtained ratios of A260/A230 were between 0.162 - 1.363 with a mean of 0.425, indicating there could be some contaminants in the samples (but this did not influence subsequent sequencing or PCR).

3.3 Control amplification of bacterial DNA

Primers that target the V3-V4 regions of the 16S ribosomal RNA (rRNA) were used to confirm by PCR amplification of microbial DNA in the DNA extracts, and samples with a strong positive signal were used for the microbiome library construction (Klindworth et al. 2013) at the sequencing company. Control amplification of the DNA was conducted in 20 µl PCR reactions containing 2 µl of DNA (extracted from the samples and diluted to 10 ng/ µl), 0.5 µl of the dNTPs (stock of 10mM each), 1.25 µl of each primer at 10uM, 2 µl of DreamTaq buffer, 0.2 µl of Dream Taq DNA polymerase and 17.3 µl of milli (mQ) water. Cycling conditions were 5 min at 95ºC, 35 cycles of 20 s at 95ºC, 20 s at the optimized annealing temperature for this primer pair (50ºC) and 20 s at 72ºC, followed by 5 min a 72ºC. The amplified target bands of approx. 500 base pairs (bp) were then observed in 1% agarose gels.

3.4 Sample selection and shipping to the sequencing company.

DNA samples were selected for microbiome library construction (n=3 DNAs / experimental group) based on i) the concentrations and ratios determined using a Nanodrop, ii) their integrity in gel electrophoresis (Figure 3) and iii) on good amplification of the 16S rRNA gene in the control PCRs. Selected samples were then shipped to the chosen sequencing company (Laragen, USA), refrigerated with ice packs. We choose to analyze 2 storage times for the Control and the HPP treated samples at 3 different pressures (300, 450 and 600 MPa), with a focus on 2 of the 3 processing times (2 and 5 mins, not analyzing the 3 mins) (Figure 1). For each of these 14 groups we selected 3 DNAs to produce libraries and so triplicate samples were analyzed for each treatment condition.

The samples selected for the libraries where the ones present in Table 1

Table 1 – Selected samples for the microbiome libraries construction (42 samples with n=3 DNAs per group). For the detailed experimental design, Figure 1 can be consulted.

Group Names	Sample	Group Names	Sample
Control 0min 1day	1.3	Control 0min 11day	11.4
	1.4		11.5
	1.5		11.6
300MPa 2min 1day	2.1	300MPa 2min 11day	12.2
	2.4		12.5
	2.6		12.6
300MPa 5min 1day	4.5	300MPa 5min 11day	14.5
	4.6		14.6
	4.8		14.7
450MPa 2min 1day	5.2	450MPa 2min 11day	15.2
	5.3		15.3
	5.4		15.5
450MPa 5min 1day	7.3	450MPa 5min 11day	17.1
	7.4		17.5
	7.5		17.7
600MPa 2min 1day	8.3	600MPa 2min 11day	18.6
	8.4		18.7
	8.7		18.8
600MPa 5min 1day	10.2	600MPa 5min 11day	20.2
	10.3		20.5
	10.6		20.8

3.5 Microbiome library construction, sequencing, and microbiome data analysis

Microbiome library construction and sequencing were conducted by Laragen, USA, using the 16S rRNA Illumina Library prep kit (Illumina 16S Metagenomic protocol). High-throughput sequencing was carried out using an Illumina MiSeq v3 sequencer, using 2 x 300 base paired-end (PE) reads for an estimated (ordered) number of filtered PE reads of 150,000 (75,000 each direction).

We used the open-source bioinformatics pipeline QIIME1 (Quantitative Insights into Microbial Ecology) (http://qiime.org/tutorials/tutorial.html) as the data analysis

software (Caporaso et al. 2010) and the SILVA database (silva_132_97_16S version), commonly used for metagenomics, in collaboration with Dr. Tania Aires and Dr. Aschwin Engelen (CCMAR).

A total of 3.3 million raw PE read sequences (part of the 16S rRNA gene) were obtained from the 42 libraries and the bacterial community analyses were performed using several scripts included in the QIIME pipeline as is now outlined. First, the sequences were screened (quality control) and filtered to eliminate short reads and those of bad quality, with more than 2 undetermined nucleotides. Quality filtering resulted in 3.1 million high-quality sequences, ranging from 32,804 to 103,919 reads per sample.

The high-quality, filtered, and paired sequences were then clustered into distinct Operation Taxonomic Units (OTUs), using the de novo OTU picking method (with Qiime command pick_open_reference_otus.py) by comparison with the SILVA_132 database files. Reads were clustered into a total of 5,388 OTU and representative sequences for each OTU were selected by the most abundant ones. Using QIIME script filter_otus_from_otu_table.py, we proceeded to clean up the OTU table by removing OTUs assigned to chloroplasts, unassigned sequences and singletons and doubletons (OTUs with less than 3 counts in any of the libraries). The general OTU table was obtained for the original clean data and for the rarefied data (rarefied by Qiime to have the same number of sequences for all libraries, based on the library with the smallest number of sequences), at the general OTU level or at the genus (L6) level. Alpha-diversity measurements were also obtained using QIIME.

3.6 16S qPCR tests

For the quantitative estimation of the total bacterial load of each sea bass fillet DNA sample, we used the primers recommended by the Earth microbiome project (515F–806R; https://earthmicrobiome.org/protocols-and-standards/16s/) that target the V4 region of the 16S rRNA gene, under quantitative real-time PCR (qPCR) conditions. qPCR reactions were carried out in duplicate using the relative standard curve method and were run on n=5-7 selected DNA samples for each group, including three used in metagenomics sequencing and additional DNA extracts considered of enough quality after QC). In each plate, we included at least two negatives (NTC) to ensure absence of DNA contaminations and one standard curve (prepared from serial dilutions of a

quantified plasmid preparation of a 1,000 bp fragment of the 16S rRNA gene of *Mycoplasma* bacteria). The standard curve allowed the estimation of the efficiency obtained in this qPCR, which was approx. 85% and an R^2 of 0.998. Specificity of qPCR reactions for 16S was verified by the presence of single peaks in the melt curves of each sample, although in the samples with lower bacterial detection (threshold cycle, CT > 30) secondary peaks also appeared that after sequencing were shown to results from cross-reaction with the host DNA. In general, CTs in the DNA samples ranged between 24-34. PCR products were analyzed by 2% agarose gel electrophoresis and sequenced to confirm identity. The thermal profile used was the following for the 16S rRNA gene qPCR (quantification of all bacteria):

Table 2 – Thermal profile for the 16S rRNA gene quantitative PCR

Procedure	Temperature	Time
Hot start	95ºC	2 minutes
X 40	95ºC	5 seconds
	50ºC	10 seconds
	72ºC	10 seconds
Melt Curve	60ºC	10 seconds
	95ºC	Increment +0.5ºC each 10 seconds until reaching 95ºC

We used 2x 96-well plates for the quantification of 16S in all the samples (in duplicate), which were loaded with 10 µl of the reaction mix indicated in the following table, prepared in a big master mix providing the volume required for all the reactions of the two plates. The qPCR reactions were performed in a Bio-Rad CFX96 Touch Real-Time PCR Detection System.

Table 3 – Composition of the mix used for the 16S rRNA gene quantitative PCR

qPCR MIX	1x (µl)
Forget-Me-Not™ EvaGreen® qPCR Master Mix BIOTIUM	5
Primer Fw (10µM)	0,3
Primer Rv (10µM)	0,3
DNA template	2
mQ-$H^2$0	2,4

The qPCR data analysis was run using CFX Maestro 1.1 software (v. 4.1.24333.1219; Bio-Rad) and included verification of replicates in the standard curve or unknown samples and using a standardized curve for the two qPCR plates. The 16S copy number for the samples was determined by comparing the average Ct value per duplicate reactions per sample to the standard curve, which allowed copy number to be related to Ct, 10 ng of DNA were used for all biological replicates and the detected copy number per sample was expressed as 16S copies / 10 ng DNA.

Copy number of the 16S rRNA genes were calculated using the following equation: number of copies = (X ⁄ NA) / (Y x 1×10^9 x 650), where X is the initial template amount (ng of the amplicon fragment), NA is Avogadro's number, Y is the template length (bp of each amplicon), and 650 (Da) is the average weight of a base pair (Martyniuk et. al. 2009)

3.7 Statistical analysis

Rarefied and treated files were obtained from QIIME and used on the microbiome analyst site (https://www.microbiomeanalyst.ca/), (with some tweaks so that the files were compatible with the required format for the site). With these analyses the rarefaction curves were obtained (to help us see the diversity, and that the sequencing depth obtained in this study was enough to cover most of the diversity of the samples) and the relative abundance of the bacteria of the microbiome of each sample, helping us see the effects of the treatment in the samples as well as the means to compare them and see the changes in the microbiome.

Permutational analysis of variance (PERMANOVA) using storage time, treatment time and processing time as factors were carried out to detect significant ($p < 0.05$) changes in the microbiome profiles obtained by metagenomics (using the rarefied OTU table at the genus level), or on the bacterial load quantified by the 16S qPCR, using the software PRIMER 6 & PERMANOVA+ v.1.0.1 (Primer-e, New Zealand). The PCoA 2D that helped us confirm the results obtained with the relative abundance at genus level was also obtained in PRIMER 6 & PERMANOVA+ v.1.0. In some cases when ANOVA did not detect significant differences by permutation, we used the Monte Carlo permutation test values to define the significant differences (used when unique permutation values are low).

4. Results and discussion

4.1 Microbiological analysis and sample selection

The HP experiment and sampling was carried out by our collaborators in Greece, (see Figure 1), and included traditional microbiological analyses and sensorial tests with the processed sea bass fillets. The traditional microbiological analyses (using culture methods) were used to determine total viable counts (PCA), and to estimate the levels of Pseudomonas, lactic acid bacteria, *Brochothrix thermosphacta*, H2S-producing bacteria, *Enterobacteriaceae*, and total yeasts and mold counts (CFC, MRS, STAA, IA, VRBD and RBC tests). These results are not presented in the thesis as they make part of a bigger study still under analyses by our collaborators in Greece. Nevertheless, the obtained microbial count values were very close to the ones obtained in the previous HPP study (Tsironi et al. 2019). The HPP-treatment reduced the microbial load and almost all values were below the detection level of the assay even with a storage time of 11 days, when compared to the Control samples.

The sea bass fillet samples intended for microbiome analysis, sampled under aseptic conditions at the surface of control or processed fillets, rapidly frozen and shipped in dry ice, were received at CCMAR, and allowed to proceed with the metagenomics experiment of this thesis. The DNA was extracted from the samples using the DNeasy Blood & Tissue Kits (from Qiagen) after optimization of disruption conditions and DNA quality and integrity evaluation by Nanodrop (Table 3) and gel electrophoresis (Figure 3) confirmed all DNAs yielded a good amount of DNA with acceptable purity (Table 4). The DNA concentrations ranged between 3.2-130 ng/µl, all DNAs yielded a good amount of DNA with acceptable purity (Table 4).

Table 4 – DNA concentration and purity of the chosen samples for the microbiome libraries (three individual fillets for control or for HP groups with three different pressures and two processing times, stored for 1 or 11 days). This were two of the parameters used to select these samples for sequencing, together with the integrity observed by electrophoresis gel.

Group Names	Sample	Concentration (ng / µL)	R260/280 (purity)	Group Names	Sample	Concentration (ng / µL)	R260/280 (purity)
Control 0min 1day	**1.3**	12.53	1.09	**Control 0min 11day**	**11.4**	28.61	1.47
	1.4	42.70	1.02		**11.5**	132.98	1.51
	1.5	8.88	1.05		**11.6**	21.34	1.51
300MPa 2min 1day	**2.1**	32.49	1.01	**300MPa 2min 11day**	**12.2**	34.42	1.57
	2.4	13.04	1.06		**12.5**	56.09	1.73
	2.6	47.00	1.90		**12.6**	38.57	1.76
300MPa 5min 1day	**4.5**	12.21	1.53	**300MPa 5min 11day**	**14.5**	36.51	1.42
	4.6	33.04	1.58		**14.6**	19.99	1.67
	4.8	15.58	1.57		**14.7**	28.36	1.44
450MPa 2min 1day	**5.2**	55.19	1.55	**450MPa 2min 11day**	**15.2**	40.01	1.46
	5.3	40.35	1.68		**15.3**	58.22	1.33
	5.4	72.57	1.44		**15.5**	34.89	1.30
450MPa 5min 1day	**7.3**	26.38	1.22	**450MPa 5min 11day**	**17.1**	36.78	1.81
	7.4	51.16	1.22		**17.5**	21.77	1.72
	7.5	100.51	1.22		**17.7**	34.21	1.72
600MPa 2min 1day	**8.3**	28.64	1.61	**600MPa 2min 11day**	**18.6**	23.36	1.36
	8.4	33.03	1.57		**18.7**	12.55	1.36
	8.7	41.85	1.56		**18.8**	22.13	1.64
600MPa 5min 1day	**10.2**	15.40	1.22	**600MPa 5min 11day**	**20.2**	12.63	1.52
	10.3	19.13	1.55		**20.5**	23.83	1.17
	10.6	19.35	1.47		**20.8**	8.41	1.00

The choice of samples for the microbiome libraries was made based on the integrity, purity, and DNA amplification of 16 S rRNA in the samples with a conventional 16S PCR (data not shown).

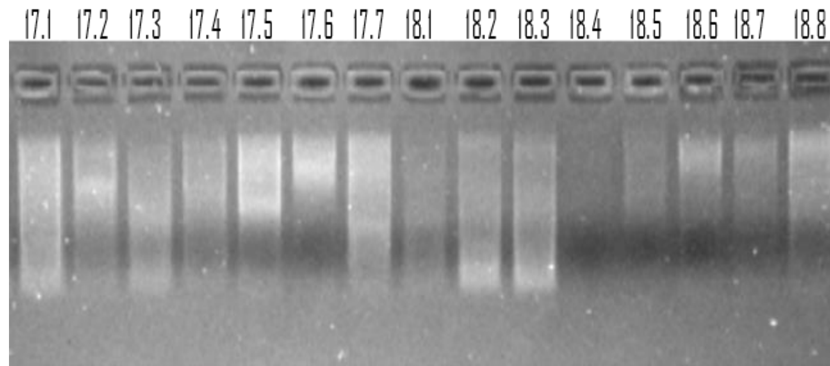

Figure 3 –Representative 1% agarose gel where the integrity of part of the extracted DNAs was analysed. This electrophoresis gel presents the analysis of all extracted individual DNAs from group 17 (450MPa.5min.11d) and group 18 (600MPa.2min.11d), which are representative of other groups that presented similar integrities in gel electrophoresis. This was one of the parameters for selection of the samples sent to sequencing, together with concentration and purity obtained using Nanodrop.

4.2 Sequencing results and alpha-diversity

From the 42 sequenced libraries, a total of 3.3 million raw read sequences were produced, which included 2 reads of approx. 300 base pairs from the two ends of each sequence (R1 and R2). After quality control and merging the reads R1-R2, these originated 3.1 million filtered reads with approx. 467 bases and good sequence quality. With QIIME we were able to calculate several indexes of alpha diversity (the diversity within each sample), including the Shannon diversity index (SHI), and the number of observed species (Table 5).

The diversity of the bacterial population present in our samples were compared between HPP and control libraries and the average SHI indexes per group are summarized in Figure 4 while the rarefaction curves of all libraries are presented in Figure 5.

In general, the rarefaction curves suggest an increase in bacterial diversity when comparing the samples stored for 11 days with those at the initial storage time of 1 day.

Table 5 – Shannon diversity index (SHI) and observed_species values for each sample, used to indicate the bacterial diversity between samples, which was higher in most groups after 11 days storage.

Group Names	Sample	Shannon	observed _species	Group Names	Sample	Shannon	observed _species
Control 0min 1day	**1.3**	2.26	493	**Control 0min 11day**	**11.4**	5.20	889
	1.4	3.34	685		**11.5**	5.02	767
	1.5	3.39	750		**11.6**	4.73	911
300MPa 2min 1day	**2.1**	3.84	603	**300MPa 2min 11day**	**12.2**	3.55	767
	2.4	3.43	653		**12.5**	2.99	598
	2.6	1.67	302		**12.6**	4.33	851
300MPa 5min 1day	**4.5**	2.33	472	**300MPa 5min 11day**	**14.5**	4.78	959
	4.6	2.70	521		**14.6**	4.01	771
	4.8	2.54	514		**14.7**	0.50	199
450MPa 2min 1day	**5.2**	2.59	601	**450MPa 2min 11day**	**15.2**	5.36	904
	5.3	2.34	511		**15.3**	5.35	954
	5.4	2.16	478		**15.5**	5.31	852
450MPa 5min 1day	**7.3**	3.41	554	**450MPa 5min 11day**	**17.1**	5.28	971
	7.4	4.04	790		**17.5**	4.83	828
	7.5	4.03	745		**17.7**	5.02	932
600MPa 2min 1day	**8.3**	5.64	1171	**600MPa 2min 11day**	**18.6**	4.78	723
	8.4	5.79	1251		**18.7**	5.05	830
	8.7	5.79	1251		**18.8**	4.70	803
600MPa 5min 1day	**10.2**	3.66	662	**600MPa 5min 11day**	**20.2**	5.26	977
	10.3	2.23	433		**20.5**	4.89	836
	10.6	2.45	486		**20.8**	5.32	1080

The rarefaction curves also indicated that the sequencing depth obtained in this study was enough to cover most diversity of the samples, because in general all curves reached a plateau. When analyzing the statistical differences between average Shannon values between groups (Figure 4), it was confirmed that in most groups the diversity was higher after 11 days storage.

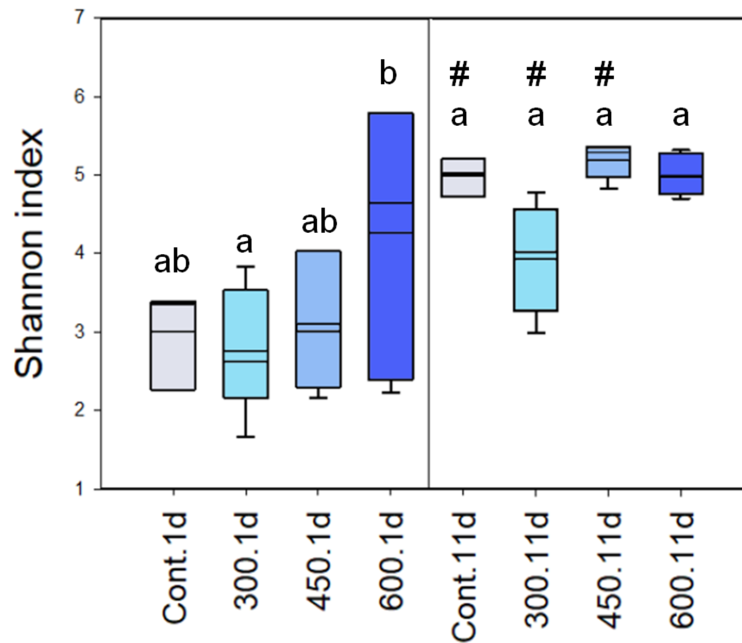

Figure 4 – Shannon index divided by groups (control or different pressures) and separated by storage time in the two panels. For the detailed experimental design please consult Figure 1. For each combination of pressure group and storage time, the values obtained for the two processing times 2 or 5 minutes were combined. In each graph (within each storage time), statistical differences between groups are denoted with letters, while # indicates significant differences between 11 and 1 day of storage within each group (evaluated by PERMANOVA $p<0.05$, as detailed in the methods).

The only exception in relation to diversity were the fillets treated with 600MPa, in which there was a great variation in diversity at day 1 of storage, which appeared to be on average higher than control fillets and the other treatments, but the apparent increase in diversity after 11 days was not significant.

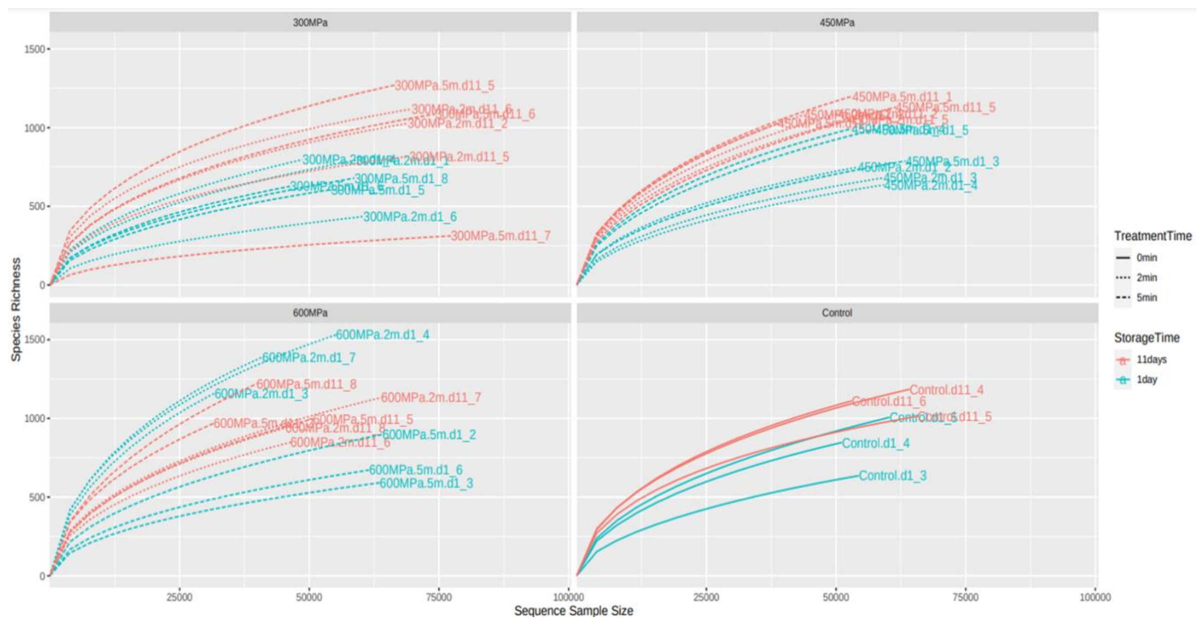

Figure 5 - Rarefaction curves to evaluate samples diversity and depth of analysis. Curves were divided in panels by treatment, while storage times are represented in different colors (11 days in red and 1 day in blue) and treatment time by different line types (0 mins in a straight line, 2min dotted line, and 5mins with dashed line) – see legend in the right.

4.3 Relative abundance and general microbiome composition

The comparison of the clustered OTU sequences to the SILVA database allowed the identity of the main bacterial genera present in the control and HP-processed fillets to be established so that their relative abundance could be compared (Figure 6, bar plot analysis). Statistical analysis of their microbiome profiles indicated that the initial microbiomes of the control and HPP fillets were in general similar, except for the fillets processed at 600MPa for 2 mins and stored for 1 day, and the results were significantly different from the control and other processing pressures (Figure 7). Generally, in the initial groups (1 day) *Massilia* was the dominant genera (representing 60-80% of the microbiome) except in the 600 MPa 2 min group where *Massilia* "shared" dominance in the microbiome with *Shewanella,* and other genera were present at lower proportions.

In the samples analysed after 11 days storage, there was significant differences in the microbiome composition between the control and all processed groups as is evident in Figure 6. The most dominant genera swapped from *Massilia* to *Shewanella* (representing 60-75% of the microbiome) in the control group after 11 days of refrigerated storage. In the groups processed at 300MPa for 2 or 5 mins, which were significantly

different from the control, the most abundant genera shifted from *Massilia* to *Carnobacterium* (representing 60-90% of the microbiome).

The microbiome of fillets processed at 450 or 600 MPa, for 2 or 5 min, was statistically different from the control fillets after 11 days of storage but did not differ between these groups. Their microbiome was mainly represented by *Massilia* and *Shewanella* bacteria, while having other genera with lower abundances (Figure 6).

The detected bacteria are common food spoilage genera that have been previously reported in fresh fish fillets stored under refrigeration (Chaillou et al. 2015). *Shewanella* is a genus implicated in fish spoilage and *Carnobacterium* is known to cause disease in fish (Leisner et al. 2007). *Janthinobacterium* was also a prevalent genus in some samples, namely those processed with 300MPa for 2 mins and stored for 11 days and was approximately 20 % relative abundance in the replicates of this group. This bacterial genus was interesting because it is reported to have some anti-viral, anti-bacterial, anti-tumoral and anti-fungal properties (Avguštin et al. 2013; Pantanella et al. 2007). Maybe these anti-fungal properties of Janthinobacterium could lead to a reduction of the mould, yeasts, or bacterial load present in the samples. This genus was also found in the previous study carried out on sea bass filets in the group processed at 600MPa for 5 mins after 67 days of storage but was not found in the control sea bass fillet samples or in the samples maintained for a shorter time in refrigeration (Tsironi et al. 2019).

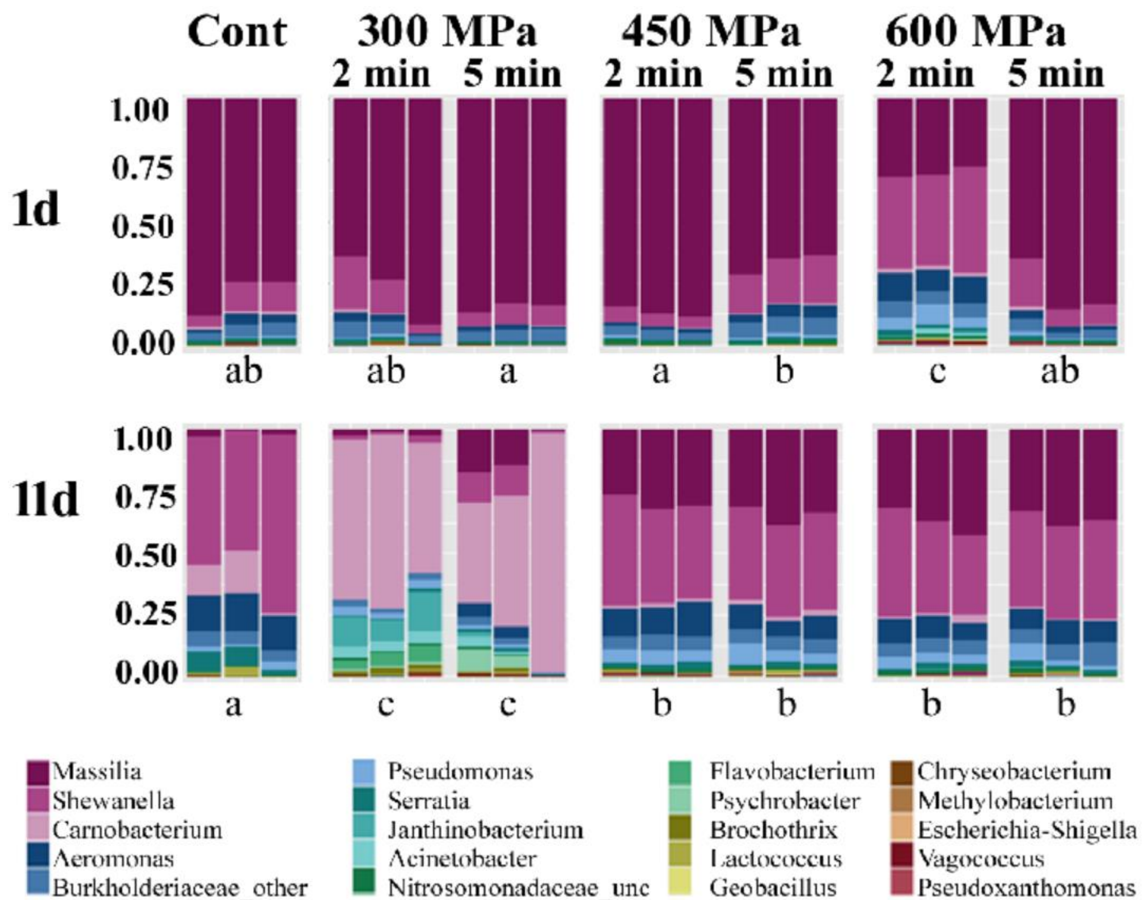

Figure 6 - Relative abundance of the main genera detected in all analysed libraries, separated by storage time in two different panels and then by treatment group including control and different high pressure processing pressures and times (for detailed experimental design and groups designation, Figure 1 can be consulted). Different letters represent statistically different groups (PERMANOVA, $p<0.05$) within each storage time.

The general microbiome composition results obtained with the relative abundance at genus level were confirmed with the PCoA graph, that permitted the estimation of the distance between samples, or the beta diversity (Figure 7). The PCoA confirmed that the groups that diverged most were those exposed to 300MPa for 2 or 5 minutes and stored for 11 days, which were in a cluster apart from the other groups. There was also a small divergence in the Control group of 11 days storage (Figure 7), reflecting their different composition compared to the groups processed with 300 MPa and those of 450 and 600 MPa, which were in general similar between them, in agreement with the composition analysis shown in Figure 6.

Comparing with the results from the previous work (Tsironi et al. 2019), there is somewhat of a difference in the microbial diversity of the samples. In the previous work the main bacterial genera present in the HP (600MPa 5 mins) at 11 days was

Pseudomonas (±57%, relative percentage), but in the present study pseudomonas was not very prevalent and had a relative abundance of approximately 3-5% in the HP treatment. And comparing the results of the 600MPa 5 mins 11 days in the previous work with the results of the present study the presence of *Pseudomonas* was low, with a relative abundance of about 2-4%. Even *Massilia* (one of the major genera present in this work) was not that much present in the previous study. The initial composition of bacterial genera present is pretty much the same (except *Massilia*), but the values vary from study to study. This part is very interesting because the differences could maybe be derived from seasonal differences linked to when the fish was caught and processed, maybe from the alimentation or even if the database used in each study was different.

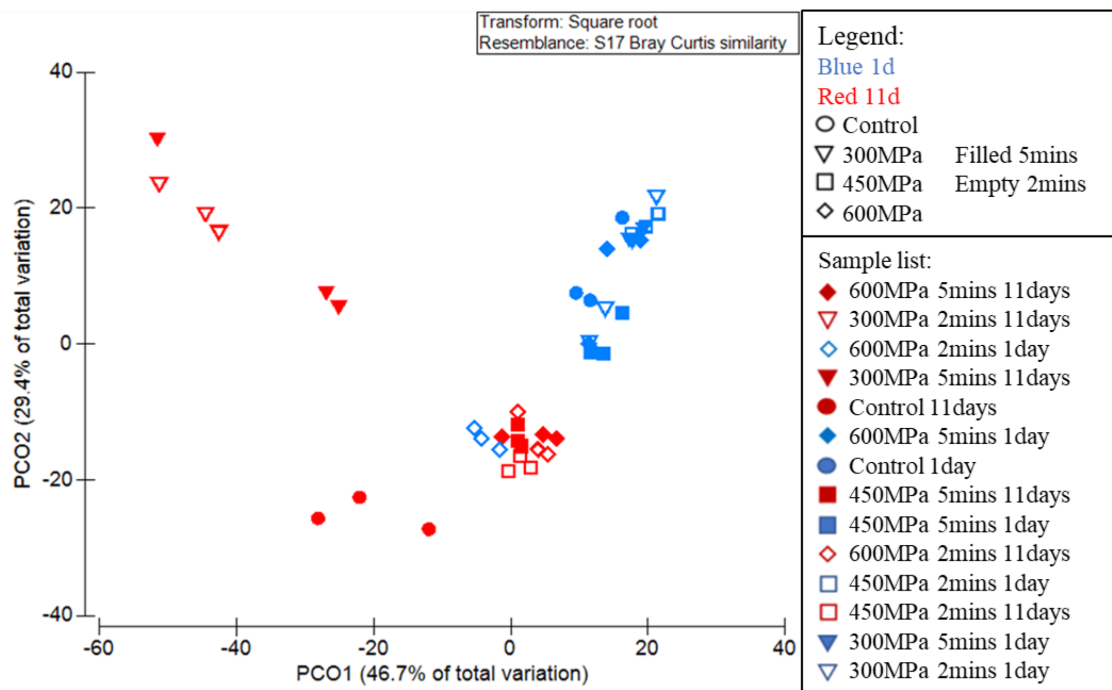

Figure 7 – Principal component analysis (PCoA 2D) representing the beta diversity of the microbiome results of all analyzed libraries, at the genus level, labeled by treatment and storage time according to the figure legend on the right. For detailed experimental design and groups designation, see Figure 1. Different storage times are represented in different colors with 1 day in blue and 11 days in red; control or treatment pressures are differentiated by shapes, with control represented with circles, 300MPa with inverted triangles, 450MPa with squares and 600MPa with diamonds. Treatment times are represented by filled (5mins) or empty (2mins) symbols.

4.4 16S gene qPCR analysis

Finally, since this metagenomics approach mainly gives relative results of bacterial proportions with the total microbiome, the last part of this thesis was dedicated at verifying the bacterial loads between different groups using qPCR for the 16S rRNA gene, while planning the quantification of specific genera of bacteria (future work in ongoing projects).

The obtained 16S quantification (Figure 8) was performed not only for the 3 individuals per group that were used in metagenomics but also for additional individuals with good quality DNA making n=5-7 individuals per group. Results revealed a bigger bacterial load in the Control 11 days when compared to the rest of the samples, thus confirming that the application of the different pressures causes an impact in the microbial load of the different samples and avoids the extensive bacterial contamination achieved in the control untreated fillets after 11 days of refrigerated storage.

There are not big differences in the results of fillets processed with pressures 450 and 600MPa, which after 11 days of refrigerated storage had values very similar of 16S quantification to the control 1 day, suggesting a significant reduction in bacterial load. Probably this suggest any of these pressures would be of potential practical application to protect fish fillets from degradation during storage, maybe preferably the 450MPa because it would cause less damage to the fillet. 300MPa was the applied pressure that after 11 days of storage led to the most different results, especially the 300MPa.2mins.11 days. When talking about 16S quantification (Figure 8) it was the only HP-processed group that did not achieve a significant reduction in bacterial detection compared to the control fillets at same storage time. When talking about the relative abundance, a major increase in *Carnobacterium* proportions, the most in any group, really differentiate this group from the other groups.

In general the reduction in bacterial 16S quantification of this study for the 450 and 600 MPa groups is in general agreement with the bacterial counts estimation (not shown) conducted by our colleges in Greece, and also to the previous study (Tsironi et al. 2019) where the application of HP (600 MPa 5 min) also significantly reduced the bacterial load of sea bass fillets sampled at 11 days of refrigerated storage compared to the respective control fillets with significant deterioration at day 11. Under development

and optimization are specific primers for the most abundant chosen microorganisms (*Carnobacterium, Massilia, Shewanella* and *Janthinobacterium)* to be used for validation or for possible markers of quality or safety of the sea bass fillets.

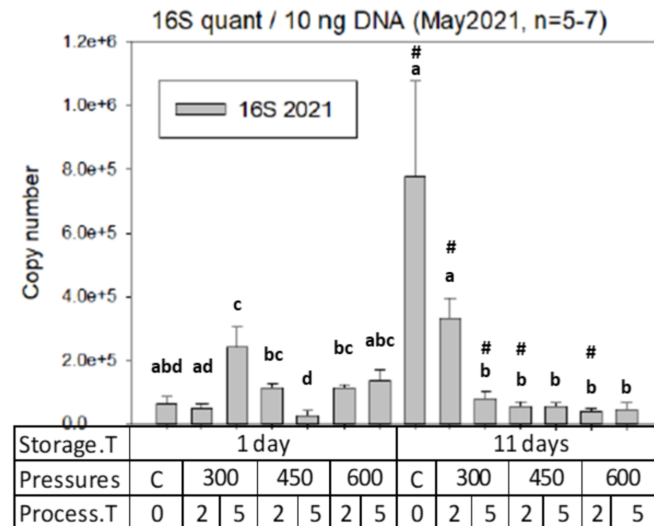

Figure 8 – Results from the quantification of 16S rRNA gene by quantitative PCR, to represent total bacterial loads in all samples. Experimental groups are indicated in the lower panel, according to the detailed experimental design in Figure. Letters and symbols represent statistically differences between groups in each storage times (letters) or between storage times for the same experimental group (#), evaluated by PERMANOVA as indicated in the methods ($p < 0.05$). Detailed average values obtained for each group were as follows: Control.1day = 6,34E+04; 300.2min.1day = 4,87E+04; 300.5min.1day = 2,42E+05; 450.2min.1day = 1,14E+05; 450.5min.1day = 2,70E+04; 600.2min.1day = 1,13E+05; 600.5min.1day = 1,36E+05; Control.11days = 7,77E+05; 300.2min.11days = 3,33E+05; 300.5min.11days = 8,10E+04; 450.2min.11days = 5,56E+04; 450.5min.11days = 5,65E+04; 600.2min.11days = 3,76E+04; 600.5min.11days = 4,60E+04.

We had good results in the 16S RNA gene electrophoresis gel at 2% (Figure 9) although in some cases there were present 2 bands, maybe it is because in the groups with lower bacterial load there was a cross reaction with the DNA from the host.

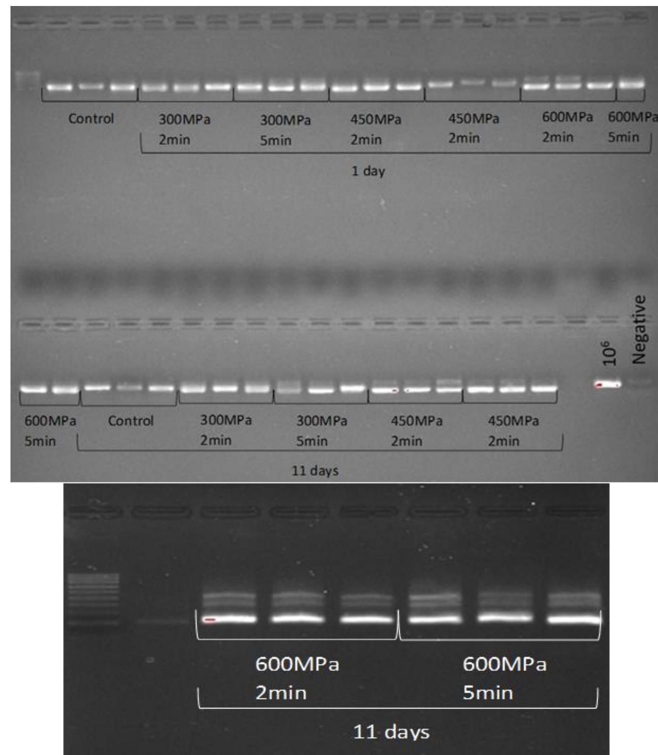

Figure 9 - Electrophoresis gel from the quantitative PCR of the 16S rRNA gene, representing all bacteria, that was run for the 42 samples selected for microbiome analysis (3 individuals for each group. Experimental groups are indicated in the gel, according to the detailed experimental design in Figure 1, including control or high pressure processed fillets at different pressures (300, 450 or 600 MPa) and processing times (2 or 5 min), stored at either 1 or 11 days.

5. Conclusion

Like in the previously published study, HPP in the present study reduced bacterial quantity while increasing the shelf life of the processed sea bass fillets (not shown), which can subsequently improve its quality and stability in refrigerated storage (Tsironi et al. 2019). This processing method and the subsequent metagenomics analyses may allow optimization of the processing conditions, and the potential to identify new monitoring tools for fish quality and safety. There were no major differences in microbiome profiles when the samples were treated for 2 or 5 minutes, this could mean that maybe a treatment time of 2 minutes could be enough for good results, since the HPP treatments should be kept short for financial feasibility reasons and to minimize the impacts on the product quality (e.g., texture or colour, previously shown to be slightly altered). Both the 450MPa

and 600MPa treatments gave good results (significant reduction of bacteria quantities after 11 days of refrigerated storage compared to the deteriorated controls and the global microbiome composition was not very different from the initial control fillets – Figs. 7-9. Maybe the 450MPa should be the optimal pressure to apply in these products since it exerts less pressure and is therefore likely to provoke less damage to the physical structure, and less difference in the taste of the sea bass fillet. This was investigated in the previous study (Tsironi et al. 2019), although only for 600 MPa, but is still under characterization in this study.

The hypotheses were confirmed that higher pressures (higher then 300MPa) will originate less microorganisms capable of spoiling the fish, with the microbial load values all being under the detection limits (spoilage level log <2 cfu/g) in most HPP groups (450MPa and 600MPa) when compared to the control. The microbiological results were confirmed by quantitative PCR of all bacteria, represented by the amplification of the 16S rRNA gene.

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