Universidade de Aveiro Departamento de Biologia
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ANA CATARINA FERNANDES MOREIRINHA

DESENVOLVIMENTO DE ESPECTROSCOPIA DE INFRAVERMELHO PARA AVALIAR A QUALIDADE BACTERIANA EM ALIMENTOS

DEVELOPMENT OF INFRARED SPECTROSCOPY FOR ASSESSING BACTERIAL QUALITY IN FOODS

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia, realizada sob a orientação científica da Doutora Ivonne Delgadillo, Professora Associada com Agregação do Departamento de Química da Universidade de Aveiro, da Doutora Adelaide Almeida, Professora Auxiliar do Departamento de Biologia da Universidade de Aveiro e do Doutor Jorge Saraiva, Investigador Auxiliar do Departamento de Química da Universidade de Aveiro

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o júri

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Doutora Maria Adelaide de Pinho Almeida
Professora Auxiliar, Departamento de Biologia, Universidade de Aveiro

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palavras-chave

Microbiologia alimentar, espectroscopia de Infravermelho, processamento por alta pressão, análise multivariada, bactérias alimentares, deterioração de alimentos, doenças transmitidas por alimentos.

resumo

A deteção rápida e específica de bactérias que podem provocar deterioração de alimentos ou doenças associadas ao seu consumo é cada vez mais importante na indústria alimentar. A deteção, identificação e classificação de bactérias são geralmente realizadas utilizando métodos tradicionais baseados em testes bioquímicos e/ou serológicos e em métodos moleculares baseados em análise de DNA ou RNA. Contudo, estas metodologias são dispendiosas, demoradas e trabalhosas. A espectroscopia de infravermelho é uma técnica confiável, rápida e económica, que pode ser explorada como ferramenta para a indústria alimentar. Nesta tese foi avaliado o potencial da espectroscopia de infravermelho para estudar a qualidade bacteriana de alimentos.

No capítulo 2, foi desenvolvido um modelo de calibração que permitiu prever com sucesso a concentração bacteriana de fiambre naturalmente contaminado, mantido em refrigeração durante 8 dias. Nesta parte, foi desenvolvida a metodologia que permitiu obter a melhor reprodutibilidade dos espectros das colónias de bactérias, com preparação mínima das amostras, que foi utilizada no trabalho subsequente. Foram realizadas várias tentativas para a obtenção de espectros de infravermelho, testando diferentes resoluções e número de scans. Os melhores resultados foram obtidos utilizando uma resolução espectral de 4 cm^{-1} e 32 varrimentos.

De seguida, no capítulo 3, foi feita uma tentativa de identificar 22 bactérias provenientes de alimentos usando a espectroscopia de infravermelho associada a análise multivariada. A análise de componentes principais, utilizada como método exploratório, permitiu a formação de grupos distintos, cada um correspondendo a um género diferente, na grande maioria dos casos. Posteriormente, foi realizada uma análise hierárquica por clusters de forma a investigar a formação de grupos e a possibilidade de distinção de espécies dentro de um mesmo género de bactérias. Observou-se que a espectroscopia de infravermelho é adequada não só para a distinção de diferentes géneros, mas também para diferenciar espécies dentro de um mesmo género, com o uso simultâneo de análise de componentes principais e análise hierárquica por clusters.

A utilização de espectroscopia de infravermelho e análise estatística multivariada foram também investigadas no capítulo 4 para confirmação da presença de *Listeria monocytogenes* e *Salmonella* spp., isoladas a partir de alimentos contaminados, após crescimento em meio selectivo. Isto permitiria a substituição dos métodos bioquímicos e serológicos que são usados para confirmar a presença destas bactérias patogénicas e que podem atrasar a obtenção de resultados por 2 dias. Os resultados obtidos permitiram a distinção de *Salmonella* spp. de outras bactérias que se possam confundir com elas.

resumo

Por fim, no capítulo 5, o processamento por alta pressão, uma metodologia emergente que permite produzir alimentos microbiologicamente seguros e aumentar o seu tempo de prateleira, foi aplicada a 12 bactérias alimentares, de forma a determinar a sua resistência e os efeitos da pressão a nível das células. Foi aplicado um tratamento de 300 MPa, à temperatura ambiente e durante 15 minutos. As bactérias de Gram-negativo foram inativadas até níveis não detetáveis, enquanto as de Gram-positivo mostraram diferentes níveis de resistência. As espécies *Bacillus cereus* e *Staphyloccus aureus* decresceram apenas 2 unidades logarítmicas enquanto a espécie *Listeria innocua* diminuiu cerca de 5 unidades logarítmicas. A espectroscopia de infravermelho foi utilizada na análise das colónias bacterianas antes e após o tratamento por alta pressão, de forma a investigar as alterações que são provocadas nos componentes celulares com este tipo de processamento. Descobriu-se que a alta pressão altera bandas espectrais correspondentes a alguns componentes celulares, de entre os quais proteínas, lípidos, oligopolissacarídeos, grupos fosfato da parede celular e ácidos nucleicos, podendo indicar rutura da parede/membrana celular.

Neste trabalho, a quantificação de bactérias e a sua classificação, bem como a análise de modificação nos componentes celulares após processamento por alta pressão foram realizados com sucesso. Assim, a espectroscopia de infravermelho demonstrou ser uma técnica bastante promissora para analisar bactérias provenientes de alimentos de uma forma simples e pouco dispendiosa.

keywords

Food microbiology, infrared spectroscopy, high pressure processing, multivariate analysis, foodborne bacteria, foodborne spoilage, foodborne illness.

abstract

Rapid and specific detection of foodborne bacteria that can cause food spoilage or illness associated to its consumption is an increasingly important task in food industry. Bacterial detection, identification, and classification are generally performed using traditional methods based on biochemical or serological tests and the molecular methods based on DNA or RNA fingerprints. However, these methodologies are expensive, time consuming and laborious. Infrared spectroscopy is a reliable, rapid, and economic technique which could be explored as a tool for bacterial analysis in the food industry.

In this thesis it was evaluated the potential of IR spectroscopy to study the bacterial quality of foods.

In Chapter 2, it was developed a calibration model that successfully allowed to predict the bacterial concentration of naturally contaminated cooked ham samples kept at refrigeration temperature during 8 days. In this part, it was developed the methodology that allowed the best reproducibility of spectra from bacteria colonies with minimal sample preparation, which was used in the subsequent work. Several attempts trying different resolutions and number of scans in the IR were made. A spectral resolution of 4 cm^{-1}, with 32 scans were the settings that allowed the best results.

Subsequently, in Chapter 3, it was made an attempt to identify 22 different foodborne bacterial genera/species using IR spectroscopy coupled with multivariate analysis. The principal component analysis, used as an exploratory technique, allowed to form distinct groups, each one corresponding to a different genus, in most of the cases. Then, a hierarchical cluster analysis was performed to further analyse the group formation and the possibility of distinction between species of the same bacterial genus. It was observed that IR spectroscopy not only is suitable to the distinction of the different genera, but also to differentiate species of the same genus, with the simultaneous use of principal component analysis and cluster analysis techniques.

The utilization of IR spectroscopy and multivariate statistical analysis were also investigated in Chapter 4, in order to confirm the presence of *Listeria monocytogenes* and *Salmonella* spp. isolated from contaminated foods, after growth in selective medium. This would allow to substitute the traditional biochemical and serological methods that are used to confirm these pathogens and that delay the obtainment of the results up to 2 days. The obtained results allowed the distinction of 3 different *Listeria* species and the distinction of *Salmonella* spp. from other bacteria that can be mistaken with them.

abstract

Finally, in chapter 5, high pressure processing, an emerging methodology that permits to produce microbiologically safe foods and extend their shelf-life, was applied to 12 foodborne bacteria to determine their resistance and the effects of pressure in cells. A treatment of 300 MPa, during 15 minutes at room temperature was applied. Gram-negative bacteria were inactivated to undetectable levels and Gram-positive showed different resistances. *Bacillus cereus* and *Staphylococcus aureus* decreased only 2 logs and *Listeria innocua* decreased about 5 logs. IR spectroscopy was performed in bacterial colonies before and after HPP in order to investigate the alterations of the cellular compounds. It was found that high pressure alters bands assigned to some cellular components as proteins, lipids, oligopolysaccharides, phosphate groups from the cell wall and nucleic acids, suggesting disruption of the cell envelopes.

In this work, bacterial quantification and classification, as well as assessment of cellular compounds modification with high pressure processing were successfully performed. Taking this into account, it was showed that IR spectroscopy is a very promising technique to analyse bacteria in a simple and inexpensive manner.

Publicações

Publicações em revistas científicas internacionais

Catarina Moreirinha, Alexandra Nunes, António Barros, Adelaide Almeida, Ivonne Deldagillo. (2014). *Evaluation of the potential of Mid-Infrared spectroscopy to assess the microbiological quality of ham*. Journal of Food Safety. Accepted for publication in 13-10-2014.

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INDEX

List of figures

List of tables

Abbreviations

API	Analytical profile index
ATR	Attenuated total reflection
BGA	Brilliant green agar
BHI	Brain-heart infusion
BPA	Baird-Parker agar
BPW	Buffered peptone water
BSA	Bovine serum albumin
CFU	Colony forming units
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamine tetraacetic acid
FT	Fourier transform
FTIR	Fourier transform infrared spectroscopy
IR	Infrared
HATR-MID-IR	Horizontal attenuated total reflection mid-infrared
HCA	Hierarchical cluster analysis
HHP	High hydrostatic pressure
HPP	High pressure processing
ISO	International organization for standardization
LPS	Lipopolysaccharides
MIR	Mid infrared
MLVA	Multiple locus variable-number tandem repeat analysis
MSA	Mannitol salt agar
OCLA	Oxoid chromogenic *Listeria* agar
PC	Principal component
PCA	Plate count agar

PCA (statistics)	Principal component analysis
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
PLS	Partial least squares
RMSEC	Root mean square error in cross validation
RNA	Ribonucleic acid
RVS	Rappaport-Vassiliadis with soya
SD	Standard deviation
SNV	Standard normal deviate
TCBS	Thiosulfate citrate bile salts sucrose
VRBD	Violet red bile dextrose
TCBS	Thiosulfate citrate bile salts sucrose
TSA	Trypticase soy agar
TTmk	Muller-Kauffman tetrathionate novobicin
XLD	Xylose lysine deoxycholate

Thesis outline

The present thesis documents the research work carried out in the scope of the development of mid-infrared spectroscopy (MIR) applications to analyse foodborne microorganisms after growth in solid media.

Nowadays, the accurate and reliable detection, identification and quantification of microorganisms in food are critical to public safety, since it is essential to avoid outbreaks caused by foodborne bacteria. Consequently, it is of extreme importance to develop rapid and inexpensive methods for the analysis of food microorganisms. Mid-infrared spectroscopy, coupled to multivariate analysis, has potential to be used as a first-screening approach and to assess the microbial concentration and classification, avoiding the traditional plating and identification methods that are time consuming, laborious and expensive.

This document is divided in six chapters.

The first chapter comprises the general introduction and objectives. In this chapter, a literature review is presented in order to provide an insight for the work carried out, and the objectives are specified.

In chapter 2, the potential of MIR spectroscopy is evaluated in order to assess the microbial quality of cooked ham. A partial least squares regression is performed to determine the microbial loads of naturally contaminated ham samples in a few minutes, using mid-infrared spectroscopy. In this chapter, it was developed the method to directly analyse the colonies, after growth in solid medium, with MIR spectroscopy, that was applied in the subsequent work.

In chapter 3 are presented the results on the attempt to identify foodborne bacteria with mid-infrared spectroscopy, using multivariate statistics. For this, bacteria isolated from fish, meat and cooked ham, as a case study, as well as other foodborne important bacteria previously isolated or obtained from culture collections, were used. Principal component analysis was performed to allow the identification of spectral groupings of the bacteria and then hierarchical cluster analysis was applied, in order to evaluate the possibility to quickly identify the studied bacteria.

In chapter 4, an alternative method for the identification of *Salmonella* spp. and *Listeria* spp. is presented, using infrared spectroscopy coupled with multivariate analysis. These are two of the most dangerous foodborne pathogens and their identification with traditional methodologies can take up to 7 days, of which 2 of them are needed for the biochemical confirmation. It was intended to assess if this final confirmation step can be

substituted with infrared spectroscopy, as long as the pathogens are plated in their specific obligatory media used in food analysis.

In chapter 5, a novel and emerging food processing technology, high pressure processing, is used in some foodborne microorganisms, in order to assess the decrease of microbial loads and understand cellular modifications triggered by pressure. For this, cells grown in agar medium were submitted to a 300 MPa pressurization for 15 minutes at room temperature. Mid-infrared spectroscopy was performed on the pressurized whole cells, for the first time, in order to quickly determine at which extent the pressure processing modified the different bacteria cellular components.

Chapter 6 presents the main conclusions of this work, as well as ideas to pursue the line of research dealt with in this thesis.

Chapter 7 presents the bibliographic references used in this thesis.

Chapter 1.

General introduction and objectives

1.1. Food spoilage, safety and shelf-life

The three main categories of food spoilage that can occur are physical, chemical and microbial spoilage. Physical damage can be caused by poor handling. Physical changes caused by moisture migration are dependant of the temperature, which is one of the more important factors that influence the rate of spoilage. Microbial growth rates, oxidation of lipids and pigments, browning reactions and vitamin losses are directly controlled by temperature. Chemical reactions that involve fats, proteins, carbohydrates and micronutrients can produce changes to the colour, flavour or texture of foods that consumers can find unacceptable [1], [2].

The microbiological quality of food became a very important factor for food industries as well as for the regulation agencies, both in terms of food spoilage and microbial safety. Food products deteriorate over time and although it cannot be totally prevented, one aim of food processing is to slow the deterioration rate by selecting appropriate processing technology, ingredient formulation, storage conditions and packaging [3].

The shelf-life of food products refers to the storage time until spoilage by microorganisms reaches a pre-determined threshold or when deterioration of one or more nutrients means that a food no longer has its declared nutritional value [2]. Microbial spoilage may be defined by a maximum acceptable microbial level or an unacceptable appearance or off-odour/off-flavour and depends on the types and number of microorganisms that are present [4].

The main factors that control the microbial type, growth and activity are the availability of nutrients in the food (e.g. carbon and nitrogen sources), pH, water activity (a_w), redox potential, presence of chemical preservatives, storage conditions (temperature, light exposure, oxygen), stage of growth of microorganisms and presence of other competitor microorganisms [5].

1.1.1. Microbial spoilage of foods

For many foods, microorganisms are the most important and rapid causes of spoilage. It is not always the quantity of microorganisms that indicates the extent of spoilage, but their activity.

Highly active microorganisms may affect the quality of the foods by one of the following mechanisms [2], [3], [6]:

- Production of extracellular hydrolytic enzymes (cellulolytic enzymes, pectinases, proteases, carbohydrases) that alter the structure of the foods resulting in softening or liquefaction;
- Production of enzymes that break down macromolecules to release for example organic acids or hydrogen sulphide. Lipases break down fats to fatty acids and volatile compounds, leading to the production of off-odours in foods, whereas non-volatile compounds may produce changes in the flavour;
- Gas production that may cause the product to swell or split or inflate the package;
- Acid production altering the colour of the food natural pigments or change the taste;
- Production of pigments altering the food colour;
- Production of polysaccharides that cause sliminess in food.

When substantial microbial growth takes place, colonies on the food can be visible. The microbial spoilage of food products includes moulds, yeasts and bacteria. There are some species of moulds frequently associated to food spoilage, such as *Penicillium, Fusarium, Rhizopus* and *Aspergillus.* The most important aspect of fungi spoilage of food is, however, the formation of mycotoxins. Nowadays, there are more than 400 known mycotoxins, being the aflatoxins the best known. These toxins can be very toxic and cause serious illnesses or even death [7]–[9]. Yeasts are used in fermentation procedures, but some species can cause food spoilage as well, being common in products with high sugar content and acidity, which restrict the growth of competing bacteria. Enteric viruses, such as Enterovirus, Hepatitis A viruses, Norovirus and Rotavirus, are also frequently transmitted by food [10].

Concerning bacteria, there are a lot of species that can cause spoilage in many different foods or cause illness associated to food consumption. Most food spoilage bacteria can grow below water activity of 0.91, being halophilic bacteria capable to grow at water activity of 0.75. The optimum pH range for the growth of most bacterial species is between 6 and 7. However, lactic acid bacteria have an optimum growth at pH 5.5-5.7 and some species can grow in foods at pH 4 or lower. The oxidation-reduction potential at which microorganisms grow determine if they are aerobic (positive oxidation-reduction values) or anaerobic (negative oxidation-reduction values). Facultative aerobes can grow at both situations [7].

Depending on the storage temperature of the food products, bacteria can grow rapidly, slowly, stop growing or even die. Mesophilic bacteria can grow between approximately 10 and 45°C and grow best between 30 and 40°C. Psychotrophic bacteria grow best between 20 and 30°C but can grow at below 7°C. Thermophiles grow at higher

temperatures and grow best between 55 and 70°C, although they can also grow at lower temperatures [1], [2], [7].

At the point of sensory rejection (spoilage), the microflora consists of microorganisms that have contributed to the spoilage and microorganisms that have grown but not caused unpleasant changes, being called specific spoilage organisms of the product. Every food product harbours its own specific and characteristic microflora at any given point in time during production and storage. This microflora is a function of raw material flora, processing, preservation and storage conditions. Regardless of the variability in all of these three parameters, some very clear patterns occur, and based on knowledge of chemical and physical parameters it is possible to predict which microorganisms will grow and dominate in a particular food product [11].

Bacterial spoilage of meat

For centuries, meat and its derived products have constituted some of the most important foods consumed around the world. Animal meats are considered to be significant reservoirs of pathogenic microorganisms. Human pathogens from animal sources continuously enter the food supply chain, where cross-contaminations during processing can occur, leading to a high rate of contamination of retail meat and meat products [12].

Meat is a highly perishable food product which, unless properly stored, packaged and distributed, spoils rapidly and becomes hazardous due to microbial growth. The combination of intrinsic and extrinsic factors determines the microbial spoilage of the meat. The intrinsic nature of most raw meats, namely their high water activity (>0.98), moderate pH (5.5-6.5), readily available sources of energy, carbon and other nutrients makes them an ideal medium to the growth of microorganisms. The primary and most important extrinsic factor that can potentiate the growth of spoilage bacteria is the temperature, being this a critical control point [13]. Meat surface is suitable for the growth of psychotrophic aerobes and Gram-negative bacteria grow easily and dominate the spoilage microflora. The main genera of spoilage bacteria found in raw meat are *Pseudomonas, Acinetobacter* and *Psychrobacter.* Other organisms such Enterobacteriaceae or lactic acid bacteria are a minor component of the spoilage microflora [5].

The initial bacterial count on meat is about 10^2-10^3 CFU/g. Ninety per cent of the initial bacteria are not able to grow at refrigeration temperatures. The heating of the meat products to temperatures above 65°C kills most of the vegetative cells. Post-heat conditions determine the shelf-life of the food products. During storage, temperature, pH, NaCl and gaseous

atmosphere select some bacteria, affecting their growth rate and activity. The maximum level of bacteria reached during refrigerated storage of meat is 10^7-10^9 colony forming units (CFU)/g and 10^7-10^8 for meat products [14].

Meat processing, in order to enhance the products quality and microbial safety, is often performed. An example of processed meat is cooked ham. Cooked ham processing involves the use of a salt brine that is either injected or infused by soaking, followed by a heat treatment, with the objective to obtain a product with high sensorial quality and microbiologically safe. The final percentage of salt content in ham after this processing is usually about 2%. Nitrite is also added (120-150 mg per kg) in order to enhance ham colour and antioxidant activity and also to preserve from pathogens [15]. However, this product is very sensitive to recontamination, mainly by lactic acid bacteria, during post-treatment handling such as slicing [16].

Bacterial spoilage of fish

The demand for fish products has been increasing in the last decades, mainly because of its role in human health. Fish is known to be a valuable food, low in saturated fatty acids, good source of protein and selenium and a good source of long-chain omega-3 fatty acids [17].

The high levels of moisture, nutrients, other nitrogenous compounds, digestible proteins and the high pH attained after *post-mortem* makes fish an easily perishable food. As fish carries a variety of microorganisms from both aquatic and terrestrial sources, microbial spoilage can occur in a short period of time even under refrigeration. In addition, seafood may contain various potential public health hazard pathogens. As a rule, fish spoil more rapidly than meat under similar chill conditions. The pH of *post-rigor* meat muscle is lower than that of fish and this contributes to the longer storage life of meat. The pH-sensitive genus *Shewanella* plays a significant role in fish spoilage but has not been reported in meat [5].

In general, microflora of fish species is usually dominated by psychotrophic Gram-negative genera like *Pseudomonas, Shewanella, Acinetobacter, Aeromonas, Photobacterium* and *Vibrio.* Gram-positive organisms such as *Micrococcus, Bacillus, Staphylococcus* are also commonly found in fish samples [18], [19]. In Europe, hake and cod are two of the more consumed fishes. Hake is usually sold on fresh ice and cod is normally salted and dried, having the necessity to be desalted before consumption. Drying and salting are long processes, highly manipulative and with high potential for environmental transfer of microorganisms to the product. Growth of the pathogenic and spoilage bacteria is repressed in low water activity environments [20]. However, some bacterial genera like *Vibrio, Listeria* and *Staphylococcus* can

survive on high salt concentration substrates (above 10% NaCl) [21]. During desalting, these bacteria could grow, causing food spoilage and threatening the consumers health, particularly because cod can be eaten raw or after a quick/soft cooking. European commission [22] established the regulation for microbiological criteria of fish products, as well as the permissible levels for several bacteria in this food product. The most common pathogenic bacteria that can cause infections and have been associated to fish and meat consumption are summarized in the next section.

1.1.2. Foodborne diseases caused by bacteria

Foodborne disease outbreaks have devastating health and economic impacts all over the world. Illness associated to food consumption is one of the most widespread health problem in the contemporary world and it has been defined by the World Health Organization as "Any disease of an infectious or toxic nature caused by, or thought to be caused by, the consumption of food or water" [5]. Foodborne diseases arise mainly from harmful chemicals or from microorganisms. They can range from relatively mild, self-limiting gastrointestinal upsets through life-threatening conditions such as botulism. Within microorganisms, there are numerous bacteria that have been associated to foodborne illness. In this section it is performed a brief description of some of the most important bacterial pathogens that can be present on fish and meat/meat products.

***Aeromonas* spp.**

Aeromonas species are Gram-negative and non-spore-forming rods of the family Aeromonadaceae. They grow optimally at 28°C but they also have the ability to grow at cold temperatures, reported as low as -0.1°C for some strains [23]. This pathogen has been found in water and in a wide range of foods, including vegetables, meat, fish and seafood [24].

Some species of *Aeromonas* can cause gastrointestinal infections. *A. hydrophila* is the most widespread representative species on the subject of foodborne illness. It causes gastroenteritis and diarrhoea, being also associated to other diseases including inflammation of the gallbladder, septicaemia and meningitis that can cause death in more than 60% of the cases [25]. The incubation time of this genus is 1-2 days and the symptoms can last from 1 to 10 days, in most of the cases [24]. The main *Aeromonas* spp. virulence factors that can be associated with foodborne diseases are the secretion of exotoxins, the endotoxin or lipopolysaccharide (LPS), the presence of S-layers, the adhesins and the production of capsules in glucose-rich medium [26].

Bacillus cereus

Bacillus cereus is a rod-shaped Gram-positive and spore-forming food pathogen of the Bacillaceae family. Its optimum growth temperature is 30°C but it can grow between 10°C and 50°C as well [27]. This species is a normal soil inhabitant and can be mainly isolated from meat, cereals, vegetables and dairy products [27].

Bacillus cereus must be present at high levels (10^5 -10^8) in food to produce sufficient toxins and lead to food poisoning. It can cause two types of food poisoning: nausea and vomiting caused by an emetic toxin produced in foods and diarrhoea and abdominal pain without vomiting caused by diarrhoeagenic toxins produced in the small intestine. The emetic type is most frequently associated with rice products, where spores survive heating and germinate after cooling product, and the diarrhoeal type of toxin is found in meat, milk and vegetables, where cells survive heat processing, multiply before consumption and produce toxins in the intestine. Usually, the illness lasts 24 hours. The incubation period of the emetic type is very short, from 30 minutes to 6 hours, and the diarrhoeal type incubation time is between 6 and 15 hours. Other *Bacillus* species also can cause food poisoning, such as *B. subtilis* and *B. thuringiensis* [28].

***Brucella* spp.**

Brucella is a genus of Gram-negative small non-spore-forming coccobacillus of the Brucellaceae family. The optimal temperature growth of this bacterium is at 37°C but it also grows between 10 and 40°C.

Brucellosis is primarily a disease of animals (zoonosis) but it was firstly recognized as a disease that manifested in humans, which is caused by three strains: *B. abortis* (from cows), *B. melitensis* (from goats and sheeps) and *B. suis* (from pigs). The disease may be mainly contracted either by consumption of milk or unpasteurized dairy products, however, *Brucella* can also be found in undercooked meat samples [29], [30].

The acute symptoms of brucellosis include weakness, fatigue, muscle and joint pain and weight loss. It may cause chronic health problems as joint inflammation, cardiovascular and neurological complications, insomnia and depression [3], [25], [31]. Following exposure, signs of illness usually appear within 3 weeks. With appropriate antibacterial treatment, it is possible to get resolution of disease in only a few weeks; however, even with treatment, symptoms may reappear and last for months or even years [32].

Brucella spp. are facultative intracellular pathogens that are able to evade the destruction by phagocytes in the host organisms. These mechanisms are not fully understood

but it has been proposed that lipopolysaccharides act as virulence factors and are important for *Brucella* replication and survival. VirB proteins that form the type IV secretion system and that are involved in intracellular replication are also considered as one of *Brucella* virulence factors. Moreover, some virulent strains contain a Cu-Zn superoxide dismutase enzyme that inhibits oxygen radicals [31], [33].

***Campylobacter* spp.**

Campylobacter is a genus of Gram-negative motile and non-spore-forming bacteria with a spiral appearance that belongs to the Camplylobacteriaceae family. These species are thermophilic (optimum growth at 37-40°C) but can survive at lower temperatures. This bacteria is sensitive to freezing, heating, drying and acidity. As this genus can be found in the intestines of many wild and domestic animals, the principal vehicles of infection are undercooked meats and contaminated water [5].

Gastrointestinal illness due to *Campylobacter* spp. is one of the most commonly reported bacterial cause of infectious intestinal disease worldwide. The most important species, which causes up to 90% of the infections caused by this genus, is *C. jejuni*. The incubation period goes from 2 to 11 days and the symptoms, in spite of lasting long (up to three weeks), are generally mild, and the patients do not require treatment except rehydration. Even though cells do not survive in foods for long periods, they are highly virulent. [3], [31]. The infection dose is low, probably much lower than the 500 CFU quoted most frequently [29]. The mechanisms of pathogenesis are unclear, but it is suggested that LPS is a virulence factor. In motile strains, flagella are also important in virulence, as non-motile strains are not able to colonize the intestinal tract. Also, the presence of plasmids play an important role in the pathogenesis [31], [34].

Clostridium botulinum* and *Clostridium perfringens

Clostridium botulinum is a Gram-positive, rod-shaped, spore-forming bacterium of the Clostridiaceae family. It is strictly anaerobic and can sporulate under adverse conditions. It includes organisms that differ in physiological properties or genetic relatedness. The optimum growth temperatures can range between 20-40°C depending on the group, but some strains can grow at temperatures as low as 3°C. Some groups have the ability to produce the most potent toxin known, the botulinum neurotoxin, with a lethal dose of less than 1 µg in humans [35]. Symptoms of botulism appear within 12-36 hours after contaminated food ingestion. They include vomiting, nausea, double vision and difficulty in swallowing, following by muscle

weakness and respiratory failure. An antitoxin has been developed and it reduces the mortality rate if it is quickly administrated, but patients may still need artificial respiration to enable recovery. Because of the severity of the intoxication, canning industry takes particular care to ensure the elimination of this pathogen. However there are cases arising from home vegetable canning, under-cooked fish or inclusion of herbs and spices in cooking oils [36].

Clostridium perfringens is, as *C. botulinum*, an anaerobic Gram-positive, rod-shaped, spore forming bacterium. This species doubles in number in about 8 minutes under optimal conditions (43-45°C), being one of fastest growing food poisoning bacteria. It can be found in cooked meats that were inappropriately cooked or refrigerated. Some isolates produce toxins that cause mild symptoms that last for about 24 hours, after an incubation period of 8-24 hours [3].

Enteropathogenic *Escherichia coli*

E. coli is a Gram-negative rod-shape and non-spore-forming bacterium from the Enterobacteriaceae family. It grows both aerobically or anaerobically with an optimum growth at 37°C but it can survive at refrigeration temperatures. *E. coli* is one of the most common bacteria in the human intestine and it is used as an indicator of faecal contamination. Regarding pathogenic strains, enteropathogenic *E. coli* is the most common and can form distinctive lesions on the surfaces of intestinal epithelial cells. Illness caused by *E. coli* is associated to the consumption of a wide variety of foods including water, vegetables, fish, and meat. The symptoms appear between 5 and 48 hours after food consumption. Some strains can cause illnesses and recently food poisoning outbreaks were associated with Vero cytotoxin-producing enterohemorragic *E. coli* O157:H7 that is responsible for significant morbidity and even mortality worldwide. In this case, symptoms appear between 10 and 24 hours after food ingestion. In industrialized countries the focus has been on the *E. coli* O157:H7, which is a threat to the public health [37]. Illness caused by *E. coli* is associated to the consumption of a wide variety of foods including water, vegetables, fish, and meat [29].

Listeria monocytogenes

Listeria species include Gram-positive and non-spore-forming bacteria belonging to the Listeriaceae family that can grow over a wide temperature range, including refrigeration temperature, and their optimal temperature is between 30 and 37°C. It is non-motile at 37°C, but forms flagella at 20-25°C. *L. monocytogenes* is the most important pathogen among the 10 *Listeria* species known. *L. monocytogenes* can infect a wide variety of foods, mainly uncooked meats and vegetables, unpasteurized milk, cheese, seafood, processed and ready to eat meats

like sausages or ham and fish [29]. Although listeriosis is rare and only causes mild flu-like symptoms or vomiting and diarrhoea in healthy adults when a large number of cells are ingested, it can be mortal in people with a compromised immune system and older people. If pregnant women become infected, *L. monocytogenes* can cause infection of the uterus, bloodstream or central nervous system, causing abortion or stillbirth [38]. Concerning the virulence of *L. monocytogenes* as a foodborne pathogen, some virulence factors have been proposed: Its capacity for intracellular growth, iron compounds, catalase and superoxide dismutase, surface components and haemolysin secretion [39], [40].

***Photobacterium* spp.**

Photobacterium species are Gram-negative and non-spore-forming bacteria from the Vibrionaceae family. They are also rod-shaped, motile and usually bioluminescent. Generally, they grow optimally between 18 and 25°C, but some species are able to grow at temperatures as low as 0°C and pressures up to 70 MPa, such as *Photobacterium profundum* [41]–[43].

Photobacterium damselae subsp. *damselae* (formerly *Vibrio damsela*) is associated with marine environments. It is a primary pathogen causing ulcers and haemorrhagic septicaemia in a variety of marine species as sharks, dolphins, and shrimps, as well as wild and cultivated fish [44]. In humans, it can cause opportunistic infections and may even cause fatal infections. Most of the reported infections in humans have their origin in wounds inflicted during the handling of fish, exposure to seawater and marine animals, and ingestion of raw seafood. In some of the human cases, the infection progresses into an extreme variant of a highly severe necrotizing fasciitis that advances following a very aggressive course leading to a fatal outcome [45]–[47]. The virulence factors of this species may include iron uptake systems, cytotoxins with haemolytic activity and other exotoxins and enzymes [47], [48].

Plesiomonas shigelloides

P. shigelloides is a gram-negative rod, non-spore-forming bacterium that belongs to the Enterobacteriaceae family. Its optimum growth temperature is 37°C but it also grows between 8 and 45°C. Cases of infection by this bacterium are more common in warmer climates or in travellers returning from warmer locations. The symptoms include a mild watery diarrhoea but can evolve to a severe colitis or cholera-like syndrome in immunosuppressed individuals [5]. Symptoms appear within 48 hours after infected food consumption and can persist for several days. Current evidence suggests that the exact mechanism of *P. shigelloides* enteropathogenicity is not fully elucidated, and that more than one mechanism may be

required to cause diarrhea in the host. However, this species motility appears to be an important factor [49], [50].

This organism is ubiquitous in surface waters and soil and fish and shellfish are a natural reservoir of the organism. However, it was also been associated to cattle, pork and poultry animals [5].

Salmonella

Salmonella spp. are amongst the most important food pathogens worldwide. They are Gram-negative, non-spore-forming and belong to Enterobacteriaceae family. The optimal growth temperature for *Salmonella* spp. is 37°C but they can grow between 6 and 46°C as well. Typically, salmonellosis symptoms appear in 6-72 hours after the food ingestion. The number of ingested cells needed to cause illness may be as low as 10-100 cells. An individual outbreak can affect several people and symptoms range from gastroenteritis to severe typhoid paratyphoid or septicaemia, causing high rates of morbidity and mortality. Nowadays, there are about 2500 serotypes known, mostly designated as the species *S. enterica* [3], [51]. *Salmonella* spp. have evolved mechanisms that allow their survival at low pH, as the production of acid shock proteins, but the exact mechanism of actuation of these proteins is not very well understood. Intestinal adhesion of cells is mediated by fimbriae or pili that exist on the cell surface. About 95% of cases of human salmonellosis are associated with the consumption of contaminated products such as eggs, meat, milk, seafood, and fresh produce [52].

***Shigella* spp.**

Shigella spp. are Gram-negative non-spore-forming bacteria that belong to the Enterobacteriaceae family. They are heat sensitive, acid resistant and salt tolerant. Their optimum temperature is 37°C but they also grow between 10 and 40°C.

Shigella spp. are waterborne pathogens, but foodborne outbreaks associated with these species are also common, in foods that are subjected to hand processing and then exposed to a limited heat treatment or eaten raw. Examples of foods from which these species were isolated are raw vegetables, fish, beef and raw oysters [53].

The genus *Shigella* comprises four taxonomic groups that are defined by the antigenicity of the somatic O antigens: *Shigella dysenteriae, Shigella flexneri, Shigella boydii* and *Shigella sonnei.* These groups differ in epidemiology, as *S. dysenteriae* is associated to epidemics, *S. flexneri* is related to endemic infection, *S. sonnei* is related to source outbreaks in

developed countries and *S. boydii* is implicated in source outbreaks in areas that have poor hygienic standards [53].

Shigella spp. multiply in the colon, invading epithelial cells and causing ulcerative lesions. The capacity to spread intracellularly and infect adjacent cells is critical in the infection process. The symptoms appear in 12-48 hours after ingestion of contaminated food.

Shigella dysenteriae produces a cytotoxin (Shiga toxin) that damage the colon cells and may also produce a neurotoxin and an enterotoxin, being this species the causative agent of shigellosis and "bacillary dysentery". The typical symptoms of infection include abdominal pain, bloody diarrhoea and fever. It may even cause convulsions and delirium and is a common death cause among immunocompromised people or infants where hygienic standards are poor. The infective dose for this species is very low, requiring just 10 cells of *S. dysenteriae* or 500 of *S. sonnei* to cause symptoms [53], [54].

Staphylococcus aureus

Staphylococcus aureus is a Gram-positive non-spore-forming bacterium that belongs to the Staphylococcaceae family. This species is able to grow in a wide range of temperatures (7-49°C) and its optimum temperature is between 30 and 37°C. This species is part of the normal human and animal flora and can be found on skin and nasal cavities. The contamination of food with this species results from inappropriate storage or cooking, poor hygiene and improper washing of food processing utensils. Foods that are manipulated and do not require additional cooking after it are the most often contaminated items [29]. Foods that have been often implicated in staphylococcal intoxication include meat, meat products, poultry, egg products, milk, dairy products, salads, bakery products, particularly cream-filled pastries and cakes, and sandwich fillings. Salted food products, such as ham, have also been associated to foodborne infection by this species [55]. When growing in food, *S. aureus* can produce more than 20 toxins and the ingestion of 94-184 ng of one toxin can result in intoxication [56]. Symptoms of Staphylococcal food poisoning include nausea, violent vomiting and abdominal pain. *S. aureus* can also produce exotoxins that are different from enterotoxins, as they do not act directly on the intestine, but act more like a neurotoxin, stimulating the vomiting centre in the brain [57], [58]. The toxins act fast and the symptoms appear in 1-6 hours after consumption of contaminated foods. Deaths due to this bacterium are rare and symptoms rarely last more than 24 hours. However, in some instances, the illness is severe enough to require hospitalization. *S. aureus* cells are destroyed by heating conditions used in food processing, but the toxins are not. These species cells do not grow under 7°C and toxins are

not produced below 10°C, thus, refrigeration is the best method of control of products that are not processed by heating [3].

***Vibrio* spp.**

Vibrio is a Gram-negative, non-spore-forming, comma-shaped bacterium of the Vibrionaceae family. The optimum growth temperature of this genus is 37°C but this bacterial species grows between 10 and 43°C as well. It is associated to seawater and seafood and fish are the most common sources of contamination. There are ten *Vibrio* species that can cause gastrointestinal illness but the most important species are *V. cholera, V. parahaemolyticus* and *V. vulnificus.*

V. cholerae may produce enterotoxins that can even cause death due to severe diarrhoea and subsequent dehydration and loss of mineral salts in immunocompromised people. Septicaemia cases are also related to this species. The incubation time of this bacteria is between 12 and 72 hours and healthy people generally recover in 1-6 days.

V. parahaemolyticus causes diarrhoea that lasts 2-3 days, rarely causing death. Infection occurs 4‑96 h after consumption of contaminated food and the illness is self-resolving in immunocompetent individuals and can be sufficiently treated with oral rehydration alone [59], [60]. It possesses a wide variety of virulence factors, including a newly discovered adhesin, toxins, and secreted effectors involved in attachment, cytotoxicity and enterotoxicity [61].

V. vulnificus causes gastroenteritis and is known to be a dangerous species because it also causes septicaemia in immunocompromised individuals, and *V. vulnificus* septicaemia is associated with a greater than 50% mortality [62], [63]. This species can also provoke wound infections and it may cause severe complications without medical treatment. This species virulence factors include secretion of hemolysins, proteinases, collagenases and phospholipases. The symptoms usually appear between 5 hours and 4 days [3], [64].

Yersinia enterocolitica

Yersinia enterocolitica is a Gram-negative, non-spore-forming, coccobacillus-shaped Enterobacteriaceae. It grows optimally between 25 and 37°C but it also grows between -1.3°C and 42°C. As it is non- heat-resistant, it is normally destroyed at processing temperatures. However, recontamination can occur because of its ability to grow at refrigeration temperatures. It is a foodborne pathogen that has been isolated from a wide variety of foods but it is mostly associated with pork consumption [3]. The incubation period for this bacterium is about 7 days and the symptoms disappear in 2-3 days generally, but may last 3 weeks in

some cases. The infection process can be done by two mechanisms. The chromosomal mechanism consists on the secretion of enterotoxins that induce diarrhoea or vomiting. The plasmid mechanism depends on antigen and calcium responses of the host cell membrane that allows the bacteria to bind to the host cell. The bacterial genome contains a gene coding for proteins that signal the invasion of epithelial cells in ileum. This in turn leads to damage of mucosal layers [29], [65]. In addition to gastroenteritis symptoms, *Y. enterocolitica* may cause autoimmune thyroid disease, liver abscesses, pneumonia or even septicaemia [3].

1.2.High pressure processing for food preservation

Food processing corresponds to the transformation of raw animal or plant materials into consumer-ready products, reducing or preventing negative modifications in their quality. There are several food processing methodologies, such as pasteurization, dehydration, smoking, baking, freezing, among others [3].

The sensorial characteristics of a food (texture, aroma, flavour, colour) are the most important attributes to the consumers. Nowadays, the demand for high quality products in terms of natural flavours, taste and nutritional value has triggered the need for the development of nonthermal approaches to food processing. High pressure processing (HPP) allows the production of fresh-like products with minimal degradation of nutritional and organoleptic properties. In this method, food is subjected to elevated pressures to decrease microbial loads or to modify food attributes in order to achieve consumer-desired qualities [66].

The pressure range used in food processing is generally between 100 and 600 MPa. This pressure range appears to have minimal effect on covalent bonds, thus, food subjected to HPP at ambient temperature, do not undergo significant chemical modifications. This means that most of the components that are responsible for the sensory and nutritional quality of foods, such as flavour components and vitamins, are not altered by HPP, being this is an important benefit for the food industry. HPP is applied in an isostatic way, in which the entire product experience an uniform pressure level, regardless of its shape, size or composition [67].

A typical high pressure system consists of a pressure vessel and a pressure generating device. Food packages are placed into the vessel, which is then closed, the air is removed and a pressure medium is pumped into the vessel until the desired pressure is reached, being these conditions maintained during a selected period of time. The pressure medium usually contains water and a small amount of soluble oil in order to transmit pressure without need for energy input. The compression during HPP processing increases the temperature of foods through adiabatic heating and the extent of temperature increase varies with the composition of the food (usually 3-9°C for each 100 MPa). The product volume reduces as well in proportion to the applied pressure, and so, the package used in HPP processing needs to be adaptable to these changes [66].

HPP technology for food preservation has been known for more than a century. The first report, showing that milk and fruits could be preserved by high pressure, dates from 1899 [68], and the first commercial products appeared almost a century later, in 1990 [69]. Since

then, the number of HPP equipments operating in the food industry for commercial applications has been increasing and actually exist more than 250 units in production (Figure 1).

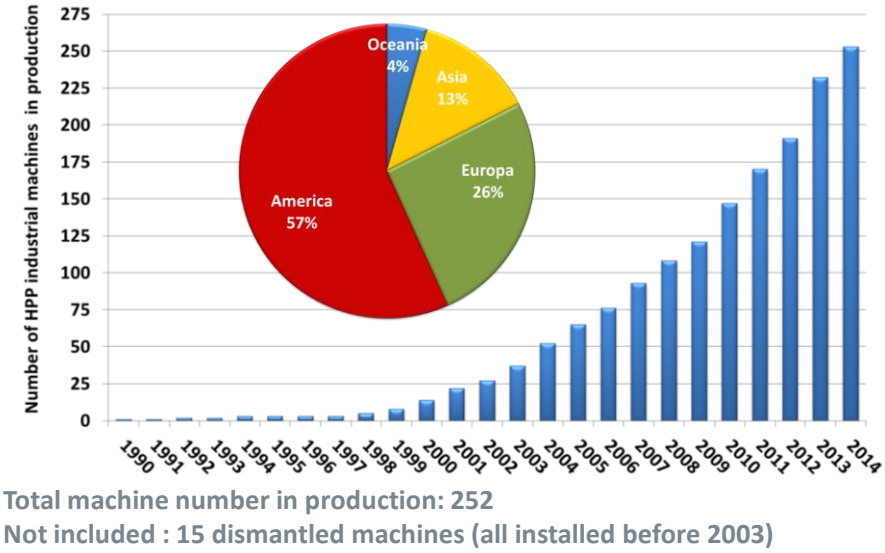

Figure 1: Evolution of total number of HPP industrial equipments in production since 1990.

(Image gently provided by Dr. Jorge Saraiva).

The most common use of HPP is found in food preservation, as it is a non-thermal processing method that can inactivate spoilage-causing organisms and foodborne pathogens. HPP treatment causes specific stress responses in microorganisms attempting to adapt and survive, affecting not only cell structural organization but also its metabolic processes. Different microorganisms react with different degrees of resistance, and high pressure usually has a higher destructive effect in organisms with a greater degree of organization and structural complexity, being prokaryotes usually more resistant than eukaryotes [70]. Viruses possess a wide range of pressure resistance, depending on their structural diversity, and enveloped viruses are usually more sensitive to the pressure than naked viruses. Spores have great HPP resistance and some of them can survive up to 1000 MPa [70], [71]. In the next section it is presented a brief review of the bacteria with great pressure resistance.

1.2.1. Bacteria with great high pressure resistance

Normally, Gram-positive bacteria are most resistant to environmental stresses than Gram-negative bacteria, and this observation applies to pressure resistance as well. Concerning to the pathogenic non-spore forming bacteria, *L. monocytogenes* and *S. aureus* are

two of the most well studied bacteria regarding HPP and the latter appears to have a high resistance to pressure. Regarding Gram-negative pathogenic bacteria, there appears to be a wide range of pressure sensitivity [67]. Some strains of *E. coli* and *Salmonella* spp. have demonstrated relatively high levels of pressure resistance, while other strains are very sensitive [72]. *V. parahaemolyticus* is substantially more sensitive to the effects of high pressure than *L. monocytogenes* and *L. innocua* [73], [74]. In addition, the bacterial endospores produced by some species are extremely resistant to high pressure and other physical treatments such as heat and irradiation, being able to survive at more than 1000 MPa [75]. *C. botulinum* spores are among the most pressure resistant ones [76], [77]. As relatively low pressures (bellow 200 MPa) can activate spore germination [78], it has been suggested to apply pressure in two stages, in which the first stage would induce spores germination and the second one would kill the germinated spores [79].

1.2.2. Inactivation mechanisms

It is known that cell membranes are primary sites of pressure damage in microorganisms [80], [81]. High pressure reduces fluidity on cell membranes due to the increasing packing of the fatty acyl chains of phospholipids. One difference observed in microorganisms adapted to pressure (piezophiles) is the increase of the proportion of fluidizing mono-unsatured and poly-unsatured fatty acids, that cannot be packed as tightly as saturated fatty acids [82]. It is also known that HPP changes the conformation of cellular proteins, as non-covalent “weak” chemical bonds, essential to maintain protein structure and function [83]. DNA changes have also been reported, as with the increasing pressure DNA molecules are stabilized, and the strand separation necessary for cell processes such as replication, translation and transcription may become more difficult [84].

It is known that old bacterial cultures are more resistant to inactivation by other food processing techniques that recent ones, but it seems that this does not significantly affect their inactivation by HPP. For example, it was reported that two *Vibrio* species that entered the dormant state of viable but non culturable cells were just slightly more resistant to pressure than control cells, and this small resistance difference did not affect the HPP inactivation result [85]. Other studies suggested that cells at exponential growth phase are inactivated under high pressure, because this procedure causes irreversible damage to cell membranes. On the other hand, cells at a stationary phase have a more robust cytoplasmatic membrane, thus being more resistant to HPP. It is a fact that cells in exponential growth showed changes in their cell enveloped that was not observed in cells at stationary phase. These alterations included

physical perturbations of the cell envelope and respective loss of osmotic equilibrium and liberation of proteins and RNA to the extracellular medium [86], [87].

Inactivation kinetics with high pressure is very complex. Plotting the log of surviving cells against time may not produce a straight line relationship. Is has been often observed that there is a linear decrease in viable cells at first, but then a decrease in the rate of inactivation leads to a "tail" of pressure resistant cells. It was reported that if this "tail" population is isolated, grown again and then once more exposed to pressure, there is no difference in pressure resistance between this population and the initial one [66]. A study in which the resistance and the recovery of growth after several consecutive cycles of HPP were evaluated in different strains of *S. aureus* shown that after 10 inactivation cycles of pressurization, the surviving bacteria did not developed resistance [88]. This effect is not fully understood, but it seems that inherent phenotypic variations in the pressure resistance of some cells or substrate growth conditions may be the main factors. Because of this, calculation of pressure decimal reduction time can be difficult and should be taken into account when the processing conditions of foods are optimized [81].

1.3. Infrared Spectroscopy

The accurate and reliable detection and identification of microorganisms in food is critical to public safety. It is extremely important to develop rapid and inexpensive methods for the detection of food microorganisms to replace traditional analysis methods that are expensive and time consuming. Currently, mid-infrared spectroscopy (MIR) is a common technique that is a powerful, fast and non-destructive tool for food quality analysis and control. Hence, it is one of the most promising techniques to the food industry and has been successfully used to study microorganisms, since each bacterial species has a complex cell membrane/wall composition which gives an unique MIR 'fingerprint'. Therefore this is an accurate method to assess the overall molecular composition of the microbial cells in a fast and non-destructive manner.

Infrared spectroscopy is an analytical methodology based on the vibrations between atoms in a molecule. An infrared spectrum is acquired by passing infrared radiation through a sample and determining what fraction of the incident radiation is absorbed at a particular energy. The energy at which any peak in an absorption spectrum appears corresponds to the frequency of a vibration of a part of the sample molecule [89].

To show infrared absorption, the electric dipole moment of a bond must change during the vibration, and so, the interaction of infrared radiation with the matter may be understood in terms of changes in molecular dipoles [90]. The larger is the change, the more intense is the absorption band.

Vibrations can involve either a change in bond length (stretching) or angle (bending). Bonds can stretch in-phase (symmetrical stretching) or out-of-phase (asymmetrical stretching) (Figure 2). Bending vibrations also contribute to infrared spectra, and can be of deformation, rocking, wagging and twisting or in-plane and out-of-plane (out-of-plane bending and in-plane bending) (Figure 3). There are many different vibrations even for fairly simple molecules and the complexity of an infrared spectrum arises from the coupling of vibrations over a large part of the complete molecule. Such vibrations are called skeletal vibrations and bands associated to them are likely to conform to a 'fingerprint' of the molecule as a whole, rather than a specific group within the molecule [90].

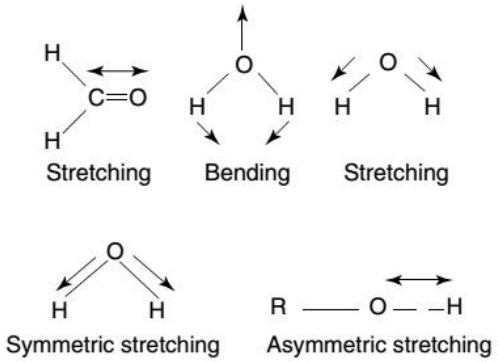

Figure 2: Stretching and bending vibrations and symmetric and asymmetric stretching (Adapted from Stuart, 2004).

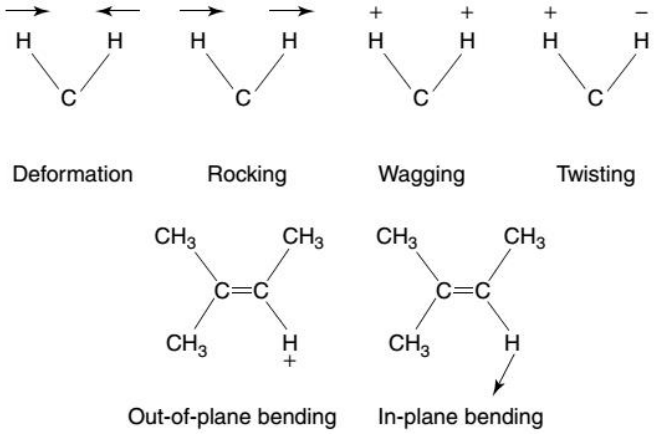

Figure 3: Different types of bending vibrations (Adapted from Stuart, 2004).

The absorbance IR spectrum of a sample is calculated from the following equation:

$$A = \log(\frac{I}{I_0})$$

Where:

A=Absorbance

I=Intensity in the sample spectrum

I_0=Intensity in the background spectrum

Absorbance is related to the concentration of the molecules in a sample via *Beer's* law equation:

$$A = \varepsilon l c$$

Where:

A= absorbance

ε =Absorptivity (L g^{-1} cm^{-1})

l=Pathlenght (cm)

c=concentration (g L^{-1})

1.3.1. Infrared spectroscopy advantages and disadvantages

Infrared spectroscopy advantages and disadvantages are summarized in Table 1.

Table 1: Advantages and disadvantages of infrared spectroscopy (Adapted from Smith, 2011).

Advantages	Disadvantages
Almost universal technique	
Spectra have much information	Cannot detect some molecules
Relatively fast and easy	Mixtures are difficult to analyse
Low-cost technique	Water can mask important peaks
High sensitivity	

IR spectroscopy is almost universal, as many molecules show strong absorbances in the mid-infrared. Thus, many types of samples including solids, liquids, semi-solids, polymers, organics, inorganics and biological materials can be measured with this methodology. IR spectra are also information rich, as the peak positions allow the identification of the structures of the molecules in a sample and the peak intensities can provide information about the concentration of the molecules in a sample. The acquisition of the spectra is relatively fast and easy and, unless the sampling technique or the nature of the sample affect the speed and ease of the analysis, spectra can be obtained in few minutes or even seconds. This methodology is also relatively inexpensive, as it usually does not require reagents to perform the analysis and the equipment is cheap compared to other laboratory instruments (Nuclear Magnetic Resonance spectrometers, Gas Chromatography-mass spectrometers, etc). Other advantage of IR spectroscopy is its high sensitivity, as it is only required a minimum amount of material to perform an analysis.

Regarding the disadvantages, the most important is that there are some materials (constituted by chemical species without vibrations) that do not produce infrared spectra, such as individual atoms not chemically bonded to anything (e.g. helium, argon) and monoatomic ions. Molecules with only two identical atoms (homonuclear) (e.g. O_2, N_2) possess a symmetric stretch vibration with a peak intensity of zero. Other disadvantage is the measurement of mixtures, where complex spectra are obtained, making it difficult the determination of what peaks are from what molecules. Spectral software can be used to facilitate spectra interpretation, by means of spectra calculation, for example, by subtracting a pure substance spectrum from the mixture spectra. The presence of liquid water is another problem, because its broad and intense peaks can "mask" peaks of interest. In this case, spectral subtraction of water spectra will not work, as the solute needs to be present in a concentration greater than 0,1% in order to be detected. A way of dealing with this is to evaporate off the water and analyse the residue [90].

1.3.2. Fourier transform infrared spectrometers

The actual generation IR spectrometer is the Fourier Transform IR that uses an interferometer instead of a monochromator. With this replacement, IR spectrometers become exceptionally powerful [91], [92].

Fourier transform infrared spectroscopy is based on the idea of the interference between two beams to yield an interferogram, which is a signal produced as a function of the change of pathlenght between the two beams. Distance and frequency are interconvertible by the mathematical method of Fourier Transform. The most common interferometer used in IR spectroscopy is the Michelson interferometer, which consists of two perpendicular mirrors, one of which can travel in a direction perpendicular to the plane (Figure 4). A beam splitter bisects the planes of these two mirrors. The two resulting beams are reflected, returning to the beam splitter, where they recombine and interfere. Fifty percent of the reflected beam from the fixed mirror is transmitted through the beam splitter while fifty percent is reflected back in the direction of the source. The beam that emerges from the interferometer at 90° to the input beam is called the transmitted beam and this is the one detected in IR spectroscopy [89].

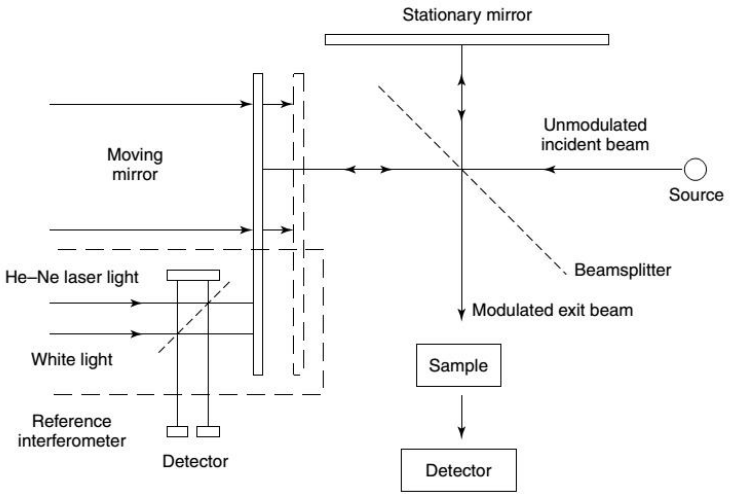

Figure 4: Schematic representation of a Michelson interferometer (Adapted from Stuart, 2004).

1.3.3. Attenuated total reflectance spectroscopy

Attenuated total reflectance (ATR) spectroscopy utilizes the phenomenon of total internal reflection. A radiation beam that enters a crystal will be submitted to total internal reflection. It happens when the angle of incidence at the interface between the sample and crystal is greater that the critical angle. The latter is a function of the refractive indices of the two surfaces. The beam penetrates a fraction of a wavelength beyond the reflecting surface and when a material that absorbs the radiation is in contact with this surface, the beam loses energy at the wavelength where the material absorbs. The resultant attenuated radiation is measured and represented as a function of wavelength by the spectrometer and origins the absorption spectral characteristics of a sample [89].

1.3.4. Mid-infrared spectroscopy

The infrared spectrum is divided in three regions: far-infrared (<400 cm^{-1}), mid-infrared (4000-400 cm^{-1}) and near-infrared (13000-4000 cm^{-1}). For general analysis, the mid-infrared spectrum can be divided into 4 regions (Figure 5):

- X-H stretching region (4000-2500 cm^{-1}): The vibrations are generally due to O-H, C-H and N-H stretching. O-H absorbs at 3700-3600 cm^{-1} and N-H is generally observed at 3400-3300 cm^{-1}. C-H stretching occurs at 3000-2850 cm^{-1} but if this bond is adjacent to a double bond or aromatic ring, the C-H stretching wavenumber absorbs at 3100-3000 cm^{-1}.

- Triple-bond region (2500-2000 cm^{-1}): C≡C bonds absorb at 2300-2050 cm^{-1}, C≡N at 2300-2200 cm^{-1}. Despite of these being the most common absorptions, there is a possibility of seeing some X-H absorptions, if X is a more massive atom such as phosphorous (absorption at 2400 cm^{-1}).

- Double-bond region (2000-1500 cm^{-1}): The principal bands are due to C=C and C=O stretching. Usually, carbonyl stretching is the most intense band in the spectrum, occurring at 1830-1650 cm^{-1}. C=N occurs at 1650 cm^{-1}, generating a strong absorption band.

- General fingerprint region (1500-600 cm^{-1}): Many vibrations may vary by hundreds of wavenumbers, even for similar molecules. This applies to most bending and skeletal vibrations, which absorb in the 1500–650 cm^{-1} region, for which small steric or electronic effects in the molecule lead to large shifts. A spectrum of a molecule may have a hundred or more absorption bands present, but there is no need to assign the vast majority. The spectrum can be regarded as a 'fingerprint' of the molecule and so this region is referred to as the fingerprint region [89].

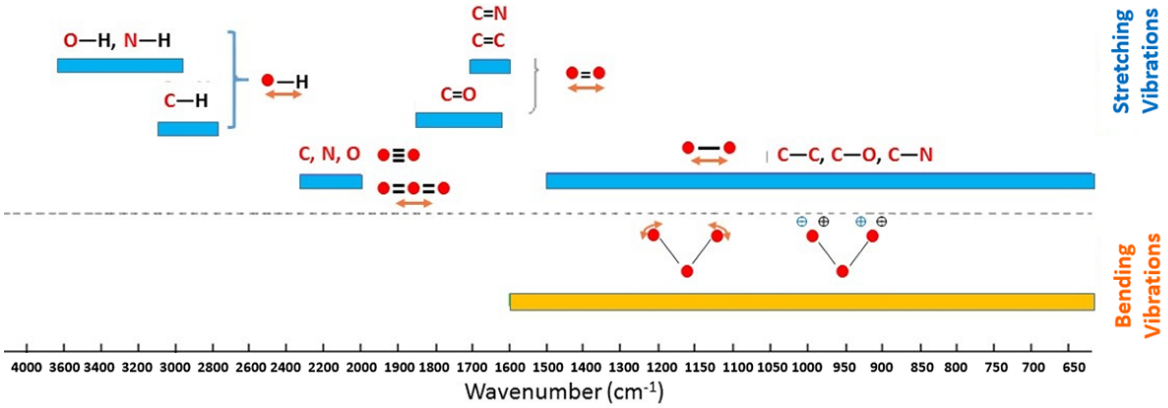

Figure 5: Group frequencies assignment in the mid-infrared spectrum.

1.3.5. Spectra pre-processing

Obtained spectra can undergo manipulations to be enhanced and facilitate the spectral interpretation and analysis. This manipulation is performed with an adequate software. Baseline correction eliminates the dissimilarities between spectra due to shifts in the baseline. Smoothing reduces the instrumental noise, improving the information content of spectra. If necessary, first and second derivatives can be applied to spectra to reduce replicate variability, amplifying spectral variations and resolve overlapping peaks. In order to eliminate the path length variation and reduce the differences between replicates, spectra are

normalized, usually to the most intense peak (typically the amide I band is an internal standard for normalization). This spectra pre-treatment is a pre-requisite for advanced statistical analysis [93].

1.3.6. Statistical analysis of infrared spectra

Data obtained from infrared spectroscopy are very often complex and, consequently, it is necessary to use chemometric techniques in order to extract as much information as possible.

The analytical information of the spectra can be interpreted using a multivariate statistical analysis that relates the spectra with the properties of the object of study, thus facilitating data interpretation [93]. While univariate analysis considers only a single property of a given object, multivariate statistics evaluate several properties at the same time.

Principal component analysis (PCA), is probably the most widespread multivariate chemometric technique, and because of the importance of multivariate measurements in chemistry, it is regarded by many as the technique that most significantly changed the chemist view of data analysis. Most chemical measurements are inherently multivariate, meaning that more than one measurement can be made on a single sample. An obvious example is spectroscopy, since a spectrum has hundreds of wavelength for a single sample, with a lot of information. PCA is one of the simplest multivariate methods that allows to explore patterns in complex data and is, normally, the first step in the data exploration that allows a visualization of the main variability aspects of a data set [93]. So, the objective of PCA is to reduce the dimensionality of a data set consisting of a large number of interrelated variables, while retaining as much as possible of the variation present in the data set. This is achieved by transforming to a new set of variables, the principal components (PC), which are uncorrelated, and which are ordered so that the first few retain most of the variation present in all of the original variables [94].

Cluster analysis aims to classify objects, i.e. the intrinsic description of the structure and property interrelationships of a given set of objects, each one defined by a multiplicity of properties [95]. Different algorithms can be used to calculate similarities between the objects. Starting from the calculated distance matrix, an attempt to distribute the objects into groups is made, such that all the objects that belong to the same cluster are as similar as possible. Cluster techniques can be divided into hierarchical and non-hierarchical procedures, being the hierarchical favoured in most cases, since it is assumed that they describe best the inherent hierarchical structure of class division within the data set [96].

Multivariate calibration has been a major basis of chemometrics. It involves connecting one (or more) sets of variables together. Usually one set (often called a 'block') is a series of physical measurements, such as some spectra or molecular descriptors, and the other contains one or more parameter such as the concentration of a number of compounds, for example. Partial least squares regression (PLS) is one of the most used algorithms for multivariate calibration model, being a procedure used to model the relationship between a set of predictor variables and a set of response variables, so that it is possible to predict the concentration of a compound in a mixture spectrum [93], [97]. PCA and PLS regression have been successfully used in the qualitative and quantitative analysis of infrared spectral data [97]–[99].

1.3.7. Mid-infrared spectroscopy as a rapid tool for food analysis

Mid-infrared spectroscopy has been widely used in food analysis, for example for the detection of adulterations in food products, as olive oils [100] or juice concentrates [101], determination of food components and contaminations, as tetracycline in milks [102] or quantification of food components, such as sugars in mango juice [99].

There are characteristic absorption bands that can be associated with major components of food, such as water (3920, 3490, 3280, 1645 cm^{-1}), C=O group (1740 cm^{-1}), acyl chain C-H (3000-2800 cm^{-1}), amide I and II (1650 and 1550 cm^{-1}), aqueous sugar molecules (1100-1000 cm^{-1}), and much more. This methodology is not only suitable for food examination but also for the analysis of microorganisms that are present in food products [103].

1.4. Infrared spectroscopy for food microbial analysis

The development and optimization of novel alternatives for the monitoring, characterisation and enumeration of foodborne pathogens is a key aspect in food microbiology and has become increasingly important for the food industry.

Classical standard microbiological methods for detecting the presence of microorganisms in foods, established by U. S. Food and Drug Administration and used worldwide, involve pre/enrichment and isolation of presumptive colonies of bacteria on several specific and/or different solid media. Additionally, a final confirmation by biochemical and/or serological procedure may be needed for the identification of the bacteria [104]. Consequently, these methods are laborious, time consuming and not always reliable (e.g. viable but non-culturable forms might not be detected). Molecular tools have emerged as a powerful fast approach in food microbiology to overcome these disadvantages. However, the efficiency of molecular methods can be negatively affected by the presence of inhibitory substances in foods, growth media, and nucleic acids extraction reagents [105], [106]. They can reduce or even block amplification reactions leading to underestimation of the bacterial load or to false negative results. Consequently, due to the demand for faster, more reliable, specific and cost-effective techniques for an effective control of food microbial quality, new methods should meet the following desired criteria: rapid and accurate identification of bacteria on small amounts, simple and uniform operating procedure, differentiation down to genus level at least, and simple standardization and complete computerization for automation [107].

Infrared spectroscopy with Fourier transform (FTIR), is one of the most promising analytical techniques in the food industry [108]. It has proven to be a valuable method for detection and distinction of microbial cells on the surface of food [109], to differentiate between bacterial colonies and even to detect differences in the colony itself [110] and in the classification of bacteria [111]. The infrared absorbance spectrum represents a 'fingerprint' that is characteristic of a chemical or biological substance. The main reasons for the wide acceptance of this method are the speed with which samples can be characterized with almost no handling, the flexibility of the equipment and the low cost of the analysis [112].

There are some studies on the detection/identification of bacteria in food samples or isolated from food products. With the advent of infrared spectroscopy and computational analysis in 1980s, Naumann and collaborators introduced infrared spectroscopy for *in situ* analysis of microbial cells, developing this methodology to identify, differentiate and classify

bacteria [111], [113], [114]. Since then, this methodology has been successfully applied for the identification, discrimination and detection of bacteria from different species, particularly foodborne pathogens such as *E. coli* [115]–[117]*, Listeria* [118], [119]*, Salmonella* [115], [120] and *Bacillus* [121].

1.4.1. Detection and quantification of bacteria from culture and food by IR spectroscopy

Detecting a microorganism in a sample by infrared spectroscopy can be performed by a direct or an indirect method. In the first case, IR spectra of culture or contaminated food are collected directly from the sample. For example, studies have differentiated and quantified different species of bacteria on apple juice using an ATR method and from an apple surface using infrared and Raman spectroscopy [107], [122]. In most cases the acquired spectra may contain noise due to food matrix or culture medium components. Indirect detection eliminates this noise by using a bacterial separation step such as filtration [115]–[117], [123] or even immunomagnetic separation [116] before spectral acquisition.

There are some studies reporting the detection of single types of bacteria [115], [124]–[126], but the detection of mixed cultures or identify a particular microorganism in a mixture is more difficult [127], [128]. IR detection of bacteria from food samples could, however, be used routinely if the spectral database for most pathogens is established.

Regarding quantification, the issue is even more complicated. A previous calibration model has to be performed in order to quantify the bacterial loads in a sample. The quantification of microorganisms in a binary mixture was performed [129] but is still difficult to predict the bacterial concentration directly in a food product.

1.4.2. Discrimination of viable, injured, and dead bacteria

The determination of bacterial viability is very important in the food industry, since it is of major concern to verify the efficacy of various treatments used in food processing in order to decrease the bacterial loads.

The conventional microbiological methods as quantitative PCR or fluorescent dye techniques are time consuming and cannot give an accurate measure of live and death cells, depending on the physiological and biochemical heterogeneity of the target bacteria and the complexity of the sample matrix [130]. IR spectroscopy, as it is based on the biochemical

composition of the cells and so less susceptible to variation, is a suitable technique for the differentiation of dead/injured and live cells. It has been used to detect *S. typhimurium* and *L. monocytogenes* injured by heat [131], *L. monocytogenes* subjected to sonication [132], chlorine-injured *E. coli* and *Pseudomonas aeruginosa* in water [133], and *Micrococcus luteus* damaged by radicals [134]. In these cases, the spectral differences between live and injured cells were minor. So, second derivative pre-processing and statistical models were used to correctly classify the live and injured cells.

1.4.3. Analysis of structural components of bacteria

As IR spectra of intact bacterial cells can be difficult to analyse due to overlapping bands, this methodology has been used to study some structural components of bacteria in order to discriminate them. It has been performed a species and strain identification of 14 Gram-positive and Gram-negative foodborne pathogens using spectra of the fatty acid methyl esters isolated from bacteria [128]. Serovars of *S. enterica* were discriminated using the outer membrane proteins [135]. As well, spectra of isolated lipopolysaccharides (LPS) from *E. coli* and *S. enterica* were successfully used for serotype level classification, achieving more than 95% of correct classifications [136], [137].

1.4.4. Taxonomic classification of bacteria

Taxonomic classification of foodborne microorganisms is of major importance for epidemiological investigation, pathogen control and outbreak detections. Classical methods used in microbial taxonomy are staining techniques, microscopy, biochemical assays, serological tests, etc. These methods are time consuming and laborious. More recent molecular subtyping methods as for example pulsed-field gel electrophoresis (PFGE) or multiple locus variable-number tandem repeat analysis (MLVA) are used to perform effectively taxonomic classifications, but they are expensive, time consuming and require trained personnel. As infrared spectra represent phenotypic and genetic fingerprints of microorganisms, they allow the differentiation of bacteria at different taxonomic levels. There are several studies on the discrimination and classification of a variety of microorganisms that were thoroughly reviewed [138]–[140]. Several chemometric approaches and different spectral pre-treatments were used in order to better analyse the data to obtain the desired results.

1.4.5. IR spectra of bacteria

Each bacterial species has a complex IR spectra due to stretching and bending vibrations of molecular bonds or functional groups present in the cell wall/membrane composition in proteins, nucleic acids, lipids, sugars and lipopolysaccharides (LPS). The molecular composition varies from species to species and even at strain level. This originates a unique and characteristic spectrum, making possible the identification of the microorganisms [140].

In order to analyse IR spectra, it is important to know some fundamental characteristics of cell surface and their composition. Gram-positive bacteria have a thicker and rigid layer of peptidoglycan (40-80% of the weight of the cell wall) than Gram-negative bacteria (10% of the weight of the cell wall). The primary of peptidoglycan structure consists of parallel polysaccharide chains of alternating N-acetylglucosamine and N-acetylmuramic acid residues linked by glycosidic bonds. In turn, the parallel chains are joined by penta or tetrapeptides, which aminoacid composition varies in different species of bacteria. Also, Gram-positive cell walls contain teichoic acids covalently bounded to the peptidoglycan. On the contrary, Gram-negative ones do not contain teichoic acids and have lipoproteins covalently linked to the peptidoglycan. Moreover, Gram-negative cells have an outer membrane outside of the peptidoglycan layer containing phospholipids and proteins in the inner part and phospholipids, proteins and LPS in the outer part. LPS consist of three basic regions: an *O*-specific side chain (heteropolysaccharide responsible for antigenic properties), the inner and outer core of oligosaccharides and a lipid anchor called lipid A [140]–[142]. *O*-antigens are used for typing bacteria. In addition to this, Gram-negative cells also contain other antigens like K-antigens (associated to the capsule) and H-antigens (associated with flagella). Differences in sugars composition and organization of phospholipids, proteins and LPS and other antigenic structures help in IR identification of bacteria.

For bacterial identification, in the absence of water, five distinct major absorbance regions should be taken into account when analysing the spectra [113]: 3000-2800 cm^{-1} region of fatty acids; 1700-1500 cm^{-1} region of amide I and amide II bands of proteins; 1500-1200 cm^{-1} region of mixed fatty acids bending vibrations, proteins and phosphate-carrying compounds; 1200-900 cm^{-1} region of absorption bands of microbial cell walls carbohydrates and 900-700 cm^{-1}, containing weak but very characteristic absorbance to specific bacteria.

Spectra interpretation and peak assignments are crucial steps for the infrared analysis of microorganisms. Even though most bacterial spectra look very similar by visual inspection, a closer observation can show subtle differences. The wavenumber positions of the bands, their

widths and intensities are very useful for functional group, cell component and sample identification [140], [143]. The peaks that appear on a bacteria IR spectrum represent functional group vibrations in the main biomolecular constituents like nucleic acids, proteins, fatty acids and carbohydrates (Table 2).

Table 2: Assignment of functional groups associated with major vibration bands in the mid-IR bacterial spectra. (Adapted from Davis and Mauer, 2010).

Wavenumber (cm^{-1})	**Functional group**
3200	N-H stretching in proteins
2995	C-H asymmetric stretching of $-CH_3$ in fatty acids
2930	C-H asymmetric stretching of $>CH_2$ in fatty acids
2898	C-H stretching of ≥C-H of aminoacids
2870	C-H symmetric stretching of $-CH_3$ in fatty acids
2850	C-H symmetric stretching of $>CH_2$ in fatty acids
1740	>C=O stretching of lipid esters
1715	>C=O stretching of ester, in nucleic acids and carbonic acids
1695-1675	Amide I band components of proteins
1655	Amide I of α-helical structures of proteins
1637	Amide I of β-pleated sheet structures of proteins
1550-1520	Amide II band of proteins
1515	Tyrosine band
1468	C-H deformation of $>CH_2$ in lipids/proteins
1415	C-O-H bending in Carbohydrates, DNA/RNA backbone, proteins
1400	C=O symmetric stretching of COO- group in aminoacids, fatty acids
1310-1240	Amide III band components of proteins
1240	P=O asymmetric stretching of phosphodiesters in phospholipids
1200-900	C-O-C, C-O dominated by ring vibrations in various polysaccharides
1085	P=O symmetric stretching in DNA, RNA and phospholipids
720	C-H rocking of >CH2 in fatty acids, proteins

1.5. Objectives

The main objective of this work was to evaluate the potential of MIR spectroscopy to analyse foodborne bacteria, with minimum sample preparation, to:

-Discriminate different colonies grown in solid media

-Predict the bacterial concentration in cooked ham using a multivariate statistical approach;

-Identify foodborne bacteria from meat, fish and cooked ham using different multivariate approaches, both in a general medium and in some differential/selective media;

-Confirm the presence of *Listeria monocytogenes* and *Salmonella* spp. isolated from food samples in order to substitute the traditional biochemical and serological confirmation methods;

-Assess the impact of an HPP treatment in some foodborne bacteria by assessing the decrease of microbial concentration and evaluating the spectral changes after pressurization

-Understand some modifications in the bacterial cell components triggered by HPP by observing the infrared spectra.

Chapter 2.
Evaluation of the potential of Mid-infrared spectroscopy to assess the microbiological quality of ham

Evaluation of the potential of Mid-infrared spectroscopy to assess the microbiological quality of ham

Catarina Moreirinha[ab]; Alexandra Nunes[c]; António Barros [b]; Adelaide Almeida[a]; Ivonne Delgadillo[b]*

[a] *Department of Biology, CESAM, University of Aveiro, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal*

[b] *Department of Chemistry, QOPNA, University of Aveiro, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal*

[c]*CBC, University of Aveiro, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal*

*Corresponding author. Tel. +351 234370718

E-mail address: ivonne@ua.pt

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2.1. Abstract

The accurate reliable detection and identification of microorganisms in food is critical to public safety. Consequently, it is extremely important to develop rapid and inexpensive methods for the detection of food microorganisms in order to minimize or even replace the traditional analysis methods that are expensive and time consuming. In this study, the potential of mid-infrared spectroscopy was evaluated, for the first time, to detect changes in colony forming units of microorganisms in freshly cut ham along the time. A partial least squares regression model was performed and a good linear relationship was obtained between spectra information and microbial load. It was concluded that infrared spectroscopy easily and quickly allows the separation of ham samples according to their microbial content and could be used to predict the microbial concentration from the spectra, using the fingerprint region (1200-950 cm^{-1}), without sample preparation or handling.

Keywords

MIR; infrared spectroscopy; ham; microbial quality; multivariate analysis.

2.2. Introduction

Microorganisms in food may cause food deterioration or illnesses associated with consumption. Meat and its products are classified in the category of perishable foods. The large amount of water and nutrients found in these products make them an excellent medium for microbial growth [5]. Modifications produced by microorganisms include changes in appearance, odour and flavour [7].

It is crucial to detect microorganisms early on, to prevent their transmission, avoiding infections and/or food poisoning, but the traditional methods used to assess the microbiological quality of foods are time consuming, hence faster methods of screening are sought.

Mid infrared has potential to be used as a rapid method to assess the microbiological quality of food. The infrared spectrum can be considered as a 'fingerprint', which is characteristic of a chemical compound or biological system [144].

Principal component analysis (PCA) and Partial least squares (PLS) regression have been successfully used in the qualitative and quantitative analysis of infrared spectral data [145]. This methodology is relatively cheap and the analysis cost of a sample is lower compared to traditional methods [89].

MIR technique proved to be quite advantageous in studies of quality and composition of various foods. It was also used for the detection of microbiological spoilage in meat by measuring the biochemical substrate [146]. In addition, MIR has also proved to be a valuable and rapid method of detection and distinction of microbial cells on food surfaces [109] and to classify different taxonomic levels of bacteria without requiring a pre-selection of strains [111] showing to be sensitive to differentiate between bacterial colonies. Previous reports on the use of MIR for microorganism studies include sample pre-treatments such as: alcohol pre-treatment, use of specific membranes to form bacterial films [124], overnight drying of colonies obtained from plates [147] or colonies grown in liquid medium [148]. The use of MIR microscopy is also mentioned [149]. Concerning the data treatment, in previous works, the 1st or 2nd derivatives transformation of the spectra were used in order to enhance the results [111], [114], [148], [150].

The main objective of this study was to evaluate the potential of MIR to assess the microbiological quality of ham. Firstly, the spectra from different microorganisms isolated directly from single colonies growing on fresh pork ham surface were acquired in order to have an idea of the similarity/dissimilarity between their spectra. Then, the liquid/viscous layer from

the ham surface was analyzed to assess if MIR could detect spectral differences as function of the increasing microbial concentration along the time. Finally, a calibration model to predict the microbial concentration in the ham samples, directly from spectra, was built.

2.3. Materials and methods

2.3.1. Isolation of microbial colonies from ham samples for MIR analysis

A 0.5 cm-thick slice of fresh pork ham (5 x 5 cm) was prepared and stored at about 20°C for 5 days, under aseptic conditions. At the end of this time several colonies of microorganisms with different morphologies (differences in color, shape, size and margins) were already visible.

Five well isolated single colonies were selected on the ham surface and purified by streaking. Each colony was streaked on plate count agar medium, incubated overnight at 37°C and streak again on the same medium two times more. 3 replicate plates were done.

2.3.2. Ham microbial analysis on freshly sliced pork ham

Fresh pork ham, from two brands (A and B), were sliced at the moment of the purchase and immediately transported in a plastic wrapper. Ham slices with 0.5 cm of thickness and 5 x 5 cm were aseptically prepared and cut, and 2 cuts were placed in each one of the 25 Petri plates (5 replicates per analysis time), per brand.

All Petri plates (unless the correspondent to initial time T0) were incubated at 4°C during 2, 4, 6, and 8 days (T0, T2, T4, T6 and T8). After the incubation time was achieved, one cut of ham per Petri plate was used for microbiological examination, the second one was reserved for infrared analysis. For microbiological assessment each cut was homogenized with an Ultra-Turrax (T25, Janke & Kunkel - IKA Labortechnik) in Ringer's Solution (Merck) in a ratio of 1:10. A standard volume (100 µL) of serially diluted samples was pour plated in duplicate in plate count agar medium. After 3 days of incubation at 37°C the number of colonies (CFU) was counted in the most convenient dilution series and it was determined the concentration of total aerobic bacteria per gram of ham (CFU g^{-1}). The weight of the ham slices was determined aseptically (Mettler Toledo ± 0.0001 g).

2.3.3. Mid-infrared spectroscopy

Spectroscopic acquisition was performed in an infrared spectrometer (Perkin Elmer Spectrum BX) with a resolution of 8 cm^{-1} and 32 scans, in the mid-infrared region (4000-600 cm^{-1}). Analyzes were performed in a room with controlled temperature (25°C). Microbial colonies were collected with a loop and placed directly on the crystal of a 2 mm x 2 mm horizontal single reflection diamond ATR (attenuated total reflectance) accessory (Golden Gate). 3 replicate spectra were obtained for each sample. The sampling accessory was cleaned with ethanol (70%) and distilled water between each measurement.

For the freshly cut pork ham analysis, the liquid/viscous surface layer of the 5 x 5 cm ham slices were thoroughly mixed and collected with a glass rod and a small amount was placed directly on the sampling accessory and was dried under gentle cold airflow. 5 replicate spectra were obtained from each sample. The samples corresponding to T0 were analyzed about 30 minutes after the ham preparation.

2.3.4. Multivariate analysis

The spectra (obtained in OPUS format) were transferred via JCAMP.DX format to an in-house developed data analysis package (CATS build 97). Principal component analysis (PCA) was used to find the major sources of variability in data, detect outliers and detect the probable presence of clusters. Partial least squares regression (PLS) was used to capture information in spectra related to the microbial content. Previous to PCA and PLS, the spectra were standard normal deviate (SNV) corrected. The predictive power of the PLS model was assessed with cross-validation to predict microbial concentration.

2.4. Results and discussion

2.4.1. MIR analysis of microbial colonies isolated from ham

The MIR spectra of the 5 morphologically different colonies isolated from the surface of ham kept at room temperature (20°C) during 5 days, showed high reproducibility among replicates and different bacteria spectral profile (Figure 6). PCA of 1800 to 950 cm^{-1} region was performed and it was found that the sub-region located between 1200 and 950 cm^{-1} (representative of several types of compounds including polysaccharides) allowed best distinction between the different colonies. This region was also previously selected as one of the more important areas for detection and differentiation of microorganisms [151]. The spectra are dominated either by a peak around 1080 cm^{-1} or at 1040 cm^{-1}. This means that in a microbial community growing on the ham we would also expect spectra to be dominated by these two peaks, making almost impossible the identification of microorganisms in a natural mixed culture.

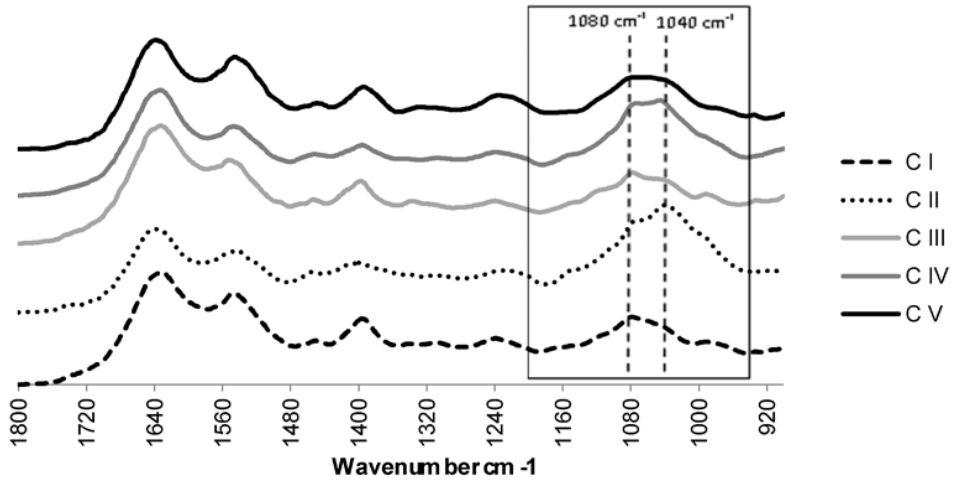

Figure 6: Infrared spectra of 5 different colonies (average of 3 replicates) of bacteria, in the 1800 to 900 cm^{-1} spectral region. CI – Colony I, CII – Colony II, CIII- Colony III, CIV – Colony IV, CV- Colony V.

2.4.2. Bacterial concentration of fresh ham

The results of aerobic plate counts expressed as colony forming units (CFU's) (Table 3) for brand A and B showed an increase in the number of the colonies as a function of time, with a greater increase between T2 (2 days after sample preparation) and T4 (4 days after sample preparation). The values are in accordance with other study in which the CFU's in ham have

been determined [152]. It is noticeable that ham from brand B showed a lower microbial concentration than the one from brand A. After T8 it was visually evident that the ham was spoiled, so it precluded the experiment follow-up. Anyway, the bacterial concentration of T10 and T12 was determined (data not shown) and it was found that the microbial counts exceeded 10^8 $CFUg^{-1}$. This value is generally accepted as the point at which spoilage becomes readily detectable [4].

Table 3: Number of colony forming units for the different times of analysis (T0, T2, T4, T6 and T8) of ham, for brands A and B. Average values of 4 replicates and standard deviation.

Time (Days)	Brand A	Brand B
T0	$2.7x10^5 \pm 3.5x10^2$	$2.2x10^5 \pm 2.5x10^3$
T2	$5.8x10^5 \pm 5.3x10^3$	$3.6x10^5 \pm 2.5x10^4$
T4	$8.8x10^6 \pm 2.5x10^5$	$6.2x10^6 \pm 1.9x10^5$
T6	$9.6x10^6 \pm 4.8x10^5$	$8.2x10^6 \pm 3.8x10^5$
T8	$1.4x10^7 \pm 5.3x10^5$	$1.3x10^7 \pm 1.5x10^5$

2.4.3. MIR analysis of fresh ham

Figure 7 shows the spectra of initial and final analysis times of the superficial watery layer of the two brands of ham (T0A, T0B, T8A, T8B), normalized by the water peak at 1645 cm^{-1}. Signals obtained were consistent with those observed in the different colonies at 1080 cm^{-1} and 1040 cm^{-1} (Figure 7).

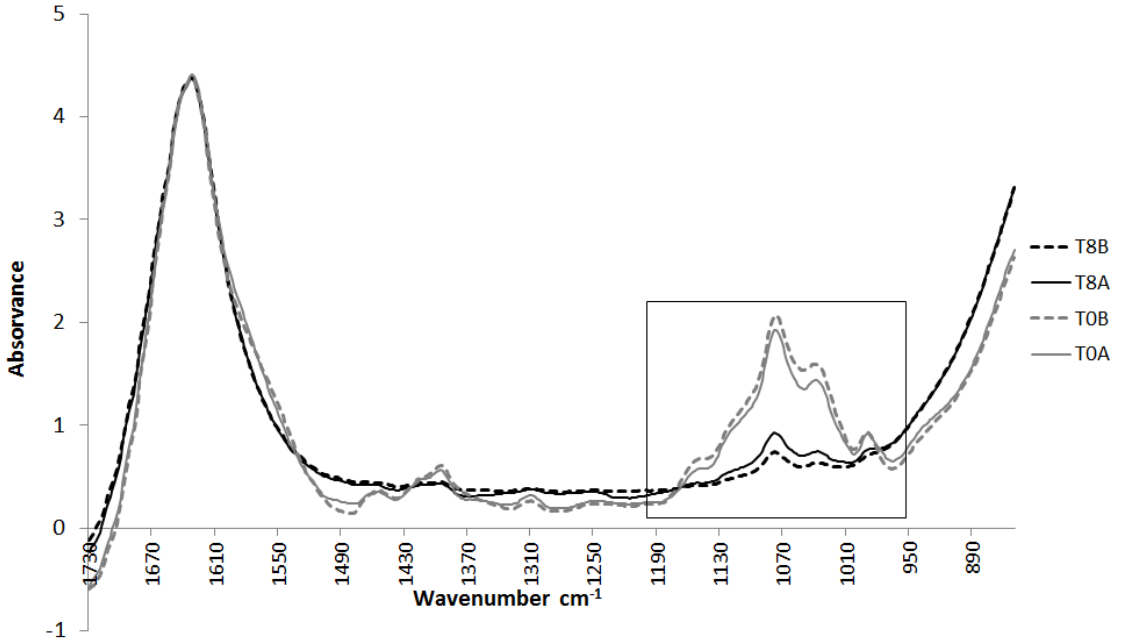

Figure 7: Infrared spectra of the 2 brands of fresh ham, in the 1800 to 850 cm^{-1} spectral region. T0A – time 0, brand A; T0B – time 0, brand B; T8A – time 8, brand A; T8B – time 8, brand B.

Visual inspection of the spectra reveals that the spectrum of water increasingly dominates the spectra. Shapiro [153], using electron microscopy to study the surface of colonies of *E. coli*, found that colonies which have grown for more than 24 hours secrete extracellular material that is deposited as a cover on its surface. This layer is rich in extracellular polysaccharides [154]. These exopolysaccharides are able to retain water as a way of self-protection against desiccation [155]. The production from polysaccharides, which retain water is consistent with the fact that, with the time, the smears from the ham surface presented increasing viscosity and were, as well, gradually more difficult to dry on the crystal. It can be observed, in Figure 6, that the spectra corresponding to 8 days show lower signal in the region between 1200 and 950 cm^{-1}. There is, clearly, an increase of water in the system.

Figure 8 shows the results for the PCA of all analyzed times for both brands. The spectral region exhibiting a better description of the system was between 1200 and 950 cm^{-1}. Samples T0A, T0B, T2A, T2B and T4B are located in the negative PC1 region. The determination of bacterial concentration revealed that the replicates of T4 from brand B had lower CFU counts than T4 from brand A (Table 3), which is in accordance with the PCA distribution. The PC1 loadings plot shows that these samples are associated to a band at 1080 cm^{-1}. In positive PC1 are found samples with microbial loads above 6.2x10^{6} CFU g^{-1} (Figure 7, Table 3). The

loadings plot of PC3 (Figure 8) shows, also, the band at 1080 cm^{-1} associated to the samples from T0 in quadrant IV.

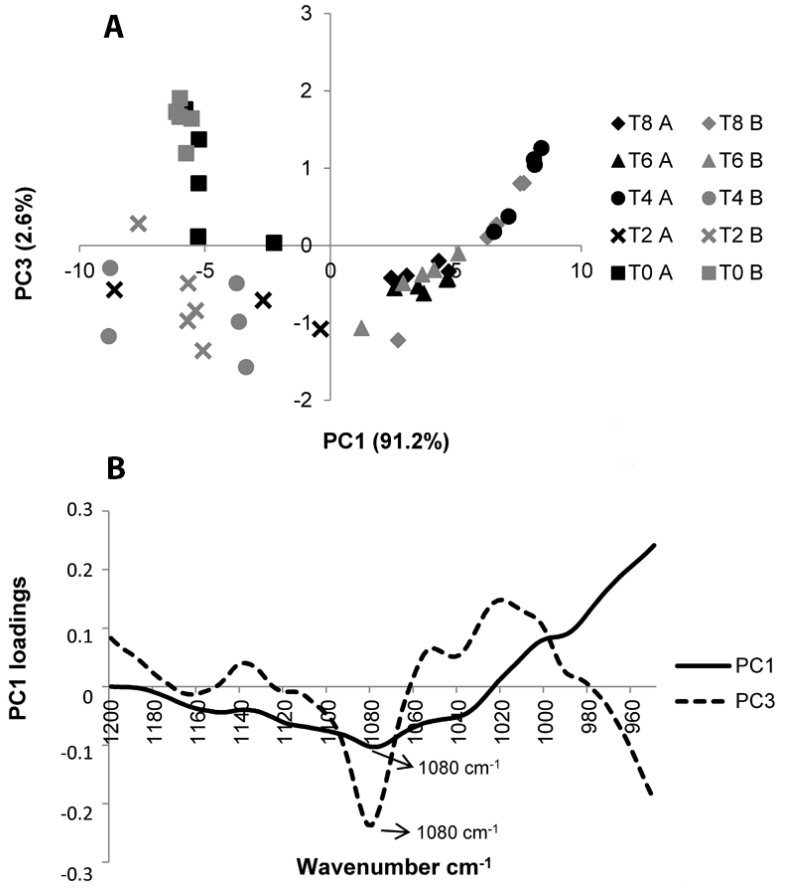

Figure 8: Scores scatter plot (A) and loadings plot profile (B) of brand A and B for fresh ham for days 0, 2, 4, 6 and 8 of analysis.

The global interpretation from the PCA suggest the peak at 1080 cm^{-1} (which is decreasing) as a reference for the increasing quantity of water associated to the microbial growth/exopolysaccharide production. The behavior from the samples in PC1 positive is consistent with the progressive increase from a unique compound: water.

2.4.4. Microbial concentration quantification by PLS-FTIR

Figure 9 represents the relationship between the observed CFU counts and the values estimated from the spectra. It was used the spectral region between 1200 and 950 cm^{-1} because it was the one that allowed the best results to construct the quantification model. It can be observed in figure 9 a good linear relationship between the predicted and the observed

values for the samples microbial concentration (Log CFU g^{-1}). This suggests that this model could be applicable to predict, from the spectral data, the CFUs of ham samples.

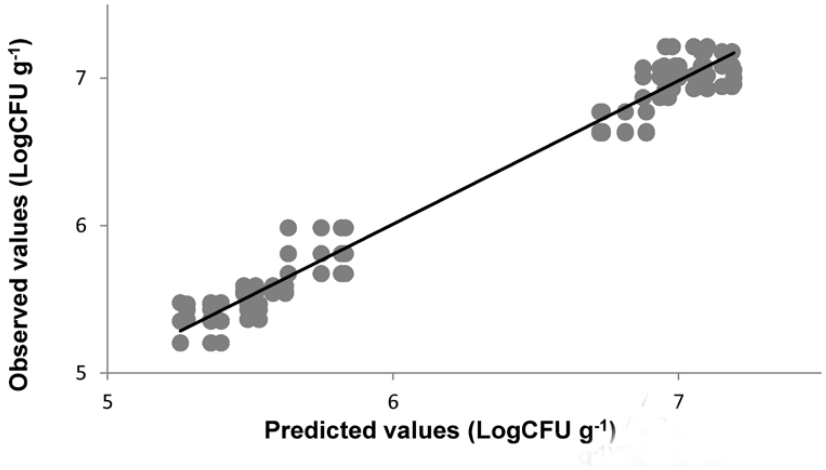

Figure 9: Calibration curve for microbial concentration obtained by PLS of the MIR spectra of the ham samples (Log CFU g^{-1}). R^2=0.97, LV=10, RMSEC%=1.91.

According to the bibliography, when leaving the factory, ham may present counts of 10^5 CFU g^{-1}, being $5x10^6$ CFU g^{-1} the borderline limit of bacterial concentration admitted (EC 2073/2005)**.** Beyond this value the ham should be rejected. So, in our study, MIR combined with PCA is able to distinct samples with satisfactory microbiological quality from ones that are approaching and exceeding the maximum acceptable limit. Moreover, the microbial content expressed as CFU can be easily determined.

The results of this study showed that MIR can be a valuable approach to detect different levels of contamination in ham. Overall, this analysis method allowed the distinction of ham samples with different degrees of contamination, in a fast and simple way (samples placed and read directly on the sampling accessory), using the fingerprint region (1200-950 cm^{-1}). Furthermore, the attempt to construct a calibration model that gives us an assessment of the bacterial concentration of ham brought good results. The total aerobic plate count can provide a general indication of the microbiological quality of a food and is one of the first and more widely used analysis to be performed in food industry. It takes at least 24 hours of incubation to know the results, however, MIR can provide these results within few minutes.

Considering that nowadays exist portable mid infrared spectrometers with excellent signal stability and at very reasonable prices, this kind of application could be very useful for the food industry, control organisms and consumer associations, since it is a very fast method that permits to screen, making the preliminary assessment and monitor the bacterial quality of ham in a factory, storage centers or even at supermarkets. Moreover, provided a model was

developed for each product, this methodology can be suitable to assess the microbial quality of other food products, such as, other meat products, fish samples or any other product that may get contaminated at the surface.

Taking further the applications of IR, and as shown in the initial part of this work, different colony types have specific and unique spectra, which can be used as a fingerprint, this technique is also useful in identifying strains, allowing to confirm the presence of different types of bacteria, which detection is required by legislation, including pathogenic bacteria (work in progress and to be published in another article) which makes more rentable the investment in this technology.

Chapter 3.

Mid-infrared spectroscopy as a rapid method to assess bacteria present in food products

Mid-infrared spectroscopy as a rapid method to assess bacteria present in food products

Catarina Moreirinha[ab], António Barros [b], Jorge A. Saraiva, Adelaide Almeida[a], Ivonne Delgadillo[b]*

[a] *Department of Biology, CESAM, University of Aveiro, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal*

[b] *Department of Chemistry, QOPNA, University of Aveiro, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal*

[c]*CBC, University of Aveiro, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal*

*Corresponding author. Tel. +351 234370718

E-mail address: ivonne@ua.pt

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3.1. Abstract

Nowadays, the early identification of foodborne bacteria is very important, in order to avoid food spoilage and particularly infections associated to food consumption. The traditional methodology for food microbial analysis, using different culture media and posterior confirmation by biochemical and/or serological methods, is laborious and time consuming, as several days are needed to have the final results. DNA/RNA based methods, despite of being more rapid, are expensive, require trained personnel and the results can be influenced by the food matrices leading to incorrect results. Taking this into account, the development of new approaches to identify foodborne bacteria on time is of crucial significance. Infrared spectroscopy is a rapid, easy to perform and inexpensive technique that can be used in microbial analysis. In this work, we were able to distinguish simultaneously 22 different bacteria using two different multivariate statistical analysis techniques. We were also able to distinguish bacteria grown on some selective media used in food microbial analysis. This makes IR spectroscopy a suitable technique with advantages on the actual used methodologies, allowing to obtain the final results 2 or more days earlier and in an inexpensive way, as there is no necessity to use the reagents routinely employed to confirm the colonies in solid media.

Keywords

Infrared spectroscopy, MIR, principal component analysis, hierarchical cluster analysis, foodborne bacteria, bacteria identification.

3.2. Introduction

The high levels of moisture, nutrients, and the high pH attained after *post-mortem* makes fish and meat easily perishable foods. Fish may contain various potential public health hazard pathogens [22]. Several bacterial genera like *Pseudomonas, Bacillus, Acinetobacter, Flavobacterium, Micrococcus, Staphylococcus, Flavobacterium* and *Vibrio* are commonly found in marine fish samples [18], [19], [156]. In Europe, hake and cod are two of the more consumed fishes. Hake is usually sold fresh and cod in Portugal and Spain is normally salted and dry, having the necessity to be desalted before consumption. Drying and salting are long processes, highly manipulative and with high potential for environmental transfer of microorganisms to the product [20]. Some bacterial genera like *Vibrio, Listeria* and *Staphylococcus* can survive on high salt concentration substrates and grow during desalting, causing food spoilage and threatening the consumers health [21]. A wide range of microorganisms from different sources can contaminate meat muscle during primary and further processing. Pathogens can include *Clostridium perfringens, Staphylococcus aureus, Salmonella* spp., pathogenic *E. coli, Campylobacter* spp., *Listeria monocytogenes* and *Aeromonas hydrophila* [14], [157]. Meat products that are cured with salt, such as ham, are often eaten raw after processing, which make them a hazardous vehicle for the transmission of salt tolerant bacteria as *S. aureus* and *L. monocytogenes* [5].

A rapid and accurate detection and identification of microorganisms in food is very important for human health. Classic microbiological methodologies are time consuming and have inherent limitations, requiring enrichment, isolation and bacterial confirmation by biochemical/serological identification after growth on selective media. There are also molecular tools that can be used to identify foodborne microorganisms. These methods, despite being rapid, are very expensive and can be inhibited by the presence of certain substances in food products. This can lead to an underestimation of bacterial concentration or false negative results [22], [152], [158]. Taking this into account, it is very important to develop faster methods to ensure the safety of food products, by detecting/identifying microorganisms in order to prevent illnesses associated to food consumption.

Mid-infrared spectroscopy (MIR) is one of the most promising techniques to the food industry. It is based in the observation of molecule vibrations that are excited by an infrared radiation beam in the spectral region between 4000 and 400 cm^{-1}. This method has been successfully used in food industry, namely to identify microorganisms, since the infrared spectra of microorganisms are fingerprint-like patterns characteristic of each bacteria [111].

MIR allowed to analyse foodborne pathogens such as *Listeria*, *Salmonella* and *Staphylococcus* [115], [118], [159]. The main reasons for the wide acceptance of this method are the speed and low cost of the analysis and the flexibility of the equipment. In addition to this, the samples usually do not need previous preparation and a very little amount of sample is enough [140]. However, the data obtained with infrared spectroscopy are very complex and it is necessary to use chemometric analysis in order to extract the information from the spectra, facilitating the data interpretation [93]. Principal component analysis (PCA) is a method that allows the compression of the data without losing their variability. It is very useful to identify patterns in data, highlighting the similarities and differences between samples [94]. Hierarchical cluster analysis (HCA) allows to classify samples, using different metrics and procedures to compute similarities between them, based on a distance matrix, and perform their distribution into groups [95].

The main objective of this work was to identify bacteria present in fish (hake and cod), meat (pork, chicken and cattle) and processed meat (cooked ham) using mid-infrared spectroscopy coupled to chemometric analysis, and to study the suitability of this technique to shorten the time of microbial identification necessary in the traditional microbiological analysis of foodstuffs avoiding the expensive and time taking confirmation steps.

3.3. Material and methods

3.3.1. Food sample preparation

Three pieces of hake (*Merluccius merluccius*), 3 pieces of dried salted cod (*Gadus morhua*), 3 pieces of pork meat and pork ham (*Sus scrofa domesticus*), 3 pieces of chicken meat (*Gallus gallus domesticus*) and 3 pieces of cattle meat (*Bos taurus*) were obtained in 3 different commercial surfaces.

Cod was desalted in sterile distilled water (fish:water ratio, 1:10) during 24 hours at 4°C before microbial analysis and the water was changed 3 times (every 8 hours) to simulate the soaking method adopted by the consumers. Cod was analysed immediately after desalting procedure. Hake, pork, chicken and cattle meat and ham were analysed immediately after the sample acquisition. A total of 18 randomly selected sub-samples were aseptically cut: 3 samples of hake, 3 samples of cod, 3 samples of pork meat, 3 samples of pork ham, 3 samples of chicken meat and 3 samples of cattle meat.

3.3.2. Bacteria used

Bacteria that are considered relevant for fish, meat and ham were included in this study: some of them were isolated from the fish, ham and meat samples, and bacteria from culture collections and other relevant bacteria previously isolated in our laboratory from fish farming waters were also used (Table 4). Bacteria were isolated from fish, meat and ham, as follows: each food sub-sample was aseptically homogenized with an Ultra-Turrax (T25, Janke & Kunkel - IKA Labortechnik) in Ringer's Solution (Merck) (1:18 weight: volume). One hundred microliters of serially diluted samples were pour plated, in duplicate, in Trypticase soy agar (TSA) medium (Merck), Violet red bile dextrose agar (VRBD) medium (Merck), Baird-Parker agar (BPA) and in Thiosulfate citrate bile salts sucrose agar (TCBS) medium (Merck). Cod samples were additionally pour plated in TSA medium supplemented with 3% NaCl. After 3 days of incubation at 37°C, some colonies presenting different morphologies (colour, shape, size and density) were selected. The colonies were purified by repeated streaking on TSA plates. *Aeromonas hydrophila* from the collection was named as (2) and the isolated as (1). These two members of *Aeromonas,* were maintained because they were apparently different in BOX-PCR electrophoresis gel (data not shown).

Table 4: Bacteria isolated from meat, fish and ham, from culture collections and isolated from other samples.

Isolated from meat, fish and ham	Culture collections	Isolated from other samples
Aeromonas hydrophila (1)	*Vibrio anguillarum* (DSM 21597)	*Psychrobacter*
Acinetobacter	*Vibrio parahaemolyticus* (DSM 27647)	*Shewanella*,
Bacillus cereus	*Listeria monocytogenes* (NCTC 1194)	*Sphingomonas*
Enterobacter	*Listeria innocua* (NCTC 11288)	*Micrococcus*
Klebsiella	*Citrobacter freundii* (NCTC 6272)	
Leucobacter	*Photobacterium damselae* (DSM 7482)	
Pseudomonas	*Salmonella* Nottingham (NCTC 7832)	
Staphylococcus aureus	*Shigella flexneri* (DSM 4782)	
E. coli	*Aeromonas hydrophila* (ATCC 7966) (2)	

3.3.3. Molecular identification of bacteria isolated from food samples

Bacterial DNA was extracted using the Instagene Matrix (Biorad, USA). 16S rDNA was amplified using the universal 27f forward primer (5'-AGAGTTTGATCCTGGCTCAG-3') and 1512r reverse primer (5'- CGGCTACCTTGTTACGACT-3'). The reaction occurred in a Multigene Gradient Thermal Cycler (MIDSCI). The reaction mixture contained 1 µL of bacterial DNA, 3.75 µL of $MgCl_2$, 2.5 µL of KCl buffer, 2.5 µL of dNTP, 0.25 µL of each primer, 0.5 µL of BSA, 1 µL of Taq polymerase (MBI Fermentas, Lithuania) and Milli-Q water (Millipore). The PCR running conditions included a 5 minutes initial denaturation of template DNA at 94°C, 25 denaturation cycles at 94°C for 1 minute, annealing at 55°C for 2 minutes, extension at 72°C for 2 minutes and a final extension at 72°C for 10 minutes.

In order to detect bacterial clones, a BOX-PCR was performed using reverse primer Box-A1R (5'-CTACGGCAAGGCGACGCTGACG-3'). The reaction mixture contained 1 µL of bacterial DNA, 1.25 µL of NH_2SO_4 buffer and 1.25 µL of KCl buffer, 2.5 µL of DMSO (5%), 3 µL of $MgCl_2$, 2.5 µL of dNTP, 2 µL of primer, 1 µL of Tap polymerase and 10.5 µL of Milli-Q water. The PCR running conditions included a 5 minutes initial denaturation of template DNA at 94°C, 30 denaturation cycles at 94°C for 1 minute, annealing at 53°C for 1 minutes, extension at 65°C for 8 minutes and a final extension at 65°C for 18 minutes. PCR products were visualized in a standard agarose gel electrophoresis with ethidium bromide staining and the bacterial clones detected were discarded. The selected PCR amplicons were purified using Ron's PCR-Pure

purification kit (BIORON, Germany). Automated DNA sequencing was performed by GATC biotech (Konstanz, Germany) and the sequences were analysed using BLAST database.

3.3.4. Mid-infrared spectroscopy

The bacteria were grown on some of the media normally used in a control laboratory: TSA, VRBD, TCBS and Baird-Parker agar (BPA). After 18 hours of incubation at 37°C the colonies were examined by infrared spectroscopy.

Spectroscopic acquisition was carried out in a MIR (Bruker ALPHA FTIR Spectrometer) with a resolution of 4 cm^{-1} and 32 scans, in the mid-infrared (region between 4000 and 600 cm^{-1}). Microbial colonies were collected with a loop and placed directly on the crystal of a horizontal single reflection platinum ATR accessory. The colonies were air-dried and then measured. At least five replicate spectra were obtained for each sample.

3.3.5. Multivariate analysis

Data pre-processing/pre-treatment: Previously to the multivariate analysis of the spectra of the whole dataset of bacteria grown in TSA, several spectral pre-processing and different pre-treatment were assessed. It was found that the best approach for sample and spectral traits analysis was based on the spectral first derivative using a Savitsky-Golay procedure [160] with a 2nd degree polynomial and a 15 points wide-window. In addition, each spectral signal was normalized by standard normal variate (SNV). Spectra from the bacteria grown in VRBD, TCBS and BPA were only normalized by SNV, without any additional treatment.

Principal Components Analysis (PCA): The main idea behind this unsupervised method is to recover the main directions of largest variability in the data (defined as principal components). The method starts by finding the largest variation and places an axes along that direction, then, looks to the variation that remains and finds another axis that is orthogonal to the previous one, and cover as much variability as possible. All the principal components recovered are, therefore, orthogonal (i.e., they are uncorrelated). These principal components are ordered as a function of the decreasing amount of variability. This approach allows to perform data compression or dimensional reduction, projecting the high dimensional data into low dimensional spaces, retaining at the same time as much variability as possible [94].

Hierarchical Cluster Analysis (HCA): This method is based on the analysis of a distance matrix between all samples. The method could start with all samples in separated clusters (or

all samples in the sample cluster) and search for those that are most similar (or dissimilar), then, those similar (dissimilar) samples are merged (separated) and the distance matrix is updated accordingly. This approach basically allows to build a tree (dendrogram) that reflects the distance between all the samples. For the current work the distance matrix was built upon on the Euclidean distance, the agglomerative procedure was based on complete linkage. The distance matrix was computed over the full rank principal component scores space.

Both data analysis approaches (PCA and HCA) were performed in the R language environment [161].

3.4. Results and discussion

A PCA was performed on the first derivative from all spectra from the bacteria grown in TSA in order to do a preliminary data exploration and visualization (Figure 10). The scores scatter plot from PC1 *vs* PC2 shows that bacteria present in positive PC1 are better discriminated than the ones in negative PC1. Bacteria from the same genera are grouped together. It is noticeable that the two *Listeria* species are placed together, as well as the two *Aeromonas hydrophila*. All of the Gram-positive analysed bacteria are present in positive PC1. It was expectable that the Gram-positive bacteria were easily distinguished from the Gram-negative, as their external envelops present differences in structure and composition. Gram-positive bacteria have a thick cell wall composed by peptidoglycan, whereas Gram-negative cell have an outer membrane of phospholipids, lipopolysaccharides and proteins covering the thin peptidoglycan layer from the cell wall. However, it has been previously found that spectra from Gram-negative and Gram-positive bacteria are very similar, suggesting that the main functional chemistry groups of both bacterial surfaces are similar [162].

However, and despite the good group formation obtained in the exploratory PCA, it would be difficult to predict accurately an unknown bacteria which would appear in negative PC1.

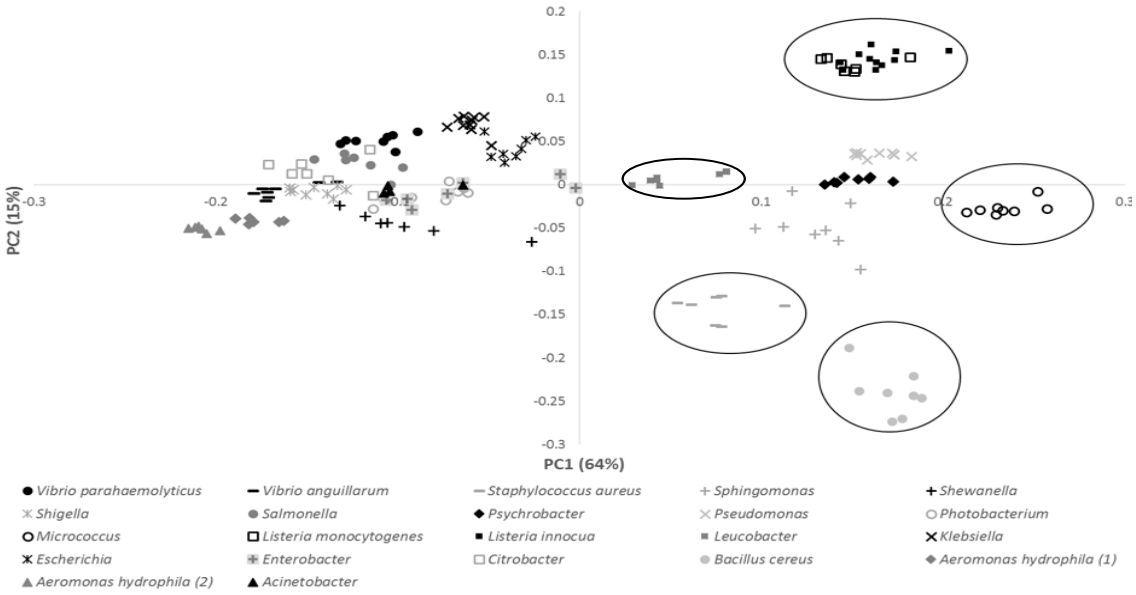

Figure 10: Principal component analysis scores scatter plot of the analysed bacteria (PC1 vs PC2). The bacteria were obtained from TSA agar and the spectra were treated with first derivative. The spectral region used was between 3000-2400 cm^{-1} and 1900-700 cm^{-1}. Bacteria surrounded by a circle are Gram-positive and all the others are Gram-negative.

Despite of the promising results from PCA and aiming to a better discrimination and insights into bacterial genera and species, a hierarchical clustering analysis was performed (Figure 11). A good separation of the different genera and species is observable. *Vibrio parahaemolyticus* is separated from *Vibrio anguillarum, Listeria monocytogenes* is separated from *Listeria innocua* and even the two species of *Aeromonas hydrophila* are placed in different clusters. Therefore, using these two approaches, a successful distinction of nearly all the bacteria analysed was accomplished with exception of *Photobacterium* and *Acinetobacter* which were placed in the same cluster node. These two microorganisms were overlaid in the PCA scores plan (Figure 10). In order to find a distinction between them, diverse spectral ranges were tested in PCA. The region between 1480 and 1200 cm^{-1} shows a better separation in this particular case (Figure 12). In principle, with these three statistical approaches (PCA, HCA, PCA) it was possible to distinguish all of the 22 studied bacteria, without the necessity of using selective media and biochemical/serological confirmation or molecular approaches.

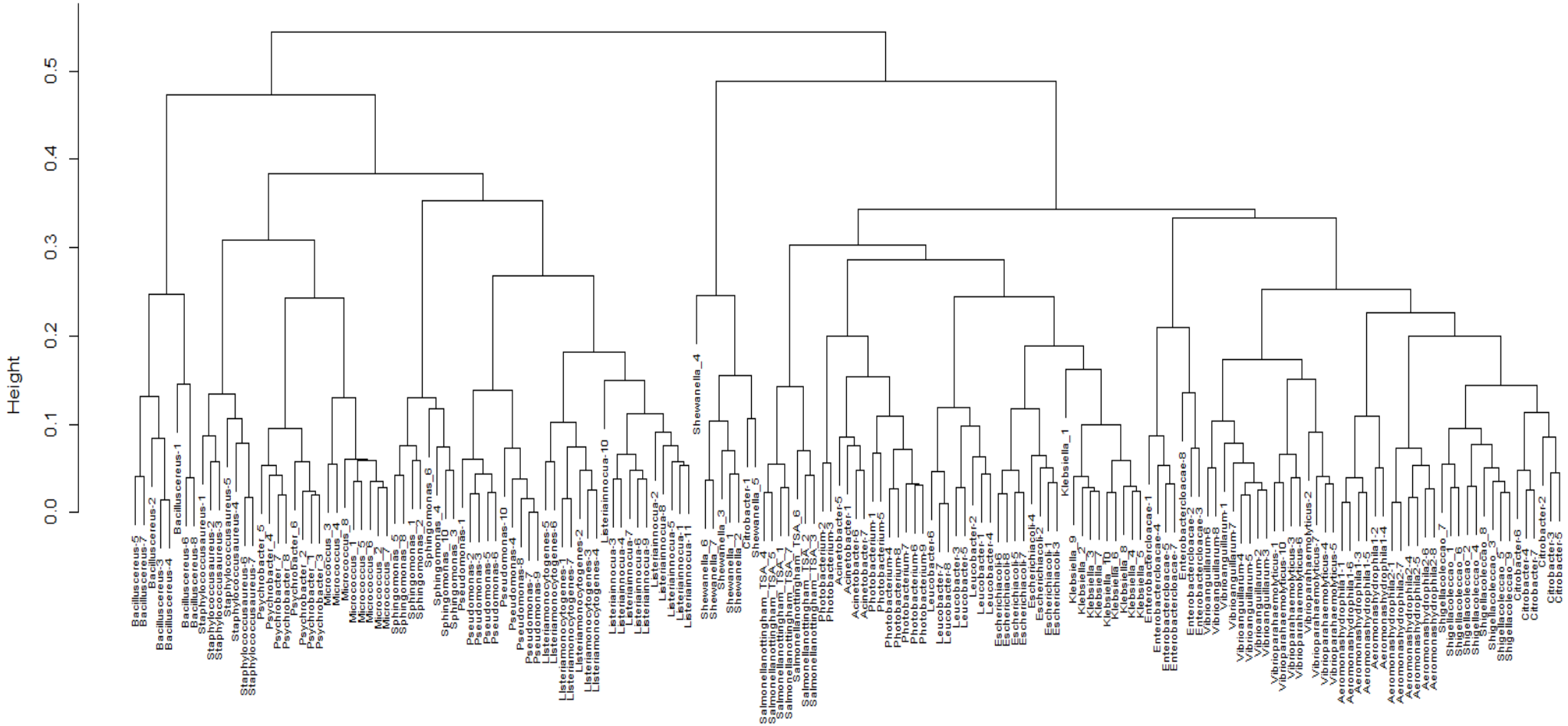

Figure 11: Hierarchical cluster analysis dendrogram of spectra obtained from each bacteria in the spectral region of 3000-2400 cm^{-1} and 1900-700 cm^{-1}.

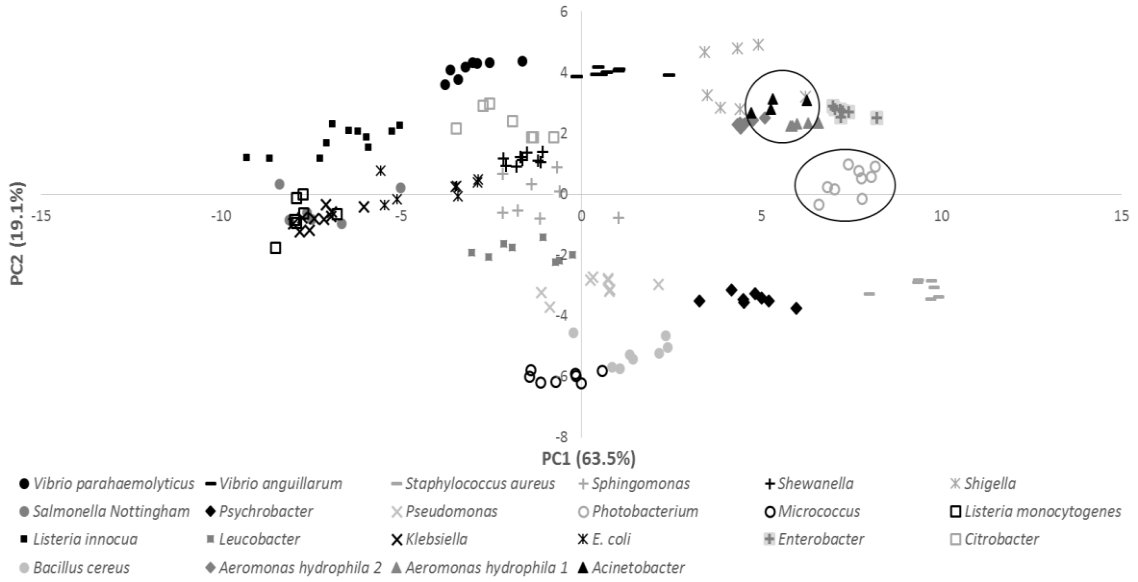

Figure 12: Scores scatter plot (PC1 vs. PC2) in the spectral region of 1480 – 1200 cm^{-1}, of bacteria grown in TSA agar. Acinetobacter and Photobacterium are surrounded by a circle.

In order to further develop the discrimination between bacteria, these were grown in selective solid media used in food industry, as follow: VRBD (selective growth medium for enterobacteria), BPA (detection of *S. aureus*) and TCBS (isolation of *Vibrio* spp.). PCA from the spectra were performed after spectra obtainment.

Current European legislation uses the Enterobacteriaceae as a parameter in process hygiene criteria, referring ISO 21528 [163] as mandatory analytical method. Suspect bacteria grown in VRBD medium have to be confirmed, and this step includes a streaking of the colonies on nutrient agar for 24 hours and then the realization of the biochemical tests (oxidase and glucose fermentation step), of which the glucose fermentation step takes 24 hours more. In this way, the confirmation step takes 2 additional days of analysis, becoming also more expensive because there is need to use more reagents. PCA of the spectra shows that all bacteria grown in VRBD (Figure 13) are well grouped and discriminated. Therefore, MIR spectroscopy can be used to discriminate the enterobacteria grown in VRBD agar, instead of the time taking and expensive confirmation steps used in food control laboratories to detect enterobacteria.

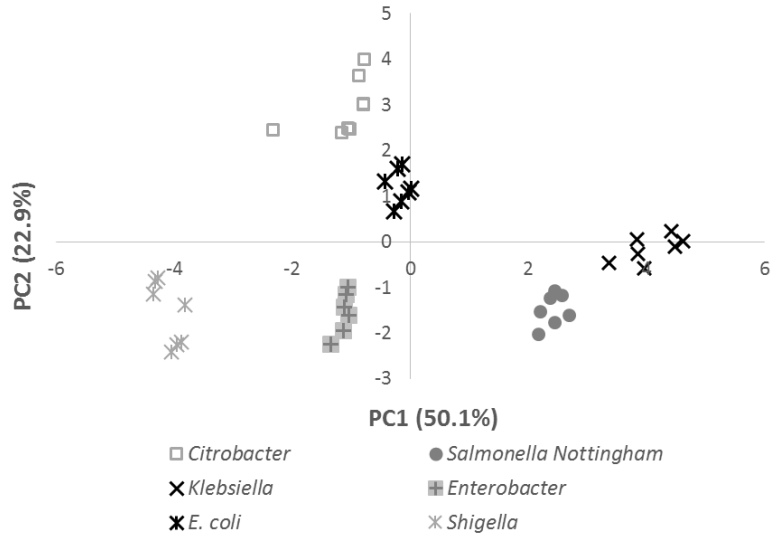

Figure 13: Scores scatter plot (PC1 vs. PC2) in the spectral region of 3000-2400 cm^{-1} and 1900-700 cm^{-1}. Bacteria grown in VRBD agar.

BPA agar is used in food industry in order to assess the presence of *S. aureus.* This species can cause gastroenteritis due to production of enterotoxins in foods by improper handling and subsequent storage at high temperatures. The illness is usually self-limiting but in some cases it can require hospitalization. This bacterium grow as grey-black colonies surrounded by an opaque halo and a clear zone. According to ISO 6888 [164], the suspect colonies have to be confirmed, following some steps, including anaerobic incubation in mannitol salt agar (MSA) and brain-heart infusion (BHI) agar. Additional confirmation has to be performed and the suspect colonies grown on MSA and BHI are tested for coagulase and thermo nuclease activities. These confirmation steps last for 3 additional days, after the step of BPA agar [165], being time and reagent consuming. In order to assess if MIR spectroscopy allows to discriminate between *S. aureus* and other species able to grow also on BPA agar, a PCA was performed on the obtained spectra (Figure 14). Once again, the results demonstrate that this technique allows to identify bacteria grown in BPA media instead of the confirmation steps.

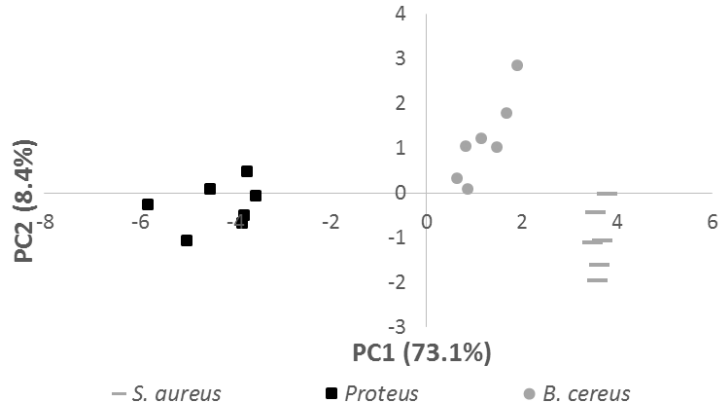

Figure 14: Scores scatter plot (PC1 vs. PC2) in the spectral region of 3000-2400 cm^{-1} and 1900-700 cm^{-1}. Bacteria grown in BPA agar.

Enteropathogenic *Vibrio* spp. can cause gastrointestinal illness associated to consumption of contaminated seafoods and, in severe causes, some species can even cause septicaemia. According to ISO 21872 [166], its detection includes an initial enrichment step followed by streaking in two selective media: thiosulphate citrate bile salts sucrose agar (TCBS) and triphenyltetrazolium chloride soya tryptone agar (TSAT). After 1 day of incubation, presumptive colonies (green on TCBS and red on TSAT) have to be confirmed, which takes 2 or 3 additional days. The 2 *Vibrio* species and *Klebsiella* and *Enterobacter,* which also grow in this media, were well discriminated by mid-infrared spectroscopy, forming individualized groups (Figure 15).

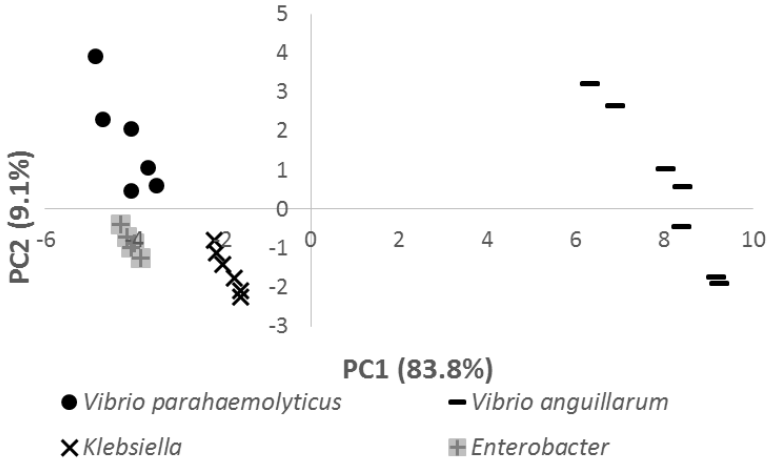

Figure 15: Scores scatter plot (PC1 vs. PC2) in the spectral region of 3000-2400 cm^{-1} and 1900-700 cm^{-1}. Bacteria grown in TCBS agar.

3.5. Conclusions

Mid infrared spectroscopy in tandem with multivariate analysis (PCA and HCA) has been successfully used for identification of bacteria. In this work we present results that show the high potential of these hyphenated techniques and the possibilities of exploring the spectral information as maximum as possible. As in the case of the microbiology work at the laboratory, using different kinds of media for confirming the identification of the bacteria, it is possible to use, as well, different multivariate treatments to further distinguish and finally identify the microorganisms. It is absolutely indispensable because the more bacteria species/serovars are involved, more challenging is the distinction with just one multivariate analysis tool. The spectra may be acquired directly from the colonies obtained in a given growth media and then different multivariate tools/wavelengths ranges can be applied to the spectra. These different tools will help to confirm or distinguish between bacteria that are overlaid in one or two of the other approaches. With these results we suggest that the confirmation of bacteria can be performed immediately after isolation in general purpose solid media: TSA for all genera, VRBD for enterobacteria, BPA for *S. aureus* and TCBS for *Vibrio* spp. Taking this into account, the confirmation of bacteria can be shortened in two or more days than with the traditional confirmation methods and in an inexpensive way than with molecular methods, as no reagents are needed after the growth of bacteria in solid media.

The use of MIR spectroscopy for discriminating bacteria immediately after growth in general purpose media or selective/differential media could be very valuable for food industry, as it would allow the immediate identification of several bacterial species, without the necessity to perform the confirmation steps that are time and reagent consuming, providing rapid and accurate results.

Chapter 4.

MIR spectroscopy coupled with multivariate analysis as alternative method for further detection of *Salmonella* spp. and *Listeria monocytogenes*

MIR spectroscopy coupled with multivariate analysis as alternative method for further confirmation of *Salmonella* spp. and *Listeria monocytogenes*

Catarina Moreirinha[a,b], Joana Trindade[c], Jorge A. Saraiva[b], Adelaide Almeida[a], Ivonne Delgadillo[b]

[a] *Department of Biology, CESAM, University of Aveiro, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal*

[b] *Department of Chemistry, QOPNA, University of Aveiro, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal*

[c]*Labinter - Laboratório Alimentar, Lda. Alameda Pêro da Covilhã, Lote 4 – R/C Dto 6200-507 Covilhã, Portugal*

*Corresponding author. Tel. +351 234370718

E-mail address: ivonne@ua.pt

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4.1. Abstract

Listeriosis and Salmonellosis are two of the most common foodborne diseases, and so, early and accurate detection of *Listeria monocytogenes* and *Salmonella* spp. in food products is a critical concern in public health policies. After growth in selective media, the process of final biochemical and serological confirmation for these pathogens delays the attainment of the results and is a laborious, expensive and time consuming procedure. So, it is of extreme importance to develop rapid, easy and cheap methods in order to replace this currently used methodology. Mid-infrared spectroscopy has been successfully used to confirm *Listeria* species and to confirm the presence of *Salmonella* within other bacteria that also grow in the same selective media. This methodology showed to be very sensitive and rapid as an alternative to detect these important pathogens, allowing to obtain results in a few minutes after previous growth in selective media.

Keywords

Infrared spectroscopy, MIR, Principal component analysis, *Listeria*, *Salmonella.*

4.2. Introduction

The microbiological quality of food has become a very important factor for food industries as well as for the regulation agencies. The accurate reliable detection and identification of microorganisms in food is critical to detect microorganisms early on to prevent their transmission, avoiding infections and/or food poisoning.

Listeria monocytogenes and *Salmonella* spp. are some of the most important and common foodborne pathogenic microorganisms, causing public health problems in almost all industrialised countries.

The genus *Listeria* is constituted by six species: *L. monocytogenes*, *L. innocua, L. ivanovii*, *L. seeligeri*, *L. welshimeri* and *L. grayi*. All of them are widespread in the environment and *L. monocytogenes* is considered an opportunistic serious foodborne pathogen for humans, causing listeriosis, which is a significant public health hazard [167]. Although rarely, human infections due to *L. ivanovii* and *L. seeligeri* have also been reported [168]–[170]. Identification of *Listeria* at the species level in routine laboratories is time-consuming, laborious and expensive. Selective primary and selective secondary enrichment followed by isolation in selective differential media and suspect colonies confirmation by biochemical tests is needed. Biochemical methods such as sugar fermentations, API system and CAMP test are used to confirm the presence of *Listeria*. However, the results are only available after 7 days, of which 2 days are required for the confirmation procedures [171]. In addition to this, the incidence of false-negative results is still a considerable problem, despite of the recent advances in these methodologies [172], [173].

The genus *Salmonella* includes two species: *S. enterica* and *S. bongori*. *S. enterica* is further divided into six subspecies, including *S. enterica enterica*, that includes more than 2500 serovars [174], [175]. Salmonellosis disease typically resolves in about 6 days and does not require treatment with antibiotics. However, bacteremia occurs in 3 to 10 percent of reported culture-confirmed cases and is particularly common among patients at the extremes of age and those who are immunocompromised [176], [177]. Cultivation methods for the detection of *Salmonella* are also laborious, time consuming and expensive. A non-selective pre-enrichment is needed, followed by a selective enrichment and posterior plating in selective and differential solid media. Posteriorly, suspect colonies have to be confirmed by biochemical or serological tests as referred in ISO 6579:2002 [178], [179]. Biochemical substrate utilization is the basis of species identification for *Salmonella*, even though considerable variation can be observed in

the biotyping pattern. The majority of *Salmonella* are recognized as non-lactose fermenters (Lac^-) and hydrogen-sulphide producers (H_2S^+), thus, confirmatory testing of all H_2S^+ and/or Lac^- colonies is required, which extends the time for identification. The majority of the H_2S^+ and Lac^- colonies turn out not to be *Salmonella enterica*, but related species such as *Citrobacter* [180]. This severely limits the effectiveness to provide a rapid response to the presence of this pathogenic bacterium. The results are only available after 5 days, of which 2 days are necessary for the confirmation steps.

During the last years, molecular and immunological procedures have been developed to detect the presence of *Listeria* and *Salmonella* in food, but most of them are limited [174], [181]–[184]. These methods, despite being rapid, are yet expensive, requiring high skilled personnel, and can be affected by the presence of certain substances in food products. This can lead to an underestimation of bacterial concentration or false negative results [152], [156]. Moreover, the legislation requires that positive results for the presence of *Salmonella* and *Listeria* in foods must be confirmed by traditional culture methods and further confirmation [171], [179]. Taking these aspects into account, it is very imperative to develop sensitive and specific faster methods to detect/identify these two foodborne pathogenic microorganisms in order to prevent illnesses associated to food consumption.

Vibrational spectroscopic techniques, infrared spectroscopy (IR) and Raman spectroscopy, have been used since the 1980s as complementary methods for bacteria differentiation owing to their rapid “fingerprinting” capabilities and the molecular information that they can provide. These techniques present several advantages in the microbiological classification and identification fields. They are fast (requiring virtually no sample processing), non-destructive, multi-purpose (e.g., detection, enumeration, classification, identification) and discriminating at different taxonomic levels (serotype, strain, species or genus) [185]–[188].

Identification of *Listeria* species and *Salmonella* serovars using FTIR spectroscopy has been undertaken previously [118], [119], [189], [190]. However, these studies included some time consuming cell pre-treatments, such as growth of the bacteria in liquid medium with subsequent centrifugation and washing with distilled water. In this study, for the first time, it was evaluated the potential of mid infrared spectroscopy (MIR), to confirm the presence of *Listeria monocytogenes* and *Salmonella* spp. in food products, after plating in the selective recommended media. For this, *Listeria a*nd *Salmonella* (identified by API system) isolated from food products (cheeses, sausages and prepared dishes) were used. The colonies that grew in OCLA medium (*Listeria*) and XLD medium (*Salmonella*) were directly analyzed by Horizontal attenuated total reflection Mid-infrared spectroscopy (HATR-MID-IR). Multivariate analysis of

the spectra was used to discriminate the different *Listeria* species. Moreover, the potential to discriminate *Salmonella* from other bacteria that grew on XLD *Salmonella* selective medium and could be mistaken for *Salmonella* spp. (e. g. *Shigella flexneri, Pseudomonas aeruginosa* and *Citrobacter freundii*) was also evaluated.

4.3. Materials and methods

4.3.1. Bacterial strains

Listeria spp. and *Salmonella* serovars were isolated from food products (cheeses, sausages and prepared dishes) according to ISO 6579:2002 and ISO 11290-1:1996, respectively.

Briefly, for the isolation of *Listeria* spp., a primary enrichment of the samples in half-Fraser broth (1:10), for 24h at 30°C was done. Then, a secondary enrichment was performed by transferring 0.1 mL of the previous culture to a tube with Fraser broth that was incubated for 48h at 37°C. The cultures obtained in the primary enrichment were transferred to *Listeria* identification agar base (PALCAM, Oxoid Lda., UK) or Oxoid chromogenic *Listeria* agar (OCLA, Oxoid Lda., UK) medium. PALCAM and OCLA plates were incubated at 37°C and examined after 24h or, if necessary, 48h to check for the presence of *Listeria* characteristic colonies. Confirmation of the presence of *Listeria* spp. was carried out by selecting 5 presumed *Listeria* colonies and plated in tryptone soya yeast extract agar (TSYEA, Oxoid Lda., UK). The plates were incubated at 37°C during 18-24h. Typical colonies, colourless with an opaque halo, were then confirmed with catalase test. If the morphological and physiological characteristics indicate the presence of *Listeria* spp., a hemolysis test was performed in Columbia agar 5% (Oxoid Lda., UK) to investigate which species of *Listeria* was present. Biochemical API *Listeria* and RAPIDEC *L. monocytogenes* identification kits (Biomerrieux, France) were also used to identify the *Listeria* species.

For *Salmonella* spp. isolation, a pre-enrichment in BPW (buffered peptone water) for 18h at 37°C was made. Then, 0.1 mL of the previous culture was transferred to Rappaport-Vassiliadis with soya agar (RVS, Oxoid Ltd., UK) and 1 mL to Muller-Kauffmann tetrathionate novobiocin (TTmk, Oxoid Ltd., UK). Samples were incubated for 24 h at 42°C (RVS) and 37°C (TTmk) respectively. After incubation, samples were plated in xylose lysine deoxycholate agar (XLD, Merck, Germany) and brilliant green agar (BGA, Merck, Germany) in order to select typical colonies of *Salmonella* spp. which are red with a black centre. The positive colonies were identified by API system (API 20E, Biomerrieux, France). Finally, OMNI-O antiserum test (Bio-Rad, USA) was performed in which *Salmonella* spp. shows positive agglutination.

Three different species of *Listeria*: 4 isolates of *L. monocytogenes,* 2 isolates of *L. ivanovii* and 4 isolates of *L. innocua* and three serovars of *Salmonella enterica*: 2 isolates of

serovar *S.* Nottingham, 2 isolates of serovar *S.* Anatum and 2 isolates of serovar *S.* Liverpool were obtained from different food products (cheeses, sausages and prepared dishes).

Shigella flexneri DSM-4782, *Citrobacter freundii* NCTC-6272 and *Pseudomonas aeruginosa* isolated on our laboratory [191] were also used in this study, as they are food pathogens that also grow in XLD agar and can be mistaken for *Salmonella* due to possible colour similarities.

For subsequent analysis, *Salmonella* isolates, *C. freundii*, *P. aeruginosa* and *S. flexneri* were plated in XLD agar and *Listeria* sp. in OCLA agar. *Listeria monocytogenes* and *Salmonella* Nottingham were also plated in Trypticase soy agar (TSA, Merck, Germany) for a preliminary analysis. Plates were incubated at 37°C for 18 hours.

4.3.2. Mid-infrared spectroscopy

Spectroscopic acquisition was performed in an infrared spectrometer (Bruker Alpha Platinum) with a resolution of 4 cm^{-1} and 32 scans, in the mid-infrared region (4000-600 cm^{-1}). Analyses were performed in a room with controlled temperature (25°C) and humidity (29%). Microbial colonies grown in agar medium: TSA for *Salmonella* Nottingham and *L. monocytogenes* preliminary analysis, XLD for *Salmonella*, *Shigella, Citrobacter and Pseudomonas* and OCLA for *Listeria*.

The colonies were collected with a loop and placed directly on the crystal of a 2 mm x 2 mm horizontal single attenuated total reflectance (ATR) accessory and were dried under gentle cold air flow for 10 seconds. At least three replicate spectra were obtained for each sample. The sampling accessory was cleaned with ethanol (70%) and distilled water between each measurement.

4.3.3. Multivariate analysis

The spectra (obtained in OPUS format) were transferred via JCAMP.DX format to an in-house developed data analysis package (CATS build 97) (Barros 1999). Principal component analysis (PCA) was used to find the major sources of variability in data, detect outliers and detect the probable presence of clusters. Previous to PCA, the spectra were standard normal deviate (SNV) corrected.

4.4. Results and discussion

4.4.1. Mid-infrared spectra of the bacteria

Observing the spectra of *L. monocytogenes* (Gram-positive) and *S.* Nottingham (Gram-negative) grown in TSA agar (Figure 16) we can see that both spectra are apparently very similar, dominated by bands from proteins and carbohydrates. A previous study, using infrared spectroscopy, reported that spectra of Gram-positive and Gram-negative intact cells were very similar, as in our case, and suggests that the bulk functional chemistry group of both bacterial surfaces are similar [162]. This fact does not agree with the general understanding that the external coat from Gram-positive and Gram-negative bacteria have significant structural and chemical differences between them.

In Gram-positive bacteria, peptidoglycan is a major component of the cell wall and corresponds to the external layer, whereas Gram-negative bacteria exhibit an additional external membrane, denominated outer membrane, which is an asymmetric membrane, the inner leaflet containing only phospholipids, while the outer leaflet contains lipopolysaccharides with Lipid A and the various pore-forming proteins, the porins [96]. It is generally assumed that mid-infrared spectroscopy (MIR) is a surface sensitive technique and it has been reported that the spectra from the intact bacterial cell may represent the surface macromolecular composition [42]. The apparent similarities between the obtained spectra, do not mean that the chemical composition of the outer layers is exactly the same, and it is evident that, in general, the spectral profile shows small but consistent differences along the spectra. These differences may be explained taking in account the chemical composition of the cell wall from Gram-positive and of the external membrane of the cell wall from Gram-negative bacteria.

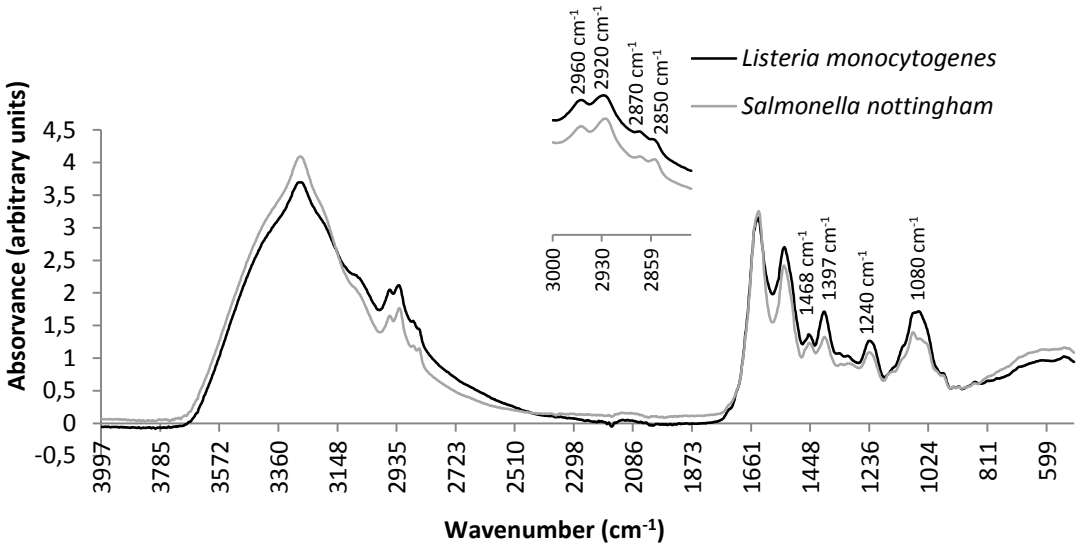

Figure 16: MIR average spectra of L. monocytogenes and S. Nottingham *obtained from TSA agar in the spectral region between 4000 and 500 cm^{-1}.*

We studied the case of a Gram-positive (*L. monocytogenes*) and a Gram-negative (S. Nottingham) bacteria in order to perceive the spectral differences between them.

The cell wall of *L. monocytogenes* is composed of a thick peptidoglycan layer, without outer membrane, formed by glycan chains containing alternating units of the dissacharide N-acetylmuramic bound to a stem peptide containing L-alanine-γ-D-glutamic-acid-*meso*-diaminopimelic acid-D-Ala-D-Ala [192]. Isolated dry cell walls of *Listeria* species consist of about 35% peptidoglycan [40]. This peptidoglycan layer contains two types of anionic polymers: the teichoic acids, which are covalently linked to the peptidoglycan and the lipoteichoic acids which are polymers of phosphoglycerol substituted, with a D-alanyl-ester or a ribitol residue [193], [194]. On the other hand, *S.* Nottingham is a Gram-negative bacterium that possess an outer membrane, in which the LPS consists of lipid A covalently bound via 3-deoxy-D-mannooctulosonic acid to a polysaccharide domain which is subdivided into an oligosaccharide portion (core) and a chain of polymerized repeating units of O antigen [141]. LPS in an amphiphilic molecule with several hydrophobic fatty acyl groups in the hydrophobic lipid A and with the hydrophilic polysaccharide part possessing negatively charged phosphate and carboxyl groups present mainly in the inner core polysaccharide. LPS is firmly linked to the surface of Gram-negative bacteria, being lipid A the anchor of LPS in the bacterial envelope. LPS-phospholipid and LPS-LPS hydrophobic bonds, as well as the chelating effects of divalent cations (e.g. Ca^{2+}), stabilize the outer membrane structure [195].

Observing the spectra (Figure 16) one can notice that *L. monocytogenes* spectrum presents more CH_3 signals visible at 2960, 2870 and 1397 cm^{-1} comparing with *S.* Nottingham which shows to have more CH_2 groups (2920, 2850 and 1468 cm^{-1}). This is consistent with the fact that the chemical structure of the outer membrane lipid A presents numerous CH_2 groups when compared with the *L. monocytogenes* cell wall which should show CH_3 signals from the numerous alanine molecules not only found in the lipoteichoic acid but also in the peptides crosslinking the peptidoglycan chains. Furthermore, differences in the spectral region under 1800 cm^{-1} are also visible, specially between 1330 and 980 cm^{-1} as it is expected, as in this region, a subtle difference in the chemical constituents can lead to a unique spectral fingerprint in the MIR region [151].

Looking to the region between 1700 and 1500 cm^{-1}, one can see clearly the amide I and amide II bands (1650 and 1550 cm^{-1}, respectively) however, the profile shows some differences, which were not unexpected. Both bacteria have peptidoglycans originating amide signals, but *Salmonella* presents additionally the mentioned external layer constituted by lipid A and porins, having both, also, amide groups. It is known that amide I and II bands are very sensitive to conformational alterations in the peptide backbone [196]. The spectral profile in the amide I and II region reflects the differences in protein composition between the two types of bacteria. Between 1500 and 1300 cm^{-1} a peak is visible at 1397 cm^{-1}, that can be assigned to the symmetric bending mode of the CH_3 in *Listeria*. The phosphate asymmetric stretching modes appear at 1240 cm^{-1} and the correspondent symmetric stretching modes should appear around 1080 cm^{-1}. This last band is very broad as it results of the overlapping of the phosphate signal with the bands arising from the saccharides in the fingerprint region from 1200 to 900 cm^{-1}. According to the spectra, *Listeria* has more phosphate groups than *Salmonella*, which makes sense looking to the chemical composition of the teichoic and lipoteichoic acids (polyglycerolphosphate) in *Listeria* [192]. However, one cannot discard the possibility that nucleic acids may be contributing also to these signals.

4.4.2. Listeria species discrimination

OCLA agar medium allows the visual differentiation of the pathogenic species (*L. monocytogenes* and *L. ivanovii*) from the non-pathogenic species (*L. innocua*). Pathogenic species colonies present blue to green colour surrounded by an opaque halo that does not exist in the case of the non-pathogenic species. However, ISO 11290:1-1996 specifies that the

biochemical confirmation based on haemolytic activity and carbohydrate fermentation should be performed. *L. monocytogenes* and *L. ivanovii* possess haemolytic activity, whereas *L. innocua* does not have this capacity. Regarding carbohydrate fermentation, it is known that in aerobic conditions *L. monocytogenes* and *L. innocua* utilize glucose, lactose and rhamnose, while *L. ivanovii* ferments xylose [197]. In this study, instead of the biochemical confirmation step, MIR was used to assess the species distinction.

The isolate bacterial strains of *Listeria* spp. obtained in OCLA agar (Figure 17) showed similar spectra to the one shown in Figure 16 for *L. monocytogenes* grown in TSA agar. Regarding species distinction, Figure 17 shows the spectral profile from the three species under study. Despite their similarity, some spectral differences are visible in the region between 1800 and 980 cm^{-1}, consequently, this was the region chosen to perform the discrimination by principal component analysis.

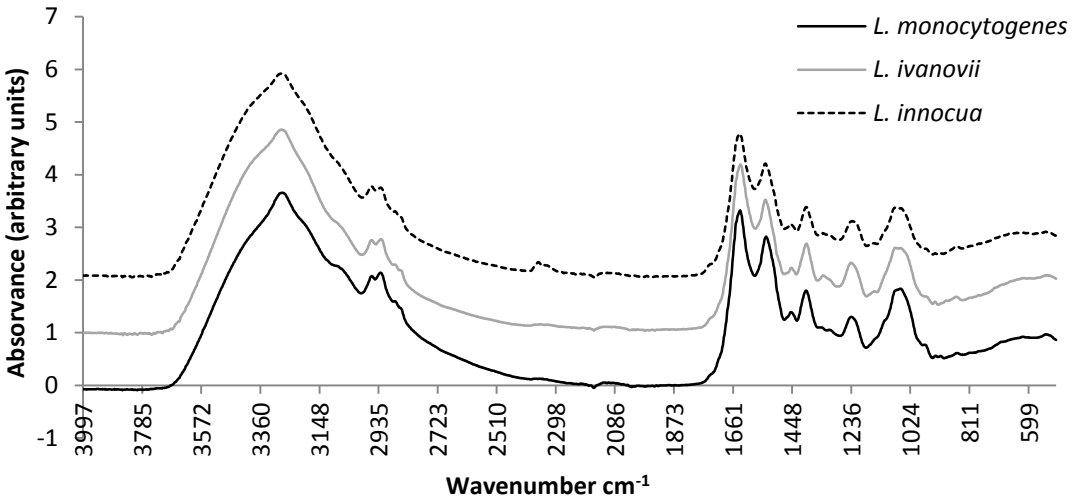

Figure 17: MIR average spectra of L. monocytogenes, L. ivanovii and L. innocua obtained from OCLA agar in the spectral region between 4000 and 500 cm^{-1}.

PCA analysis (Figure 18) revealed 3 different clusters. *L. monocytogenes* is located on negative PC1, mainly characterized by a peak at 1517 cm^{-1}. *L. innocua* and *L. ivanovii* are located on positive PC1, both characterized by a broad band between 1700 and 1570 cm^{-1} and a peak at 1200 cm^{-1}. *L. innocua* is also located at positive PC2, characterized mainly by a peak at 1666 cm^{-1} and another at 1505 cm^{-1}, and *L. ivanovii* is located at negative PC2, with the most significant peaks at 1615 cm^{-1}, 1330 cm^{-1}, 1240 cm^{-1} and 1035 cm^{-1}.

In this case, it was possible to discriminate the 3 different studied *Listeria* species after growth in OCLA agar. Regarding the chemical structure of the cell wall of the different species, there is few information. In consequence, it is difficult at this stage to make specific assignments to the discriminatory peaks. However, taking in account the discussion above, differences on the surface proteins, with influence on the species virulence, would contribute to the found discrimination (peaks between 1700-1500 cm^{-1}). It is in accordance with studies showing that surface protein patterns are specific for species and even serovars [198]. These observations indicate observed that not only the proteins may have a significant paper on the species distinction, but also the saccharides and phosphates were responsible for the obtained distribution changes (peaks between 1300 and 980 cm^{-1}). For example, modifications on the polyglycerolphosphate, like their substitution by D-alanyl or ribitol, their degree of polymerization and in consequence the quantity of total phosphate, would be enough to justify some of the spectral differences. Taking these results into account, MIR spectroscopy allowed the rapid differentiation of the 3 *Listeria* studied species without the necessity of performing the biochemical confirmation step, after obtaining the colonies in the selective medium.

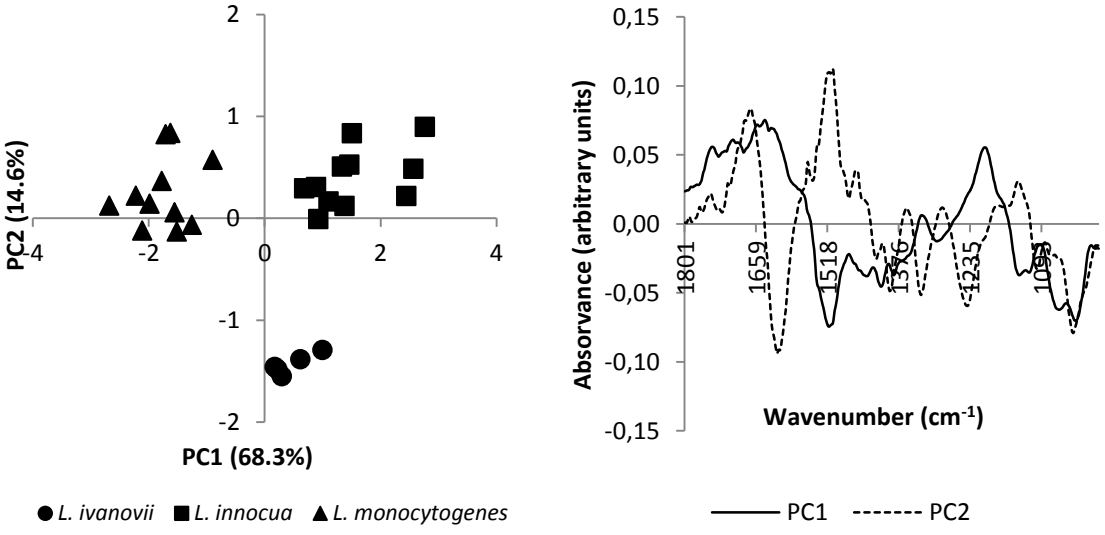

Figure 18: Principal component analysis (PC1 vs PC2) of L. *ivanovii, L. monocytogenes* and *L. innocua spectra in the region between 1800 and 980 cm^{-1}. Left: Scores scatter plot. Right: Loadings plot profile.*

4.4.3. Salmonella identification

XLD agar offers the possibility of selecting *Salmonella*-like colonies because the medium contains indicators of H_2S production and pH changes. *Shigella* spp. are genetically close to *Salmonella* spp. and *Citrobacter* spp. [199]. *Citrobacter* generally grows as yellow colonies, different from *Salmonella,* however, there are some works reporting that *C. freundii* and other *Citrobacter* species produced false-positive colonies [200], [201]. Likewise, *P. aeruginosa* and *Shigella* spp. can produce colonies very similar to those produced by some *Salmonella* species on XLD media, leading to misinterpretation of the results obtained [202], [203].

The mid infrared spectra from the isolated bacterial strains of *Salmonella* serovars, *C. freundii, P. aeruginosa* and *S. flexneri* obtained in XLD agar showed similar profiles (Figure 19). However, as in the case of *Listeria* species, some spectral differences are visible at the region between 1800 and 980 cm^{-1}, consequently, this was the chosen region to perform principal component analysis in order to discriminate them.

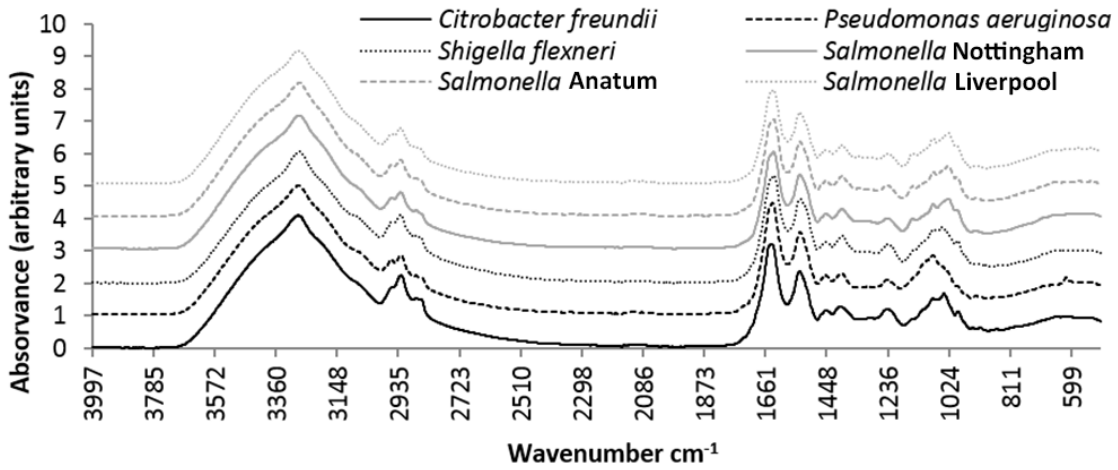

Figure 19: MIR average spectra of C. *freundii, P. aeruginosa, S. flexneri*, S. Nottingham, S. Liverpool *and* S. Anatum *obtained from XLD agar in the spectral region between 4000 and 500 cm^{-1}.*

The PCA of the three *Salmonella* serovars, *C. freundii, P. aeruginosa* and *S. flexneri* are shown in Figure 20. One can see five distinct groups: *S.* Anatum *and S.* Liverpool were grouped together and *S. flexneri, S.* Nottingham*, C. freundii* and *P. aeruginosa* formed distinct groups. *S.* Anatum*, S.* Liverpool and *S. flexneri* were located on negative PC1. *C. freundii, S.* Nottingham and *P. aeruginosa* were located on positive PC1. *C. freundii* on negative PC2 separates from *S.*

Nottingham and *P. aeruginosa,* both on positive PC2. One can observe that the most significant peaks that contributed to the distinction of the studied species were in the region between 1200 and 980 cm^{-1}, region of saccharides and phosphates, compounds that may differ at the surface of the different studied species/serovars. As is known, IR spectra of bacteria provide not only the absorption bands that describe molecular composition of the cells, but many of these bands are also sensitive to structure changes, numerous intra and inter-molecular interactions including the hydrogen bonding pattern, membrane constitution, lipid-protein interactions, and conformational states as the secondary structures of proteins [96]. So, also in this case, it is difficult to make an accurate assignment of the spectral bands to structural compounds of bacteria external membrane or cell wall.

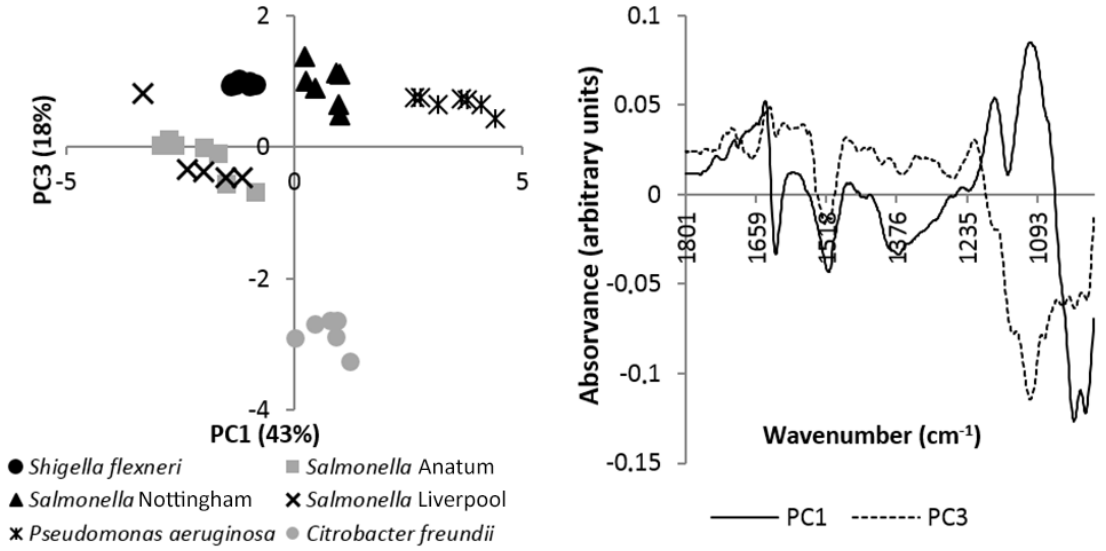

Figure 20: Figure 5: Principal component analysis (PC1 vs PC3) of S. enterica serovars (Anatum, Nottingham and Liverpool)*, S. flexneri and C. freundii and* P. aeruginosa *spectra in the region between 1800 and 980 cm^{-1}. Left: Scores scatter plot. Right: Loadings plot profile.*

Despite the existence of distinct groups, it was intended to obtain a better separation of *Salmonella* serovars and *S. flexneri*. So, as the latter is distributed in the same quadrant that *S.* Anatum and *S.* Liverpool, a PCA with *Shigella* and *Salmonella* serovars was performed, and it was found a good separation between *Shigella* and *Salmonella* (Figure 21).

S. flexneri is separated from the other bacteria in the negative PC2. *S.* Anatum, *S.* Liverpool and *Shigella* are separated from *S.* Nottingham in the positive PC1. Loadings plot profile shows that the distribution of the species is mainly characterized by peaks in the region

between 1700-1500 cm^{-1} and 1200-980 cm^{-1}, that may have the contribution of protein, phosphate and saccharide differences between the studied species/serovars.

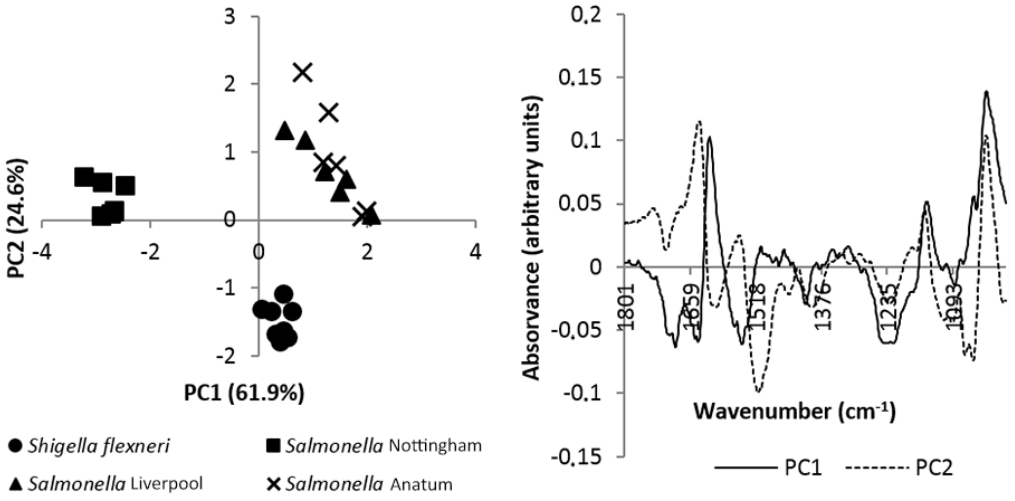

Figure 21: Principal component analysis (PC1 vs PC2) of S. enterica serovars (Anatum, Nottingham and Liverpool) *and S. flexneri spectra in the region between 1800 and 980 cm^{-1}. Left: Scores scatter plot. Right: Loadings plot profile.*

4.5.Conclusions

In general, the studied bacteria were discriminated by peaks corresponding mainly to polysaccharide (1200-980 cm^{-1}) and protein (1700-1500 cm^{-1}) spectral regions. This was expectable, as the cellular surface of the different species and serovars studied differ in these compounds. Some signals, corresponding to DNA phosphates, could, also, be important in the discrimination of the bacteria.

So, in this study, mid-infrared spectroscopy has proved to be a very useful method for the confirmation of *Salmonella* spp. and *Listeria* spp. By currently used methods, this confirmation step is expensive and takes another 2 days of analysis using the traditional biochemical and serological methods. The methodology used in this work is very easy to perform (the colonies obtained in specific agar media are directly placed on the MIR sampling accessory without any handling), rapid (a few seconds to obtain a spectra) and considerably inexpensive, taking in account that MIR spectrometers from very good quality and not expensive are available nowadays. The other advantage is to avoid the use of additional expensive chemicals.

Under the research point of view, infrared spectroscopy, associated to multivariate analysis, can be very useful as quick monitoring technique, helping to identify targets and to follow studies on the cell wall and external membrane composition.

Chapter 5.
High-pressure processing effects on foodborne bacteria by mid-infrared spectroscopy analysis

High-pressure processing effects on foodborne bacteria by mid-infrared spectroscopy analysis

Catarina Moreirinha[a,b]; Adelaide Almeida[a]; Jorge A. Saraiva[b]; Ivonne Delgadillo[b]

[a] *Department of Biology, CESAM, University of Aveiro, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal*

[b] *Department of Chemistry, QOPNA, University of Aveiro, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal*

*Corresponding author. Tel. +351 234370718

E-mail address: ivonne@ua.pt

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5.1. Abstract

High pressure processing causes minimal changes in the characteristics of the fresh foods, while produces microbiologically safe foods and prevents food spoilage and foodborne diseases on the consumers, being an emergent technology for food preservation nowadays.

In this work, 12 different foodborne bacteria were submitted to high pressure processing (300 MPa, 15 min, room temperature) in order to assess the bacterial reduction and the alterations induced with this processing in cellular molecules. IR spectroscopy was used to investigate the differences in the spectra of the cells before and after the high pressure treatment. It was observed that all the Gram-negative bacteria were inactivated to undetectable levels while Gram-positive bacteria showed resistance to pressure, being *Staphylococcus aureus* and *Bacillus cereus* the most resistant bacteria, decreasing only 2 logs. *Listeria innocua*, in turn, showed intermediate resistance, as it decreased about 5 logs.

Regarding cellular modifications triggered by the high pressure treatment, it was possible to notice that modifications in hydrogen bonds appear to be on the basis of the modifications observed in the cell spectra after high pressure processing.

There appear to be changes in protein region, and in peaks assigned to the C-H stretching vibration corresponding to CH_3 and CH_2 of amino acid side chains, lipids and oligopolysaccharides. Bands assigned to the PO_2^- groups, which may correspond to the phosphate groups from the cell wall and to the backbone of nucleic acids, were also modified with the HPP treatment. These results indicate that HPP processing may cause cellular envelope disruption both on Gram-positive and Gram-negative bacteria, being the second more affected.

Keywords

Infrared spectroscopy, high pressure processing, foodborne bacteria.

5.2. Introduction

Fish and meat have a high nutritional value and are considered easily perishable foods, as they can be contaminated from the environment and/or during its processing. In the case of fish, the high post mortem pH attained in the flesh, the high water activity and the presence of free amino acids and nucleotides make this food a readily available bacterial growth substrate [204], [205]. The same happens in meat, in which the high water activity, moderate pH and readily available sources of energy, carbon and nutrients, make this product ideal for microbial growth [12].

Food preservation methods have been successfully used to preserve fish and meat products, such as cooking, canning, curing or freezing. However, many of the characteristics of fresh fish and meat are lost with these processing methods. High-pressure processing (HPP) is an emerging food preservation method that offers numerous advantages over other food processing procedures. This procedure inactivates or reduces spoilage and pathogenic bacteria, but nutrients, flavour and colour remain largely unaffected, allowing the production of foods with almost complete retention of their nutritional and sensory qualities. Moreover, as pressure is transmitted uniformly independent of the shape or size of the food, it is ensured that the whole food is adequately processed [3], [66]. It is known that high pressure does not alter the low-energy covalent bonds, which have low compressibility and does not break these bonds within the ranges habitually applied in high pressure processing of food. As a consequence, the primary structure of molecules such as proteins and fatty acids remains intact, however, modifications may occur in secondary, tertiary and quaternary structures, for instance in the form of protein unfolding. The resistance of microorganisms to pressure varies considerably depending on the pressure range applied, temperature and duration of the treatment, as reviewed by some authors [206], [207]. The inactivation of bacteria by HPP is the result of a combination of factors and cell membranes are the primary sites that are damaged by pressure, altering cell permeability, transport systems, loss of osmotic state, organelle disruption and inability to preserve pH [21]. There are other components and cellular functions sensitive to high pressures that are modified or inhibited, such as the ribosome, protein synthesis, and enzyme activity [71], [208]. However, nucleic acids are relatively resistant to high pressures and as the structure of the DNA helix is largely the result of hydrogen bond formation, it is also stable under pressure [81]. It is known that Gram-positive bacteria are more resistant to high pressures than Gram-negative cells [67], [75], [209]. Gram-positive cells have a thick cell wall constituted by peptidoglycan that is less affected than the thinner peptidoglycan cell wall overlaid by the outer membrane of Gram-negative cells. The cell

membrane is considered to be a primary site of pressure damage in microorganisms, which affects the cell integrity [80], [81]. Apparently, the double layered phospholipids of the external membrane are packed tightly in the compression stage, promoting the transition to a gel state. During decompression, the membrane structure is lost and pores are formed. In order to maintain its functions and properties, the membrane should preserve its fluid state, which is determined by the composition of unsaturated fatty acids. High pressure reduces fluidity on cell membranes due to the increasing packing of the fatty acyl chains of phospholipids [80], [82]. Factors such as cells growth phase or age tend to influence the resistance to high pressure as well. However, the physiological state of bacteria does not appear to be a significant factor if the HPP treatment is applied in order to inactivate all microorganisms of safety concern.

Rapid methods such as mid-Infrared spectroscopy (MIR) have been successfully used to analyse food microorganisms [128], [140]. Infrared spectra of microorganisms are very complex fingerprint-like patterns typical of each different bacteria [111], [140], [210]. This methodology studies the interaction of the infrared radiation with samples representing an "image" of their chemical composition. There are studies in which cell components can be identified by IR spectroscopy. Differences in cell lipids and proteins after ultraviolet irradiation of bacteria were investigated by this technique and spectral modifications were detected [211], [212]. The objective of this work was to use MIR in order to identify modifications in the cellular components of bacteria isolated from food samples after HPP processing. This approach intends to develop a fast and effective technique to screen food pathogens in foods before and after HPP processing, in order to understand at which extent pressure damages the cell structure in the studied bacteria.

5.3. Materials and methods

5.3.1. Isolation of bacteria from fish, meat and cooked ham

Three pieces of hake (*Merluccius merluccius*), 3 pieces of dried salted cod (*Gadus morhua*), 3 pieces of pork meat and cooked ham (*Sus scrofa domesticus*), 3 pieces of chicken meat (*Gallus gallus domesticus*) and 3 pieces of cattle meat (*Bos taurus*) were obtained in 3 different commercial surfaces.

Cod was desalted in sterile distilled water (fish:water ratio, 1:10) during 24 hours at 4°C before microbial analysis and the water was changed 3 times (every 8 hours) to simulate the soaking method adopted by the consumers. Cod was analysed immediately after desalting procedure. Hake, pork, chicken and cattle meat and ham were analysed immediately after the sample acquisition.

A total of 18 randomly selected sub-samples were aseptically cut: 3 samples of hake, 3 samples of cod, 3 samples of pork meat, 3 samples of pork ham, 3 samples of chicken meat and 3 samples of cattle meat.

5.3.2. Bacteria quantification

Each food sub-sample was aseptically homogenized with an Ultra-Turrax (T25, Janke & Kunkel - IKA Labortechnik) in Ringer's Solution (Merck) (1:10, weight: volume). One hundred microliters of serially diluted samples were pour plated, in duplicate, in Tryptic Soy Agar (TSA) medium (Merck), Violet Red Bile Dextrose Agar (VRBD) medium (Merck) and in Thiosulfate Citrate Bile Salts Sucrose Agar (TCBS) medium (Merck). Cod samples were additionally pour plated in TSA medium supplemented with 3% NaCl. After 3 days of incubation at 37°C, some colonies presenting different morphologies (colour, shape, size and density) were selected. The colonies were purified by three repeated streaking steps on TSA plates.

5.3.3. Bacteria identification

For the identification of the selected colonies, bacterial DNA was extracted using the Instagene Matrix (Biorad, USA). 16S rDNA was amplified using the universal 27f forward primer (5'-AGAGTTTGATCCTGGCTCAG-3') and 1512r reverse primer (5'- CGGCTACCTTGTTACGACT-3').

The reaction occurred in a Multigene Gradient Thermal Cycler (MIDSCI). The reaction mixture contained 1 µL of bacterial DNA, 3.75 µL of MgCl2, 2.5 µL of KCl buffer, 2.5 µL of dNTP, 0.25 µL of each primer, 0.5 µL of BSA, 1 µL of Taq polymerase (MBI Fermentas, Lithuania) and Milli-Q water (Millipore). The PCR running conditions included a 5 minutes initial denaturation of template DNA at 94°C, 25 denaturation cycles at 94°C for 1 minute, annealing at 55°C for 2 minutes, extension at 72°C for 2 minutes and a final extension at 72°C for 10 minutes. The selected PCR amplicons were purified using Ron's PCR-Pure purification kit (BIORON, Germany). Automated DNA sequencing was performed by GATC biotech (Konstanz, Germany) and the sequences were analysed using BLAST database.

Acinetobacter, Aeromonas hydrophila, Bacillus cereus, Enterobacter, Klebsiella, Staphylococcus aureus, and *Pseudomonas aeruginosa* were selected. *E. coli* NCTC 10418, *Listeria innocua* NCTC 11288, *Photobacterium damselae damselae* DSM-7482, *Salmonella enterica* sv. Nottingham NCTC 7832 and *Vibrio anguillarum* DSM-21597 from culture collections were also used as they are important pathogenic bacteria that can infect fish, meat and cooked ham. Bacteria were streaked on TSA medium (Merck) and were used after 18 hours of incubation at 37°C.

5.3.4. High pressure processing

For determination of microbial concentration before and after HPP, bacterial mass was weighted and diluted with appropriate amount of Ringer solution (Merck) in order to posteriorly determine the colony forming units (CFU) per mL. This mixture was very well mixed and 500 µL were inserted in polyethylene tubes without air. The tubes were afterwards inserted in a polyamide-polyethylene bag (PA/PE-90, Albipack-Packaging solutions, Portugal), manually heat-sealed (vacuum packager Packman, Albipack, Águeda, Portugal), in order to avoid as much as possible to leave air inside the bags. Finally, these bags were inserted in another similar bag filled with sodium hypochlorite solution to avoid contamination of the pressure vessel, in case the bag containing the bacteria leaked. For each bacteria, 3 samples were prepared. In this study, all the bacteria used were grown for 18 hours and were at the stationary phase of growth (10^8-10^9 CFU/g), at which bacteria tend to be more resistant to inactivation by most of the food processing methods [67].

For mid-infrared spectroscopy analysis, the colonies were carefully collected from the petri dishes with a sterilized loop and placed into polyamide-polyethylene bags (PA/PE-90, Albipack-Packaging solutions, Portugal), manually heat-sealed (vacuum packager Packman,

Albipack, Águeda, Portugal), in order to avoid as much as possible to leave air inside the bags. The bags were previously sterilized with UV radiation (BioSafety Cabinet Telstar Bio II Advance, Terrassa, Spain). Each bag was inserted in another bag and afterwards in a third bag, this one filled with sodium hypochlorite solution to avoid contamination of the equipment. For each bacterium, 3 samples were prepared.

HPP treatments were carried in a hydrostatic press (high-pressure system U33, Unipress Equipment Division, Poland), in a pressure vessel of 35 mm diameter and 100 mm height, at room temperature (≈ 21°C), using as pressurizing fluid a mixture of water and propylene glycol. Two independent HPP treatments were made in different days to assure that the results were reproducible, being in each day analysed 3 samples for each.

Petri dishes containing 30-300 CFU were selected for counting and the results were expressed as logarithm of CFU. The CFU were determined before and after HPP treatment in order to know the effect of the pressurization on bacterial counts.

5.3.5. Mid-infrared spectroscopy and data analysis

Spectroscopic acquisition was carried out in a MIR (Bruker ALPHA Platinum-ATR FTIR Spectrometer) with a resolution of 4 cm^{-1} and 32 scans, in the mid-infrared (region between 4000 and 600 cm-1). Microbial colonies were collected from the petri plates with TSA medium or the bags submitted to HPP with a loop and placed directly on the crystal of the horizontal single reflection ATR accessory. The colonies were gentle air-dried with a cold flow and then measured. At least six replicate spectra were obtained for each sample.

Difference spectra (HPP treated minus control) were calculated by use of OPUS 5.0 (Bruker, Germany). In the resultant difference spectra, upward moving bands correspond to bands that appear or increase intensity after the high-pressure treatment, while downward-moving bands correspond to bands that disappear or decrease intensity after the treatment. Standard deviation spectra of the samples before and after high pressure treatments were calculated in OPUS 5.0 to assess the modifications that occurred after pressurization. The relative height of the bands assigned to amide II (≈ 1540 cm^{-1}) and amide III (≈ 1400 cm^{-1}) were measured in OPUS 5.0 from the standard deviation spectra. Second derivatives of the spectra were calculated using a Savitsky-Golay procedure [160] with a 2^{nd} degree polynomial and a 15 points wide-window. Each spectral signal was normalized by standard normal variate (SNV).

5.4. Results and discussion

5.4.1. Microbial concentration before and after HPP treatment

The inactivation of bacteria by HPP is probably the result of a combination of various factors. It is assumed that HPP does not damage a unique cellular component. In fact, increasing pressure compromises several important cellular functions, leading to loss of cell viability.

Figure 22 shows the results for the total viable counts before and after high pressure processing at 300 MPa during 15 minutes at room temperature. Bacteria counts decreased to undetectable levels for all the Gram-negative analysed bacteria: *Acinetobacter, Aeromonas, E. coli, Enterobacter, Klebsiella, Photobacterium, Pseudomonas aeruginosa, Salmonella* and *Vibrio anguillarum*. On the other hand, the Gram-positive bacteria included in this study (*Bacillus cereus, S. aureus* and *L. innocua*) showed resistance to high pressure. The higher resistance of Gram-positive bacteria compared to Gram-negative cells was reviewed by some authors [67], [71], [75], [213]. We observed that *B. cereus* and *S. aureus* showed great resistance, decreasing only approximately 2 logs. These two species are two of the most well-studied bacteria regarding the use of high pressure processing and have been reported to be pressure resistant [67]. A study in which HPP was used to inactivate a strain of *S. aureus* reports that pressure treatment must be combined with high temperatures in order to effectively destroy this pathogen [214]. Another study reports that *Listeria innocua*, also a Gram-positive bacteria, showed intermediate resistance to pressure, decreasing nearly 5 logs from an initial population of 9 logs. It is in accordance with a study in which *L. innocua* could also not be totally inactivated at room temperature at 300 MPa during 15 minutes [215].

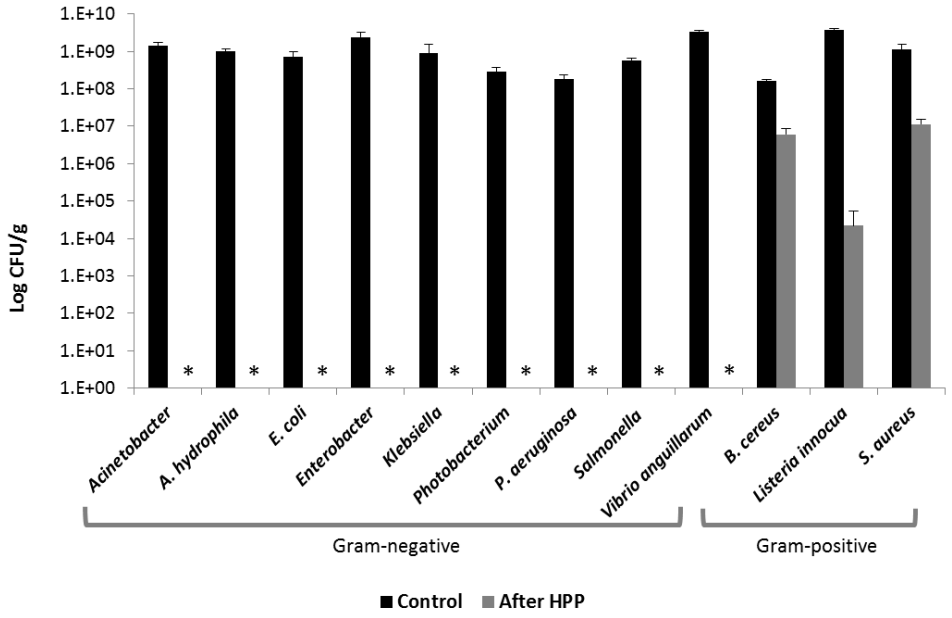

*Figure 22: Colony forming unit counts (log CFU/g) of the bacteria isolated from fish (hake and desalted cod), meat (pork, bovine and chicken) and cooked pork ham samples, before and after HPP treatments of 300 MPa during 15 minutes at room temperature. * - Bacteria inactivated to undetectable levels after pressurization.*

5.4.2. Mid-infrared spectroscopy analysis of food bacteria before and after HPP treatment

Despite of all research on the changes induced by pressure treatments on bacterial cells, the mechanisms of microbial inactivation are far from being full understood. Taking this into account, we performed a study by MIR spectroscopy to try to follow the changes in the cellular components, by means of obtaining spectra before and after HPP treatment of 300 MPa during 15 minutes at room temperature (Figure 23). Difference spectra were obtained in order to better understand the changes originated by high-pressure treatment, but as the difference spectra are quite similar among all of the studied bacteria, only the example from *Salmonella* is shown (Figure 24), in which can be observed that only the signal at 3400 cm^{-1} is decreasing whereas all others remain.

The spectra of the bacteria before and after high pressure processing (Figure 23) are very similar between them, being dominated by protein-related bands, what was not unexpected, as it is known that in stationary cells the ratio protein/lipids increase [216]. The most relevant bands are: i) a large band between 3700 and 2700 cm^{-1}, centred at 3278 cm^{-1}, corresponding to amide A and O-H from water and containing the peaks from C-H stretching vibrations in the region of 3000 to 2800 cm^{-1}); ii) a band with a maximum at around 1634 cm^{-1},

which is assigned to the amide I vibration with a component of the H-O-H deformation from water, iii) the amide II band, with a peak around 1540 cm^{-1}.

More than 95% of the amide A band (3278 cm^{-1}) is due to the N-H stretching vibration. This vibration mode is independent of backbone conformation but is very sensitive to the hydrogen bond stretching. Pressure induces a slightly decrease at 3400 cm^{-1}, and an increase around 3100-3040 cm^{-1}. This last broad peak is assigned to amide B band and together with the amide A are part from the Fermi resonance doublet originated by resonance with combinations of amide II modes [217]. The decrease in the peak of 3400 cm^{-1} would correspond to the reduction of hydrogen-bonded water, since the symmetric and antisymmetric stretching vibration of non-hydrogen bonded water absorb between 3630 and 3760 cm^{-1}. A considerable number of water molecules remain associated with the dried proteins, and it is known that in protein films with little free water, the stretching vibration from water appears at 3400 cm^{-1}, as in our case [218]. The results indicate that the pressurized cells are more dehydrated than the controls.

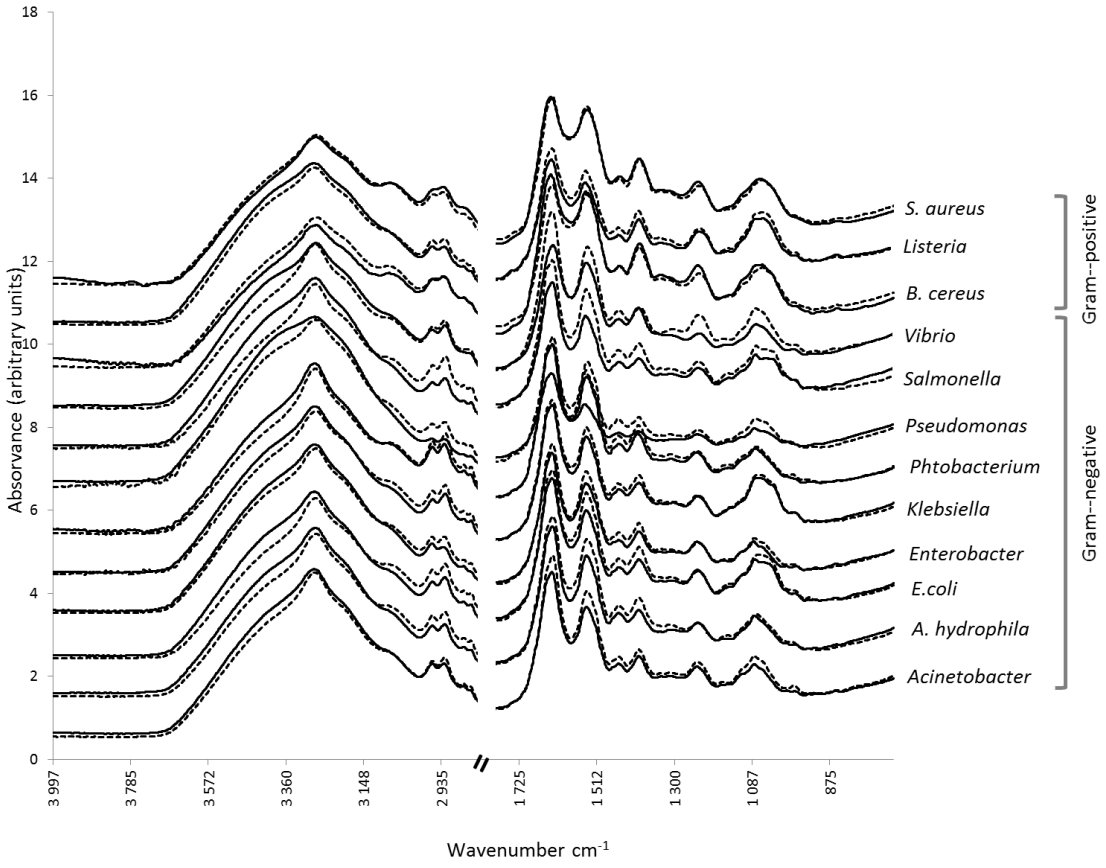

Figure 23: MIR spectra of the dried bacteria before and after high pressure processing, in the spectral region between 4000-2800 cm^{-1} and 1800-800 cm^{-1}. Black lines correspond to untreated bacteria and dashed black lines corresponds tobacteria treated by HPP.

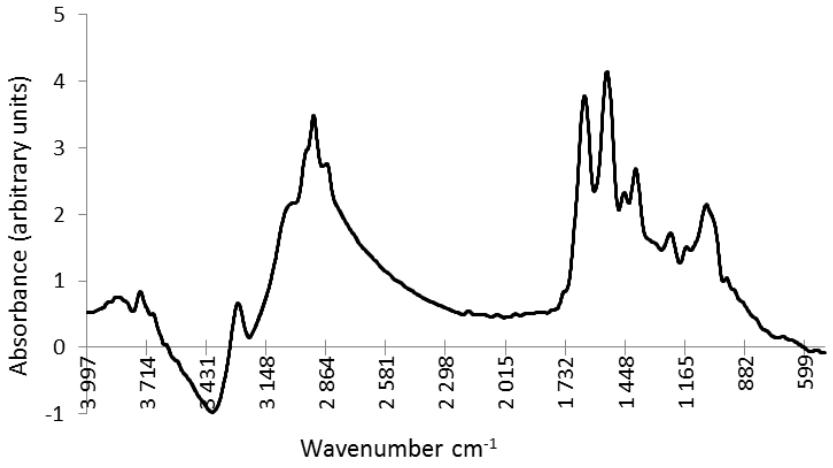

Figure 24: Difference spectrum from Salmonella between HPP treated and control, in the spectral region between 4000 and 500 cm^{-1}.

In general, spectra from all bacteria after high pressure treatment show only small changes in the protein region, between 1700 and 1500 cm^{-1}. The amide I band, reportedly centred near to 1650 cm^{-1}, is the most intense absorption band in proteins and in our case, as mentioned above, appears around 1634 cm^{-1}. In our case, cells have a mixture of proteins that cannot be considered as a single one. This signal is primarily originated by the stretching vibration of the C=O (70-85%) and C-N groups (10-20%) and is sensitive to the structure of the protein backbone and consequently to modifications in the secondary structure [218]. After high pressure treatment, this maximum shifts by about 5-6 cm^{-1} to lower frequencies. Changes of this order have been associated to the formation of protein aggregates [219]. It was previously stated that bands at a wavenumber lower than 1630 cm^{-1} are not common in native proteins, and that this kind of band found in concanavalin A was assigned to peptides with an extended configuration, with a hydrogen-bonding pattern formed by peptide residues not taking part in intramolecular β-sheet, but rather hydrogen-bonded to other molecular structures [220].

The amide II vibration of proteins is hardly affected by side chain vibrations, but the correlation between protein secondary structure and frequency is less straightforward than the amide I vibration. It has been related to hydrogen bonds, as the N-H bending contributes to amide II mode [221]. In our case, the form of the peak from amide II varies and shoulders appear at lower frequencies, but the maxima (determined by second derivative) are around 1543 cm^{-1} before and after high pressure, occurring an intensity increase in the HPP treated bacteria.

Taking a closer insight into the spectra, one can see signals from the C-H stretching vibrations from CH_3 (≈ 2960 cm^{-1}) and CH_2 (≈ 2930 cm^{-1}) corresponding to the amino acid side chains, lipids and oligopolysaccharides. These peaks are more evident in HPP treated cells. Consistently with this fact, a very small increase in the signals at 1740 cm^{-1} (C=O in esters) is visible in subtraction spectra between before and after high pressure in 6 of the bacteria (*Acinetobacter, Klebsiella, Pseudomonas, Salmonella, B. cereus* and *Listeria*). It may indicate some disruption of the outer and/or cell membrane. A study reported that pressurized exponential-phase cells from *E.coli* stained with a lipophilic dye show visible disruption of the envelopes, showing the formation of vesicles of lipidic material coming out of the cells [87]. Our cells were in stationary phase, and the time of treatment at 300 MPa was of 15 min, which is different from the 8 min used in the study just cited, however, it cannot be ruled out that in certain extend this effect can occur also in stationary phase cells, as well.

The signals under 1500 cm^{-1} are difficult to interpret. A peak around 1400 cm^{-1} is present in all of the studied bacteria and enhances with high pressure treatment. It could correspond to the amide III mode. This mode is the in-phase combination of N-H bending and CN stretching vibration with small contributions from C=O in plane bending and C-C stretching vibration.

There are also two peaks around 1240 cm^{-1} and 1080 cm^{-1} which are consistent with the bands corresponding to the antisymmetric and symmetric stretching vibrations from PO_2^- groups, respectively, which may correspond to the phosphate groups from the cell wall and to the backbone of nucleic acids. Hydrogen bonding to PO_2^- groups lowers the observed band position of the two stretching vibrations [221]. In our case a lowering around 5 cm^{-1} is observed. In this region one can expect to find, also, the peaks from polysaccharides and sugars from nucleic acids that are surely overlaid with the phosphate signals. The region between 1200 cm^{-1} and 900 cm^{-1} is very variable between the different bacteria. This region has been used to discriminate between bacteria [111], [210].

It is difficult to quantify the changes that occurred after HPP treatment, especially to differentiate the behaviour between Gram-positive and Gram-negative bacteria. When one wishes to know how similar or repeatable are the spectra from the replicates, a treatment of averaging with the functionality of storing the standard deviation (SD) spectra can be performed, which quantifies the spectral changes in an scale of dissimilarity/similarity [222], [223]. It was decided to apply this treatment to each set of bacteria in order to observe the dissimilarity between the control and HPP treated bacteria (Figure 25). The resulting standard deviation spectra were different for each bacterium, with the Gram-positive bacteria showing small standard deviations and the Gram-negative showing larger variabilities. Within Gram-

positives, *S. aureus* and *B. cereus* present the lowest peak intensities, even than *L. innocua,* being this consistent with the results from the surviving cells after pressurization.

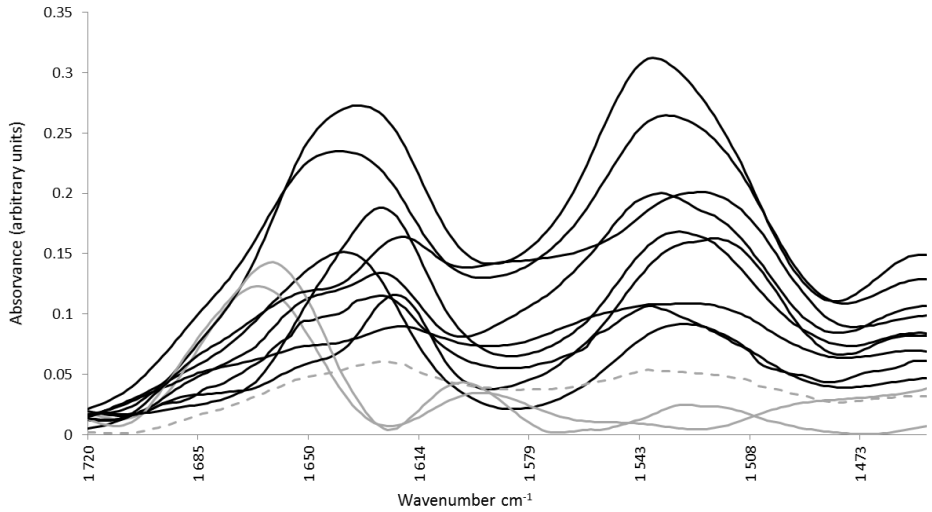

Figure 25: Standard deviation spectra of the bacteria before and after HPP treatment, in the region between 1720 and 1450 cm^{-1}. Black lines correspond to Gram-negative bacteria, grey lines correspond to S. aureus and B. cereus and the dashed grey line corresponds to L. innocua.

A detailed observation of the SD spectra showed that the amide II peak presented the largest variability in intensity, conserving its position between 1540 and 1520 cm^{-1} in all samples. In addition, the amide II peak is considered to be sensitive to the hydrogen bonding and it is not affected by water. Taking this into account, it was decided to choose this peak as marker of the total modification of the spectra between control and HPP-treated bacteria. On the other hand, the amide I peak, which is sensitive to water, appeared at different frequencies for each bacterium, between 1665 and 1610 cm^{-1}. It may indicate the modifications in the secondary structure of proteins, but it is difficult to know in which extend it may have also contributions from the residual bonded-water.

The intensities from the amide II peak and the peak at around 1400 cm^{-1} were measured from the SD spectra and are presented in Figure 26. It can be seen that the Gram-positive bacteria (*S. aureus*, *B. cereus* and *L. innocua*) show lower variation in the amide II band than the Gram-negative bacteria. This was interpreted as reflecting better stability from the Gram-positive cells towards the HPP treatment. In the case of *L. innocua,* in which about 50% of the cells survived, one can observe an intermediate behaviour when compared to Gram-negative bacteria. A study in which *L. monocytogenes* cells were submitted to 400 MPa during 10 minutes reports that some cell surface presented bud scars after the treatment and the cell

wall integrity was damaged [224]. It was also shown in this study that the membrane integrity was not homogeneous in the cellular population, although high-pressure treatments are considered to be isostatic. Apparently cellular damage is not equally withstood by all the cells, suggesting that less damaged cells are present in the pressurized bacteria.

It was observed that the peak at ≈1400 cm^{-1} showed a correlation with the amide II peak in the Gram-negative bacteria, which could indicate that this peak may correspond to amide III, which also has an N-H component. In the case of *S. aureus* and *B*. *cereus* the peak at around 1400 cm^{-1} is higher than the small peak corresponding to amide II, however, as in this region peaks from other groups appear, they may overlay the small changes from protein origin.

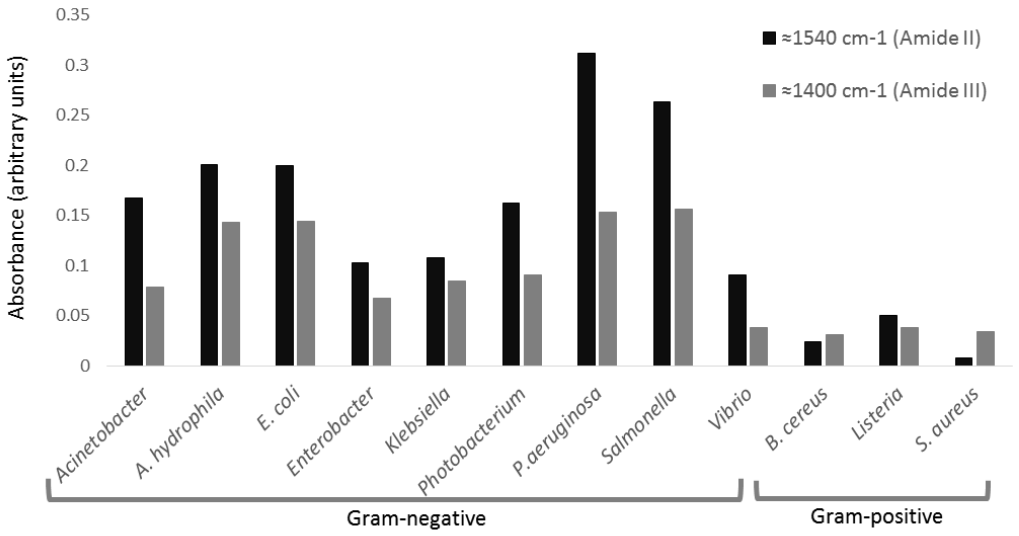

Figure 26: Relative height of the peaks of the standard deviation spectra between HPP treated and control, at around 1540 cm^{-1} (assigned to amide II) and 1400 cm^{-1} of the spectra after high-pressure processing at 300 MPa, during 15 minutes at room temperature for all of the studied bacteria.

5.5. Conclusion

In this study, as expected, all the Gram-negative studied bacteria were inactivated to undetectable levels by an HPP treatment of 300 MPa, during 15 minutes, at room temperature. On the other hand, Gram-positive bacteria showed resistance to inactivation by HPP, being *S. aureus* and *B. cereus* the most resistant bacteria, decreasing only 2 logs from an initial population of 8-9 logs. *Listeria innocua*, in turn, decreased 5 logs. Infrared spectroscopy reproduces this behaviour and the spectra showed modifications after pressure treatment. The fact that a decrease in the water signal was observable may indicate a disruption of the cell envelope/cell membrane leading to cellular content loss. The band of amide II, which is sensitive to hydrogen bonding, can be used as marker, in the SD spectra, for the magnitude of the effect of high pressure.

Looking to the overall interpretation of the results, changes in hydrogen bonds appear to occur on the basis of modifications that can be observed by mid infrared spectroscopy, which in turn should reflect the changes/interactions in the cell structure underling the inactivation of bacteria by high pressure or their resistance.

Chapter 6.
Conclusions and future work

6.1. Conclusions

In this thesis, infrared spectroscopy was explored in the analysis of food bacterial identification and concentration determination.

Regarding the determination of bacterial concentration in food, it was possible to detect different levels of contamination in cooked ham samples that were naturally contaminated. The results obtained using MIR and PLS were correlated with the total aerobic plate count in agar culture media, being the former procedure faster, easier and cheaper to apply. The total aerobic plate count takes at least 24 hours of incubation to obtain the results and is a widely used methodological analysis in food industries to obtain an indication of the quantity of bacteria in a food sample. In this work, in which the smears are taken directly from the food surface, it was shown that MIR can provide results within few minutes, for the two different brands of cooked ham analysed. This methodology can be also suitable to assess the bacterial concentration of other foods, after development of a calibration model.

As the identification of foodborne bacteria is crucial to prevent food spoilage and illness associated to food consumption, MIR, coupled with multivariate analysis, was tested in order to perform bacteria identification. This methodology allowed the classification of the analysed foodborne bacteria in groups accordingly to their genus or species. Principal component analysis (PCA) was used to perform a preliminary exploratory visualization of the data that revealed that most of the bacteria were organized by genus. Then, the hierarchical cluster analysis (HCA) allowed to classify the bacteria that were not discriminated by PCA. This suggests that PCA and HCA, used simultaneously, easily allow to classify bacteria within few minutes. Using selective/differential media, bacteria were also well grouped and discriminated, using only PCA. Taking this into account, MIR spectroscopy, in tandem with multivariate analysis, showed to be a suitable, more rapid and inexpensive technique to replace the traditional identification methodologies based in the enrichment, isolation and confirmation by biochemical/serological methods used in the food industries to identify bacteria or the molecular tools based in DNA/RNA analysis.

Additionally, *Listeria monocytogenes* and *Salmonella* spp. were also quickly confirmed by this methodology. These two pathogens obligatory need biochemical and/or serological confirmation after growing in specific and differential media, as stated in ISO 6579:2002 and ISO 11290-1:1996, which delays the obtention of the results for up to 2 days. The three *Listeria* spp. studied were well discriminated in PCA. *Salmonella* serovars were more difficult to

discriminate between them, in spite of being well separated from the other tested species. However, as all of the *Salmonella* serovars are pathogenic for humans, their distinction was not important for our purpose. Also in this case, infrared spectroscopy showed to be very sensitive and rapid as an alternative to detect these important pathogens, allowing to obtain results in a few minutes after previous growing in selective/differential media.

Concerning the cellular compounds analysis, IR spectroscopy revealed to be a suitable methodology to identify modifications in cell composition after high pressure processing. This processing technology is an emerging food preservation method that intends to satisfy the increasing demand for fresh-like products with minimal degradation of the nutritional and organoleptic properties. It was suggested that the hydrogen bonds are on the basis of the cellular modifications that occur during pressurization. There appear to be modifications in protein region, as well as in bands assigned to the C-H stretching vibration corresponding to CH_3 and CH_2 present in amino acid side chains, lipids and oligopolysaccharides. Bands assigned to the PO_2^- groups, which may correspond to the phosphate groups from the cell wall and to the backbone of nucleic acids, were also modified with the HPP treatment. These results indicate that HPP processing may cause cellular envelope disruption both on Gram-positive and Gram-negative bacteria, being the latter more affected.

Infrared spectroscopy demonstrated to be usefull to bacterial quantification, as well as for identification, confirmation and detection of the intensity of changes due to high pressure treatments. These kind of applications of mid-infrared spectrometry could be very useful for the food industry and control organisms, since it is a method that can be explored for making the preliminary assessment and monitor the bacterial quality of foods in factories, storage centres or commercial surfaces, providing rapid and accurate results, substituting laborious and expensive methods that are currently used. Moreover, nowadays exist portable infrared spectrometers at reasonable prices and, additionally, no reagents are needed to perform the analysis of these kind of samples, making this methodology relatively inexpensive. Under the research point of view, infrared spectroscopy can be very helpful in the study of cellular composition, particularly the cell wall and external membrane.

6.2. Future work

Some suggestions for future work may be raised. It is important to study other important foodborne bacteria in order to enlarge the spectra library for further identifications in a real context, using the developed methodology. Calibration methods for the determination of bacterial concentration should be developed for other foods. It may also be interesting to study the cellular compounds after high pressure with complementary methodologies to IR spectroscopy, in order to better understand the modifications that were triggered by this processing technology.

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