Universidade de Lisboa

Faculdade de Farmácia

Evaluation of the antimicrobial activity of lipids extracted from canned fish industry waste streams

Tiago Filipe Guerreiro Rodrigues

Dissertation supervised by Researcher Frédéric Bustos Gaspar and co-supervised by Professor Madalena Maria Vilela Pimentel

Mestrado em Qualidade Alimentar e Saúde

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“The most beautiful experience we can have is the mysterious”.

Albert Einstein

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Abstract

The public's negative perception towards chemical preservatives has sparked an increased interest regarding the use of alternative compounds of natural origin, which hold the potential to be used as preservatives with antimicrobial activity. Natural preservatives can be obtained from a variety of sources, including plants, natural polymers, organic acids or animals.

Thus, in this experimental work, the main objective was to evaluate the antimicrobial activity of lipids extracted from canned fish industry waste streams. Lipid extracts were obtained by using two different methods: solvent extraction (Bligh and Dyer) and supercritical CO_2 extraction. In opposition to conventional methods, which typically involve toxic and flammable solvents, using a supercritical CO_2 methodology offers a less polluting alternative, making it easier to separate the solvent from the extract as it is a gas at ambient temperatures and pressures.

A lipid characterization of the extracts with antimicrobial activity helped define the components present in their composition. This activity was evaluated by several methods such as well diffusion, disk diffusion, agar microdilution and liquid microdilution. The antimicrobial activities of lipid extracts were evaluated against several microorganisms (*Staphylococcus aureus, Escherichia coli, Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Candida albicans*) that can represent food safety issues in food matrices.

The results suggest an antimicrobial potential of these lipids for applications as natural preservatives.

Keywords

Food waste; lipids; lipid extraction; lipid characterization; antimicrobial activity.

Resumo

As perceções negativas do público acerca dos conservantes químicos têm gerado um elevado interesse no uso de compostos alternativos de origem natural, com potencial uso como conservantes com atividade antimicrobiana. Os conservantes naturais podem ser obtidos através de diversas fontes que incluem as plantas, polímeros naturais, ácidos orgânicos ou animais.

Assim sendo, neste trabalho experimental, o objetivo foi avaliar a atividade antimicrobiana de lípidos extraídos de resíduos alimentares da indústria do peixe. Foram obtidos extratos lipídicos por dois métodos distintos, extração por solvente (Bligh and Dyer) e extração por CO_2 supercrítico. O uso do método de CO_2 supercrítico oferece uma aplicação menos poluente, ao contrário de um método convencional que normalmente envolve solventes tóxicos e inflamáveis, além de que, é mais fácil de separar do extrato por ser um gás a pressões e temperaturas ambientais.

Uma caracterização lipídica dos extratos ajudou a perceber os diferentes componentes na sua composição que na sua totalidade levaram a uma atividade antimicrobiana. Esta atividade foi avaliada por diversos métodos, como a difusão em poço, difusão em disco, microdiluição em agar e microdiluição líquida. As atividades antimicrobianas dos extratos lipídicos foram avaliadas contra diversos microrganismos (*Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae, Pseudomonas aeruginosa* e *Candida albicans*) que podem representar problemas de segurança alimentar em matrizes alimentares.

Os resultados obtidos sugerem um potencial antimicrobiano destes lípidos para aplicações como conservantes naturais.

Palavras-chave

Resíduos alimentares; lípidos; extração lipídica; caracterização lipídica; atividade antimicrobiana.

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List of Abbreviations

AST – Antimicrobial susceptibility testing

ATCC – American Type Culture Collection

B&D – Bligh and Dyer

caMHB – cation-adjusted Mueller-Hinton agar

CECT – *Colección Española de Cultivos Tipo* (Spanish Type Culture Collection)

CLSI – Clinical & Laboratory Standards Institute

DHA – Docosahexaenoic acid

EFSA – European Food Safety Authority

EPA – Eicosapentaenoic acid

EUCAST – European Committee for Antimicrobial Susceptibility Testing

FA – Fatty acid

FAME – Fatty acid methyl ester

FDSS – Freeze-dried steam-cooked sample

$FDSS_C$ – Lipids obtained by conventional extraction from FDSS

$FDSS_{SC}$ – Lipids obtained by supercritical extraction from FDSS

GC – Gas chromatography

ISO – International Organization for Standardization

MBC – Minimum bactericidal concentration

MHA – Mueller-Hinton agar

MIC – Minimum inhibitory concentration

MOPS – 3-(N-morpholino)propanesulfonic acid

MUFA: monounsaturated fatty acids

PUFA – Polyunsaturated fatty acid

SFA: saturated fatty acids

SS – Steam-cooked sample

SS_C – Lipids obtained by conventional extraction from SS

Chapter 1

1. Introduction

1.1. Food contaminants

Food contaminants, either from physical, chemical or microbial origin (Figure 1), which may result in human health problems, have been shown to be a serious concern for consumers.

Consequently, there is a constant search of ways to avoid food contaminants, and even if efforts to prevent the problem are enforced, food contamination along the food production chain can still persist (1,2).

Food exposed to the organisms responsible for food spoilage can present visible alteration, however, food associated to contamination by pathogenic agents can frequently appears to be in perfect condition, hence the danger. These unidentified pathogenic agents can cause diseases, hospitalizations or deaths by food contamination (3,4). It is through constant vigilance and maintenance of high standards of hygiene that humans could avoid food contamination, which could be caused by non-microbial (chemical or physical contaminants) or microbial contaminants (4).

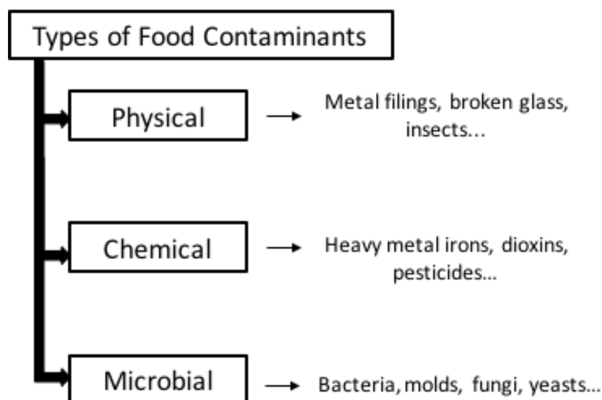

Figure 1: Types of food contaminants.

1.1.1. Non-microbial contaminants

Physical contamination of food can happen at any stage of food manufacture, where substances are non-intentionally added. These may not damage or change the food but their presence can create health hazards for the user.

Examples of physical contamination are the covering material, metal filings, broken pieces of glass, as well as rodent droppings or even insects, which can transport microbes into the food, such as flies, that can bring bacteria by contact with food (2). The risks caused by physical materials can be diminished with good agricultural and processing practices (5).

Chemical food contamination could be caused naturally or artificially. For example, heavy metals or dioxins could be naturally present in the soil or be present by anthropogenic activity, and consequently, plant tissues could concentrate those substances, resulting in food contamination (4,6).

Managing pests and insects may also lead to chemical contamination. For instance, some pesticides, such as polychlorinated biphenyls and dichlorodiphenyltrichloroethane, even if not used for several years, tend to appear in sediments and soil (7). The symptoms caused by the ingestion of these chemicals include dizziness, nausea, headache, muscle tremors and asthenia. Such symptoms could evolve to paralysis and coma, and ultimately lead to death.

Besides those contaminants, processed food mainly contains chemical preservatives to increase its shelf life. The long-term cumulative effects of the ingestion of such compounds still needs to be adequately established and the subject remains controversial (4).

1.1.2.Microbial contaminants

Microorganisms present in food could have several origins: microflora of soil, water, air, food itself, as well as microorganisms introduced during food manipulations (8).

The presence of the microorganisms is not the only explanation for food spoilage, likewise, the environmental conditions should equally be adequate to allow their growth. When in presence of favourable conditions, some microorganisms could cause food alterations and affect the quality, leading to the spoilage of the food. On the other hand, some pathogenic microorganisms could be a food safety problem only if they are present in food in any quantity, which could put in danger the health of the consumer. That is the reason why normally only one microbial species or fewer in the present microflora is responsible for undesirable food modifications, causing problems in the quality and/or safety of food (2,8).

Sometimes, even if hygiene control strategies are properly maintained in the industries, the microbial contamination could still occur, which could lead to food contamination. The microbial food contamination causes adverse effects in humans that can be distinguished by two main types, the intoxication and infection, or even an overlap of both categories. In the bacterial intoxication, a toxin is produced, which is responsible for triggering the clinical manifestation of the disease, without being necessary to ingest the viable bacteria to suffer food intoxication. On the contrary, in the bacterial infection the viable bacterium is consumed. These bacteria need to multiply in the intestine before the symptom's manifestation (2,4).

The incubation period of the disease is frequently the reflection of the nature of the food contamination. For example, in food intoxication by *Staphylococcus aureus*, the symptoms become clear between 30 minutes and 6 hours after the ingestion of the toxin. On the contrary, the medium period of incubation for *Salmonella enteritidis* is between 15 to 48 hours. This longer period of incubation represents the necessary time for bacterial multiplication in the intestine before the symptoms manifest (8).

These food contaminations are mainly caused by foodborne pathogenic bacteria, such as the already mentioned *Salmonella* and *S. aureus*, as well as *Bacillus cereus*, *Clostridium perfringens*, *Clostridium botulinum*, *Escherichia coli*, *Listeria monocytogenes*, *Campylobacter* or even by some yeasts, like *Candida albicans* (4,8,9).

1.1.2.1.Gram-positive vs gram-negative Bacteria

The bacteria phyla include a vast number of organisms that differ in their sources of energy, their shape, their metabolic pathways, the end products of their metabolism and their ability to interact with an array of different compounds and different organisms. These small sized unicellular organisms, with a diameter between 0.5 and 2 µm, could provide some benefits for the food industry, but some of them could be opportunists and even pathogenic, putting in danger the human health (8,10).

One characteristic of these organisms is the particular chemical composition of their cellular walls. Among others functions, the cell wall protects bacteria against osmotic shock as well as being responsible for the characteristic shape of the different bacterial species. One chemical component that characterizes the bacterial walls is the peptidoglycan, which forms the rigid frame of the wall (8). Other chemical compounds are part of the wall composition and vary depending on the bacterial group. After gram coloration, these differences of the wall enable or obstructs the passage of defined dyes,

which permits the distinction between gram-positive and gram-negative bacteria by microscope observation. This technique is a very important standard for bacterial distinction and characterization (8,11).

The gram-positive bacteria have thicker walls (15 to 80 nm) when compared to the thinner but more complex walls (6 to 15 nm) of gram-negative bacteria (Figure 2). The gram-negative walls are organized in several distinct layers and separated from the cytoplasmic membrane by the periplasm. Also, gram-negative cell wall has a higher abundance of lipids when compared with their gram-positive counterpart (8,11).

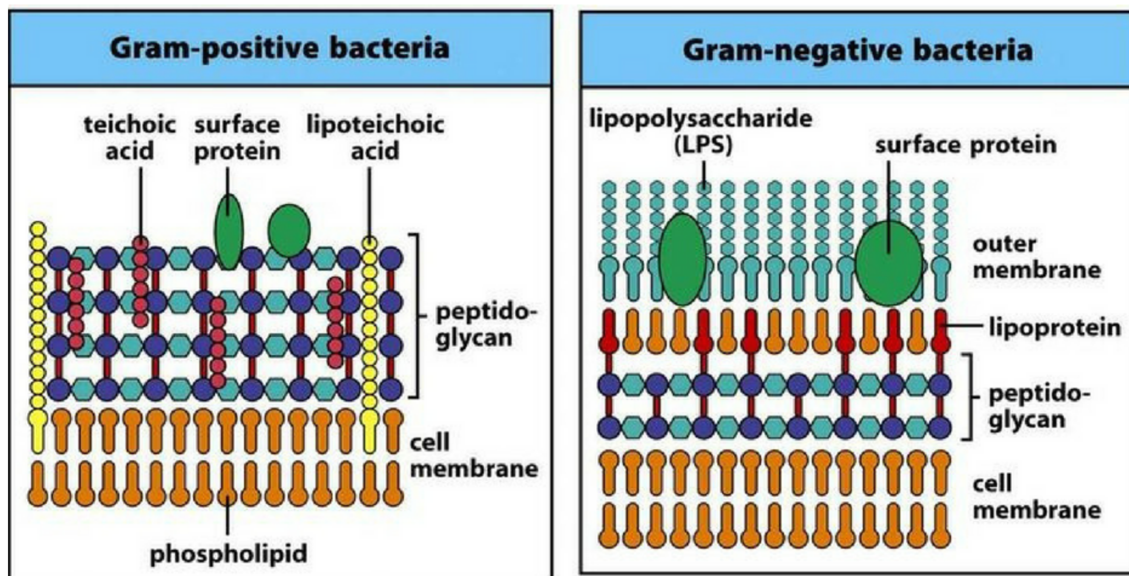

Figure 2: Cell wall of gram-positive vs gram-negative bacteria (12)**.**

1.1.2.2.Yeasts

Yeasts are single-celled fungi that can be spherical, oval, or cylindrical, with a diameter of 3 to 5 µm (Figure 3). They generally reproduce by budding, in which a small outgrowth on the cell produces a new cell. Yeasts can accumulate in their cytoplasm large quantities of food reserves, either in the form of glucose or lipids (8,13).

They can cause diseases in humans in four ways. The first way is the development of an allergic reaction to the spores. Also, a reaction to the toxins produced by yeasts is another form of causing diseases. Another way is the possibility of yeast to actually grow on or in the human body causing the disease. Finally, they can destroy the human food supply, causing starvation and death (13).

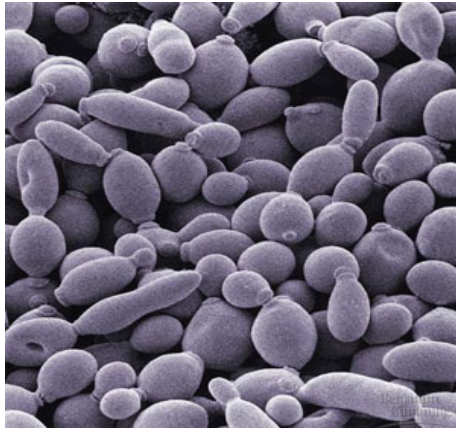

Figure 3: Yeasts representation (14)**.**

1.2. Extending shelf life

In order to extend shelf life of food, substances are added to maintain or improve the quality, taste, appearance or texture. Although the most common substances used are salt or sugar, there are several that have been developed over time to satisfy the food industry's needs.

1.2.1.Food additives

Food additives are added intentionally to foodstuffs to perform certain technological functions. Those functions are associated to several categories, such as antioxidants, colours, emulsifiers, stabilisers, gelling agents, thickeners, sweeteners and preservatives, which are identified by an E number in the European Union (Table 1) (15).

Table 1: Main categories of food additives with their assigned E number by the European Union (15)**.**

E-codes number	Additives
E-100	Colouring agents
E-200	Preservatives
E-300	Antioxidants
E-400	Thickeners, stabilizers, Gelling agents, emulsifiers
E-500	Agents for physical characteristics

E-600	Flavour enhancers

1.2.2.Food preservatives

Food preservatives are substances added to food to minimize or prevent food spoilage caused by oxidation or microbes (bacteria and fungi). They are one category of the most common additives to appear on food labels in the European Union.

1.2.2.1.Chemical food preservatives

Synthetic chemical preservatives are amongst the most effective and generally used in food preservation, such as benzoates, nitrites and sorbates. Their efficiency depends on the type of microorganism, the composition of food and concentration of the preservative. The currently authorized preservatives used in the European Union are given numbers that vary from E200 to E285 (15).

Synthetic food preservatives are subjected to a scientific risk assessment, where a health-based reference value is presented, such as the acceptable daily intake, which is compared to the predicted or measured dietary exposure. The concentration limits for food preservatives is therefore settled on the basis of their safety assessment, consistent with their technical function (16). However, consuming these food additives, even lower than the recommended limits as defined by regulatory agencies (such as EFSA), could lead to significant health risks, such as allergic reactions, gastrointestinal disorders and cancer (16,17). Likewise, another problem with processed food, that has been processed with the addition of numerous synthetic preservatives, is the slow degradation periods, which could conduct to environmental problems, such as pollution (18).

When selecting an appropriate preservative for a specific food product it is necessary to consider several factors. Those factors are the type of the target microorganism (deteriorative or pathogen), pH, composition, physical state, product shelf life, use and application. It is also important to account possible organoleptic side-effects of the preservatives in food products, such as changes in flavour or colour (19).

Nowadays, consumer preferences are moving towards foods that contain lower levels of chemical preservatives, that exhibit characteristics of fresh or natural products, and that are microbiologically safe (20).

1.2.2.2.Natural food preservatives

The negative public perception of industrially processed food has generated interest in the use of more naturally occurring compounds, where a promising antimicrobial potential of natural extracts has been demonstrated when compared with synthetic antibiotics (18,21). The challenge is to find a naturally occurring antimicrobial compound which can be added to a food product susceptible to microbiological contamination from another food product. The food product would have to contain an antimicrobial, which is completely nontoxic and highly effective in controlling the growth of microorganisms (22). This increasing demand has opened new dimensions for the use of natural preservatives derived from plants, bacteria or animals (23).

1.2.2.2.1.Non-animal derived food preservatives

Plants and their derived compounds can provide a vast source of natural preservatives, due to the wide range of bioactivities that makes them useful as natural additives in different types of food (24,25). For centuries, plants and their derived essential oils have been used for the treatment of infections and diseases worldwide. They contain a large number of secondary metabolites that are known to delay or inhibit the growth of bacteria, yeast and moulds (23). The antimicrobial compounds in plant material are commonly found in the essential oil fraction of leaves, flowers or buds, bulbs, seeds, rhizomes and fruits. These compounds may be lethal to microbial cells or they can inhibit the production of microbial cells secondary metabolites. However, they are generally more inhibitory against gram-positive than gram-negative bacteria (23,25).

Bacteria produce many compounds (bacteriocins) which can kill or inhibit bacterial strains related to or not related to the producer bacteria. The first report of bacteriocins from lactic acid bacteria dates back to 1928. These were used in cheese making, and inhibited the growth of other lactic acid bacteria strains. Bacteriocin production started to be exploited by food processors to provide an additional barrier to undesirable bacterial growth in foods (23,26). Bacteriocins use in the food industry is justified if they fulfil several requisites, such as being non-toxic to humans, not altering nutritional and organoleptic properties of the food, being effective at low concentrations, being sufficiently stable during storage and being accepted by recognized authorities for regulatory approval (26).

1.2.2.2.2. Animal derived food preservatives

There are numerous antimicrobial agents of animal origin that often evolve as host defence mechanisms. Animals are engaged in a constant battle with microorganisms, which lead to an interest in appreciating the rich diversity of simple substances (such as peptides or lipids) utilized by animals to kill microbes (23,27). Antimicrobial peptides are widely distributed in nature and are used by many life forms as essential components of nonspecific host defence systems. They are one promising solution to the problem of antibiotic resistance. Likewise, other animal products are demonstrated to have antimicrobial activity against a wide range of microorganisms, like chitosan (a natural biopolymer obtained from the exoskeletons of crustaceans and arthropods) and lipids (animal tissues) (23,27,28).

1.3. Antimicrobial activity of fatty acids

1.3.1. Lipids

Contrary to other groups of biological molecules (such as carbohydrates, proteins and nucleic acids), there is not a unique structural pattern in the molecules referred to as lipids. They are defined as molecules that are insoluble in water, due to the predominance of apolar regions, mainly aliphatic, present in their molecular structure.

Lipids are predominant in cellular energetic and synthetic metabolisms. They are the main constituents of the cell wall, a structure which dictates part of an organism's behaviour.

Lipids can be organized in 3 large main categories, such as derived lipids (the fatty acids and alcohols), the simple lipids and the complex lipids (Figure 4). The criteria to distinguish the simple from the complex lipids is the fatty acid and the additional groups content (29,30).

Simple lipids contain only esters of fatty acids with various alcohols, and the complex lipids are esters of fatty acids and alcohols containing other additional groups (phosphorous, carbohydrates, proteins or sulphate groups). Derived lipids are lipids obtained upon hydrolysis of the simple or complex lipids but still retain the characteristics of lipids (29,30).

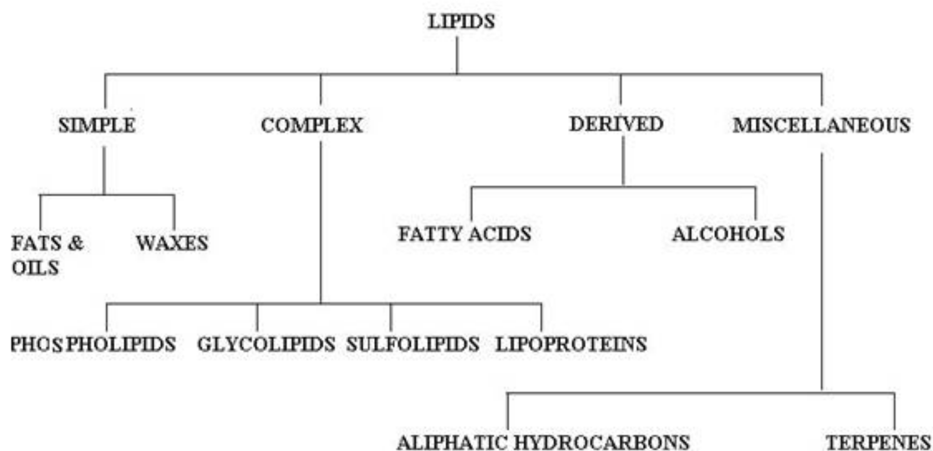

Figure 4: Classification of lipids (31)**.**

1.3.2.Fatty acids

The expression fatty acid refers to any aliphatic monocarboxylic acid that can be released by hydrolysis from any natural oils and fats. Fatty acids are biologically important for the cells, mainly because they participate in their structural organization and because they are a source of energy. They have a linear aliphatic hydrogen-carbon chain, saturated (no C=C double bonds) or unsaturated (one or more C=C double bonds) (Figure 5), containing a single functional carboxyl group. Normally, the unsaturated fatty acids are more abundant in plants (example: linoleic and oleic acid), whereas the saturated predominate in animals (example: palmitic and lauric acid). In their pure state, the saturated fatty acids are liquid at the environmental temperature if they have until 10 carbon atoms and they are solid if they have longer carbon chains (29,30).

Figure 5: Saturated and unsaturated fatty acids. Palmitic acid above and palmitoleic acid below (32).

Fatty acids can be submitted to oxidation by the addition of oxygen to the double bonds, producing epoxides, unpleasant odour acids and aldehydes, by a process denominated as rancidification. These reactions occur not only with simple fatty acids, but also with all the lipids which contain fatty acids in their composition (29).

1.3.3. Antimicrobial activity

Recently, many studies have shown that fatty acids have an antimicrobial activity against several microorganisms. For example, palmitic acid, stearic acid, oleic acid and linolenic acid have shown bactericidal effects against *S. aureus* and *Streptococcus pyogenes* (33). Another study reported antimicrobial activity of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) against several bacteria, such as *Bacillus subtilis*, *L. monocytogenes*, *S. aureus*, *Pseudomonas aeruginosa*, *E. coli*, *Enterobacter aerogen*es and *Salmonella* (34)). Additionally, there is a report showing that n-3 polyunsaturated acid (PUFA), EPA, DHA and α-linolenic acid exhibit strong antimicrobial activity against various oral pathogens (such as *Streptococcus mutans*, *C albicans*, *Streptococcus gordonii* and *Fusobacterium nucleatum*) (35).

These antimicrobial activities could be achieved through diverse modes of action (33–39). These include disruption caused by interference with the cell membrane which lead to permeability changes or interference with the activity of membrane bound enzyme complexes and events following lipid peroxidation with radical formation. The ability of fatty acids to disrupt cellular membranes has been demonstrated for both gram-negative and gram-positive bacteria, where a reduction of the permeability barrier in the outer membrane and cell wall could be caused by the acidity of the lipid (40,41).

1.4. Fish waste stream and circular economy

Fish waste is the fish tissue that is not suitable for consumption, including bones, intestines, heads and tails. These wastes have significant content of nutritive compounds, such as proteins that could be used as animal feed. They could be minced, homogenised and mixed thoroughly with other dietary ingredients. Fish waste can also be used as biodiesel/biogas obtained from the oils and fats derived from the fish or used as natural pigment such as the carotenoids responsible for the colour of many fish and shellfish. They can even be used in food industry/cosmetics where a number of useful compounds (proteins, enzymes, lipids) that have antimicrobial and antitumor capabilities can be isolated from (42,43).

Fish waste can be generated by aquaculture activity, fishing activity at sea, fish markets, retail trade and restaurants or even in the Portuguese fish processing industries. The decomposition of these wastes can produce a considerable amount of methane (a potent greenhouse gas with more global warming potential than carbon dioxide) which makes the incineration an inappropriate option. In Portugal, the food and agriculture industry, including the fish industry, is the main industrial sector, being responsible of over 16% of the manufacturing industries business (44,45).

Over the last few years, in the context of the circular bio-economy, the exploration and valorisation of the sub-products from the food industry has been attempted. To reduce and avoid as much as possible the production of waste, by reusing raw materials and recovering biomaterials has been the main focus (44).

Food waste, which is generated by the food industry during the processing of raw materials, is one of the most produced bio-wastes in the world (21,46). The inappropriate accumulation and improper elimination of industrial wastes can cause pollution problems as well as the waste of valuable biomass, which could be used as a source of bioactive compounds (21). According to the Food and Agriculture Organization (FAO), food losses

represent “a waste of resources used in production such as land, water, energy, and inputs. Producing food that will not be consumed leads to unnecessary CO_2 emissions in addition to loss of economic value of the food produced” (47).

The world economy loses $750 billion each year, and it is estimated that about 1.3 billion tons of food and one-third of the total global food production is wasted. These problems happen in the steps of production, handling, storage, processing, distribution or consumption (21,46,48). There are several reasons to develop advanced valorisation practices for residues and food waste which mainly are composed of functionalized molecules, such as carbohydrates, proteins, triglycerides or lipids (Figure 6). These molecules are so abundant, readily available, under-utilised and renewable and can be recovered, concentrated and re-used as antioxidants or antimicrobial agents in functional foods (21,49)

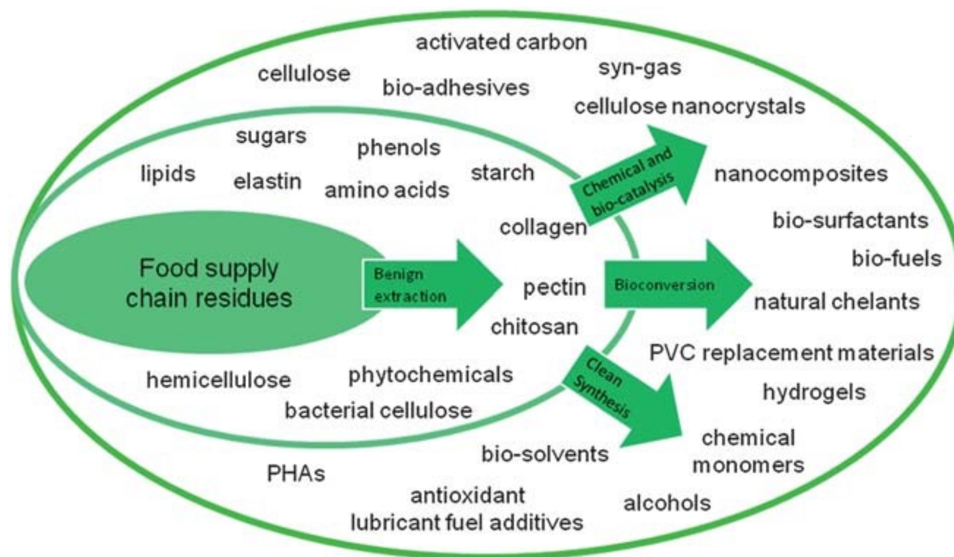

Figure 6: Components present in food supply chain residues and their uses in common consumer applications (49)**.**

Chapter 2

2. Aim of the dissertation

The main objective of the present study was to evaluate the antimicrobial activity of lipids extracted from canned fish industry waste streams. For that, two different lipid extractions of those waste streams were performed. These extractions were followed by a subsequent evaluation of the antimicrobial activity of the extracted lipids by applying different methodological approaches to different target microorganisms.

The study was divided in two tasks: an initial lipid extraction and characterization task and an antimicrobial susceptibility testing task.

The lipid extraction and characterization task focused on the following objectives:

- Selection of the waste sample: researching the fatty acids availability and composition of each available sample.
- Implementation of the conventional Bligh & Dyer and the supercritical CO_2 extraction methods in the selected waste sample.
- Comparison of the two extraction methods by analysing the mass yields and lipid composition.

The antimicrobial susceptibility testing task focused on these next objectives:

- Optimization and application of several qualitative and quantitative antimicrobial evaluation methods: well diffusion, disk diffusion, agar microdilution and broth microdilution.
- Implementation of the antimicrobial evaluation methods against target gram-positive bacteria, gram-negative bacteria, and one yeast.
- Evaluation of the antimicrobial potential of the lipid extracts obtained by two distinct extraction techniques by analysing the inhibitory halos, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values.

Chapter 3

3. Materials and methods

3.1. Lipid extractions and characterization

3.1.1.Samples and reagents

For the realization of the initial part of this study, which consists in the lipid extractions and characterization, samples and reagents were obtained from multiple companies.

Samples of sardine (entrails and heads), cod-fish (skin) and crustaceans shells (crab and shrimp) were obtained from the canned fish industry "Fábrica de Conservas - A Poveira" (Póvoa de Varzim, Portugal) and stored at -20ºC in the absence of light. The -20ºC frozen steam-cooked sample will be herein referred as SS (Steam-cooked Sample). The SS were dehydrated using a Coolsafe Superior Touch 55-80 freeze dryer (Scanvac) at -55ºC, posteriorly milled using a cutter-emulsifier CK-8 (Sammic) and stored at room temperature in the absence of light until use. The freeze-dried SS will be herein referred as FDSS (Freeze-Dried Steam-cooked Sample).

Chloroform 95%, methanol 99.9% and xylene were obtained from Carlo Erba, acetone 99.8% was obtained from Fisher Chemical, isooctane 99.8% was obtained from Merck, methanolic potassium hydroxide solution was obtained from Sigma-Aldrich, 52 fatty acid methyl esters (FAMEs) standard samples were obtained from Nu-Chek-Prep, Inc., nitrogen ALPHAGAZ™ 1 and carbon dioxide 99.99% ALPHAGAZ™ 1 were obtained from Air Liquide.

3.1.2.Waste-stream sample selection

A bibliographical research about fatty acids composition that each sample could offer was accomplished. Afterwards, the sample was selected after a comparative analysis of the compilation of results obtained from the comprehensive bibliographical research.

3.1.3.Moisture content determination

The moisture content was determined for both SS and FDSS using the conventional Dean-Stark distillation (50). Briefly, the weight of the samples used was fixed so as to obtain an adequate amount of water in the receiver (2 g of SS or 10 g of FDSS) and 75 mL of xylene were added. At the beginning of the experiment, the electrical heating was maximized until collection of the first droplets in the Dean-Stark

receiver. The heating was adapted to a slower distillation rate and continued until the water level in the receiver did not increase more than 0.1 ml in 30 min, then the distillation was stopped. The volume of water was measured and the moisture content calculated as follows:

$$\text{Moisture (\%)} = \frac{\text{Volume of water (ml)} \times 0.997\ (\frac{\text{g}}{\text{ml}})\ \text{(density of water at 20°C)}}{\text{Weight of the sample (g)}}\ \text{X}\ 100$$

3.1.4.Bligh and Dyer method: total lipid extraction and purification

The extractions were performed as described by Bligh and Dyer (B&D) (51), with a reduced amount of sample and solvent as proposed by Smedes et al. (52).

Briefly, for a sample of approximately 2 g of SS or FDSS, a step of homogenization in a vortex (WiseMix VM-10) for 2 min with a mixture of 2 ml chloroform and 4 ml methanol was initially performed. After that, a volume of 2 ml of chloroform was added for another blending of 30 s. To finalize, 2 ml of distilled water were added and blending continued for another 30 s. If necessary, the volumes of reagents used were scaled according to the initial sample weight. The homogenate was filtered through Whatman No. 1 filter paper on a Buchner flask using a vacuum pump (Büchi Labortechnik V-85). The filtrate was transferred to a test-tube, and allowed to rest a few minutes in order to completely separate and clarify both layers (chloroform layer at the bottom and methanol/water layer at the top). The methanol/water layer was removed with a Pasteur pipette along with a small volume of the chloroform layer to avoid the carry-over of water in the next steps. The chloroform layer, which contains the extracted lipid, was transferred to a tared glass bottle and the content was evaporated using a stream of nitrogen until the full chloroform evaporation. The previously described procedure of B&D is represented in Figure 7. Afterwards, the lipid content was stored at -20ºC protected from light. The weight of the lipid content was determined and the global extraction yield calculated by the following equation:

$$\text{Yield (g/100 g (SS or FDSS))} = \frac{\text{lipid weight (g)} \times 100}{\text{Sample weight (g)}}$$

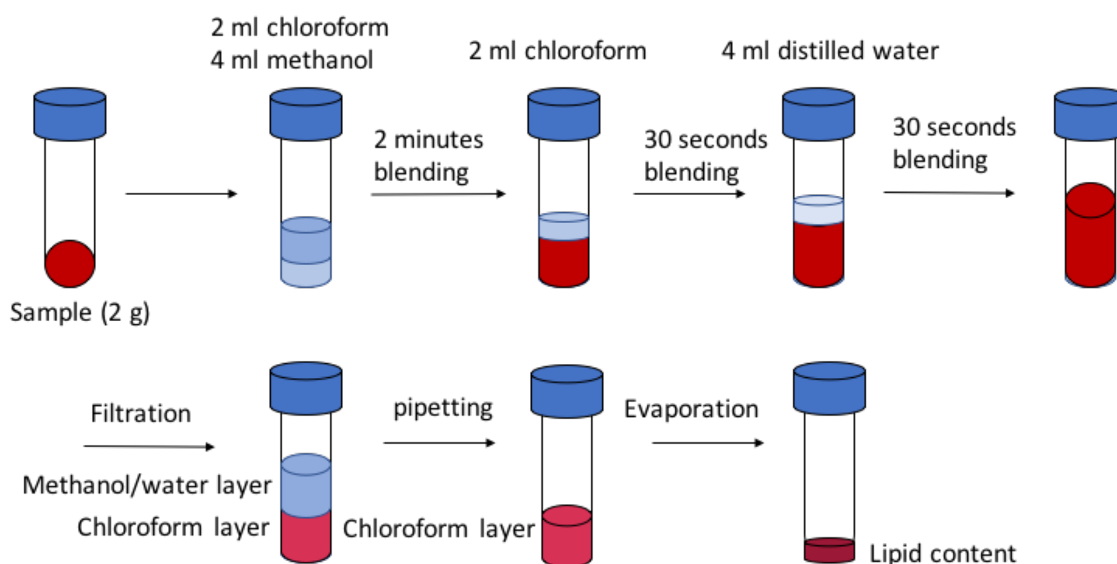

Figure 7: Schematic representation of the Bligh and Dyer method when used 2g of sample.

3.1.5. Supercritical CO_2 of lipids extraction

For this study, 9 extractions were performed in a supercritical fluid extraction system (SFE-500F-2-C50, Thar Technology) doing binomial combinations of pressure (300, 425 and 550 bar) and temperature (35, 55 and 75ºC), as represented in Table 2.

Table 2: Temperatures and pressures variations in the supercritical CO_2 extraction method.

Extraction	Pressure (bar)	Temperature (ºC)
1		35
2	300	55
3		75
4		35
5	425	55
6		75
7		35
8	550	55
9		75

This methodology was carried out as described in Sánchez-Camargo et al. (53). Initially, for each extraction procedure, the extractor was filled with 10 g of FDSS mixed with glass spheres and the remaining void volume was completed with more glass spheres.

The temperature and pressure of the extraction vessel were defined in the computer according to the values described in Table 2, with the temperature of the fraction collector 1 remaining at 25ºC and the pressure at 60 bar in all the extractions. The fraction collector 2 remained at the room temperature and pressure. The extraction vessel and fraction collectors are represented in Figure 8. The system was equilibrated until pressure, CO_2 flow rate (25 g/min) and temperature became constant to begin the extraction. The duration between the static and extraction times was fixed at 5, 10, 15, 20, 25, 30, 35, 45, 60, 75, 90, 105, 120 and 135 min, respectively. After all collections, both fraction collectors were washed with acetone, using a Pasteur pipette, to recover the retained extract. The solvent was evaporated using a stream of nitrogen and all the extracts were stored at -20ºC in the absence of light. The weight of the lipid content was determined and the global extraction yield calculated by the following equation:

$$\text{Yield}\left(\frac{\text{g}}{100\text{g}}(\text{SS or FDSS})\right) = \frac{\text{lipid weight (g)} \times 100}{\text{Sample weight (g)}}$$

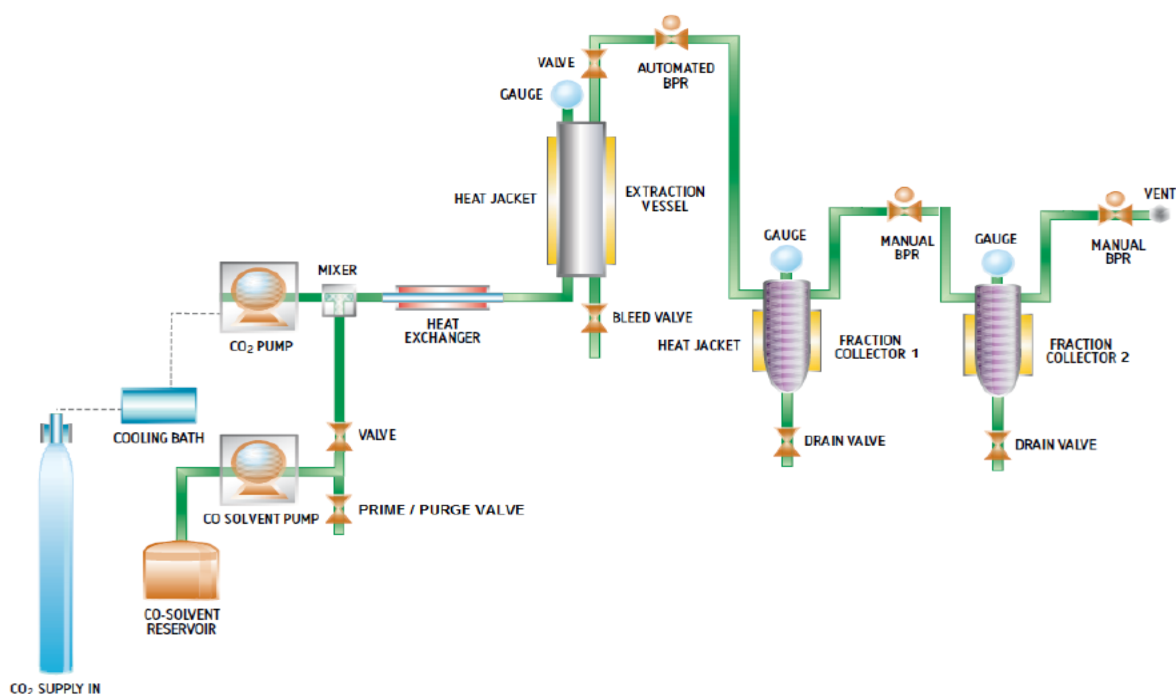

Figure 8: Schematic representation of the supercritical fluid extraction system (SFE-500F-2-C50, Thar Technology) (54).

3.1.6.Lipid characterization (gas chromatography analysis)

The following lipid characterization procedure was performed in collaboration with Instituto Nacional de Investigação Agrária e Veterinária, Unidade de Tecnologia e Inovação.

The samples underwent a transesterification in order to dissolve the sample glycerides and obtain the FAMEs by the method described in ISO 5509:2000 (55).

Briefly, 4 ml of isooctane were added and dissolved in the sample, followed by 200 µl of methanolic potassium hydroxide solution with a pipette. The mixture was shaken vigorously for about 30 s. The reaction mixture started to become clear after an initial cloudiness due to the separation of glycerol. The upper layer containing the methyl esters was collected and injected in the gas chromatographer.

GC analyses were carried out on a ThermoQuest Trace GC 2000 (CE Instruments, Ltd.) gas chromatographer operated with a flame ionization detector, a J&W DB-23 capillary column (Agilent Technologies, Inc.), 60 m x 0.25 mm I.D., 0.25 µm phase thickness and temperature program 70-195ºC, at a rate of 5ºC/min. Temperature was kept at 195ºC for 30 min, then increased up to 220ºC and kept for 65 min. Helium was the carrier gas, the injector temperature was 220ºC and the detector temperature was 280ºC. FAMEs were identified comparing the retention times with those obtained for a standard mixture of 52 FAMEs.

3.2. Antimicrobial susceptibility testing

3.2.1.Samples and reagents

For the realization of the second part of this study, which consisted in the lipid antimicrobial susceptibility testing (AST), several media and reagents were obtained from multiple companies.

The reference microbial strains used in this study were *S. aureus* ATCC 6538, *E. coli* ATCC 8739, *Klebsiella pneumoniae* CECT 8453 *P. aeruginosa* ATCC 9027 and *C. albicans* ATCC 10231, representing gram-positive and gram-negative bacteria and a yeast. Excluding *P. aeruginosa*, these strains are commonly recognized as food contaminants (section 1.1.2). The strains were obtained from the laboratory culture collection and stored at -20ºC in cryovials. *E. coli*, *K. pneumoniae* and *P. aeruginosa* strains were sub-cultured overnight in nutrient agar (Oxoid) at 37ºC and *S. aureus* in

tryptone soya agar (Himedia) at 37ºC, prior to each experiment. *C. albicans* strain was sub-cultured in malt extract agar (Scharlau) at 37ºC, 48 h before each experiment.

To perform the AST using bacterial strains, the growth media used were Mueller-Hinton agar (MHA) and cation-adjusted Mueller-Hinton broth (caMHB), obtained from BD.

To perform the AST using yeasts strains, the medium used was RPMI 1640 (with glutamine, with glucose 0,2 %, without bicarbonate, and with phenol red as pH indicator), obtained from Thermo Fisher Scientific, buffered with 0.165 mol/L MOPS, obtained from Sigma. While stirring, the pH was adjusted to 7 at 25ºC using 1 mol/L sodium hydroxide, obtained from Carlo Erba.

Sodium chloride (NaCl) was obtained from Panreac, the PrestoBlue cell viability reagent was obtained from Thermo Fisher Scientific and the absolute ethanol was obtained from Carlo Erba, Sharlau.

The antimicrobial agents used were the lipids obtained by conventional extraction from SS, herein referred as SS_C, lipids obtained by conventional extraction from FDSS, herein referred as $FDSS_C$ and the lipids obtained by supercritical extraction from FDSS herein referred as $FDSS_{SC}$.

3.2.2.Inoculum preparation

Each bacterial inoculum was prepared according to the direct colony suspension method of the EUCAST guidelines version 6.0 (56). Briefly, fresh microbial colonies were suspended in 0.85% NaCl from an agar plate, to achieve a turbidity corresponding to the 0.5 McFarland standard (Pro Lab Diagnostics, UK). This standard is approximately equivalent to 1 to 2 x 10^8 colony-forming units (CFU)/mL for *E. coli* ATCC 25922, although, the standard is appropriate to all organisms used in this study.

The tubes were compared against a Wickerham card and the turbidity was also measured using a spectrophotometer, where the absorbance at 625 nm should be 0.08 to 0.13 for the 0.5 McFarland standard.

3.2.3.Well diffusion method

This method was adapted from the EUCAST guidelines 6.0 (56), following the modifications used in Magaldi et al. (57), where the application of the disk was replaced by the removal of an agar plug in order to create a well. Briefly, MHA plates were

inoculated using a sterile cotton swab dipped into an adjusted inoculum suspension prepared as described in section 3.2.2 and used within 60 min of preparation.

The inoculation was performed by swabbing in three directions (with a 60º rotation between each swabbing), making sure the inoculum was spread on the entirety of the agar surface. Afterwards, four wells, each with a 7.5 mm diameter, were punched out of the agar with the back side of a sterile 200 µl tip. A volume of 20 µl of the antimicrobial agent dissolved in absolute ethanol (1 g/ml) was dispensed into each well, with one well containing only ethanol for blank control purpose. The plates were inverted within 15 min of volume application and incubated at 37ºC for 24 h. The inhibition zone diameters were then measured in millimetres and interpreted. The steps of this method are represented in Figure 9.

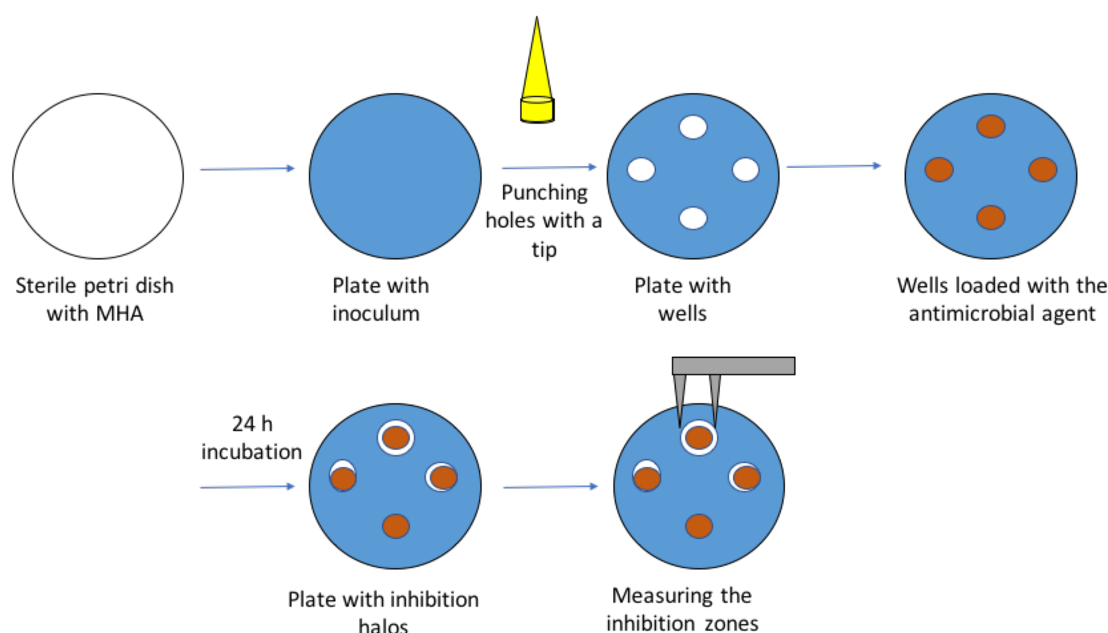

Figure 9: Schematic representation of the Well Diffusion method.

3.2.4.Disk diffusion method

This method was adapted from EUCAST 6.0 (56). Briefly, the MHA plates were inoculated as described in section 3.2.3. Afterwards, four sterile 6 mm diameter cotton disks (Fluka Analytical) were placed on the agar surface with sterile tweezers. A volume of 15 µl of the antimicrobial agent dissolved in absolute ethanol (1 g/ml) was placed on each disk with a pipette, with one disk containing only ethanol to function as a blank control. The plates were inverted within 15 min of volume application and incubated at

37ºC for 24 h (27). The inhibition zone diameters were then measured in millimetres and interpreted. The steps of this method are represented in Figure 10.

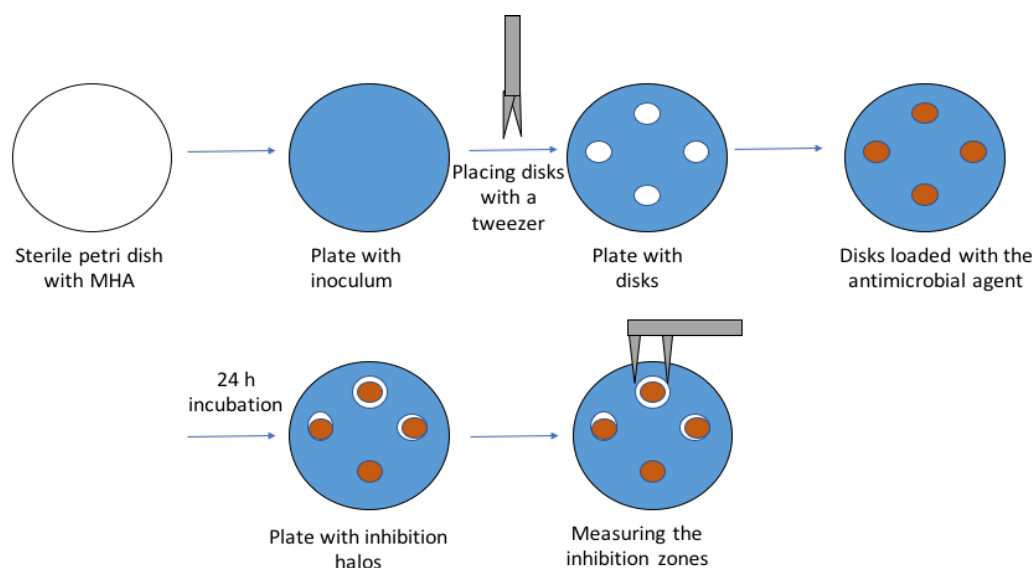

Figure 10: Schematic representation of the Disk Diffusion method.

3.2.5.Agar microdilution method

The agar microdilution method was performed as described in Golus et al. (58). The Golus et al. method is a modification of the agar dilution described in CLSI-M07-A10 (59), with the difference of being performed in a microdilution plate instead of an agar plate. Briefly, as demonstrated in Figure 11, twofold serial diluted intermediate solutions (each solution 10 times concentrated related to the target concentration) of the tested antimicrobial agent in caMHB were prepared. These intermediate solutions were added (20 µl per each replicate) to Eppendorf tubes with molten MHA (180 µl per each replicate), vortexed and kept at 50ºC in a ThermoMixer (Eppendorf 5436). Afterwards, 100 µl of each solution (trying to avoid bubbles) were dispensed into a 96-well microplate (flat-bottom) and kept at room temperature until the agar solidified.

The inoculum suspension was previously prepared as described in section 3.2.2 and then diluted 1:10 in fresh MHB to obtain a concentration of 1 x 10^7 CFU/mL. Afterwards, 2 µl were applied using a pipette to each well of the microplate, ensuring a final inoculum of 1 x 10^4 CFU per spot. After the complete inoculum absorption into the agar, the microplate was stored in a plastic bag and incubated at 37ºC for 16-20 h.

The minimum inhibitory concentration (MIC) results were obtained using the broth microdilution reference method as described by Golus et al. (58). The MIC value

was recorded as the lowest concentration of the tested antimicrobial agent that inhibited the formation of bacterial colonies. Each experiment was repeated three times, and the median MIC value was calculated (60).

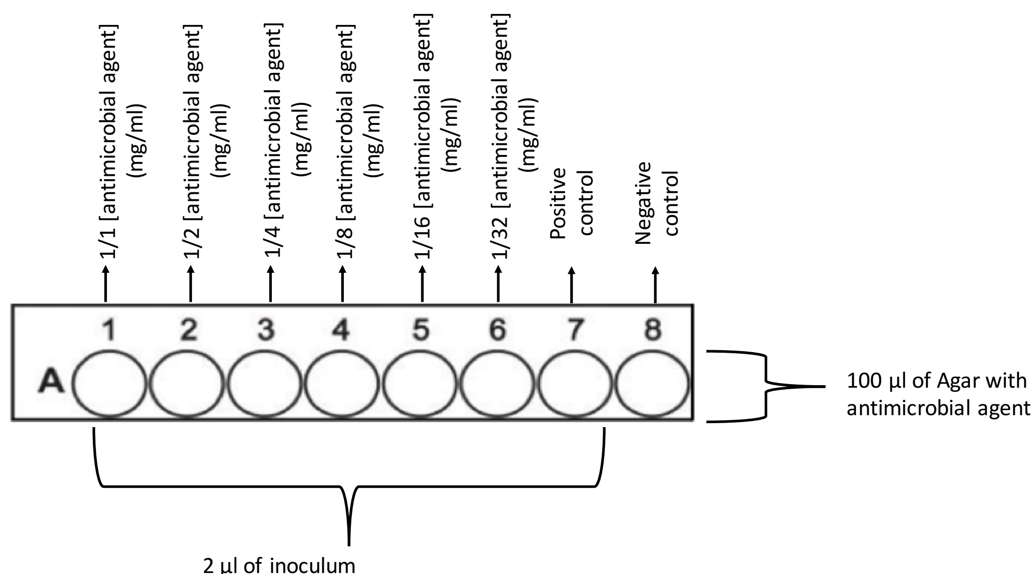

Figure 11: Schematic representation of the 96-well microplate using the agar microdilution method.

3.2.6.Broth microdilution method for bacterial strains

The broth microdilution method using bacterial strains was performed as described in CLSI-M07-A10 (59). Briefly, antimicrobial agent stock solutions were prepared in caMHB and 100 µl were placed in the first well of a row in a 96-well round bottom microplate. Afterwards, serial twofold dilutions were performed along the row of the microplate, in order to have a final volume of 50 µl of each solution in each well.

The inoculum was prepared as previously described (section 3.2.2) and diluted so that, after inoculation, each well contained approximately 5 x 10^4 CFU and a final volume of 100 µl, as shown in Figure 12. The microplate was inoculated using a multichannel pipette with 50 µl of freshly prepared inoculum, stored in a plastic bag and incubated at 37ºC for 16-20 h.

The MIC values were determined using the broth microdilution reference method as described by CLSI-M07-A10 (59). The MIC were recorded as the lowest concentration of the tested antimicrobial agent that completely inhibits bacterial growth visible to the naked eye. Each experiment was repeated three times, and then the median MIC value

was calculated (60). In case of an ambiguous MIC value reading, the cell viability reagent PrestoBlue (Invitrogen, USA) was used according to the manufacturer's instructions. PrestoBlue is a resazurin-based product (non-fluorescent blue pigment) that is reduced to resorufin (highly fluorescent pink pigment) by metabolically active cells. This reagent can therefore help detect the presence of metabolically active cells that will take a pink colouration or the absence of cellular growth or cell death as indicated by a blue coloured well.

Afterwards, the full content of each well where no growth was visible was inoculated on agar plates and incubated at 37ºC for 16-20 h.

The MBC results were reported as the lowest concentration producing a 99.9% reduction in bacterial viable count in the wells, relative to the initial inoculum.

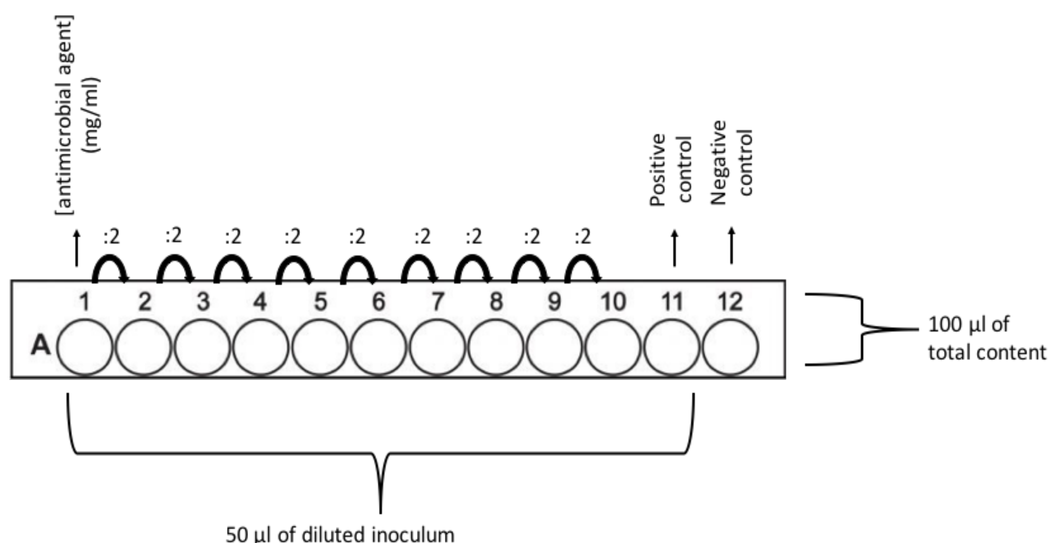

Figure 12: Schematic representation of the 96-well microplate using the broth microdilution method for bacteria strains.

3.2.7.Broth microdilution method for yeasts

3.2.7.1.Preparation of the inoculum

Each yeast inoculum was prepared according to the method described in section 3.2.2, where the 0.5 McFarland standard corresponds to approximately 1 to 5 x 10^6 CFU/mL in the case of yeasts (61).

3.2.7.2.Method

The broth microdilution method using yeast strains was performed as described in CLSI-M27-A2 (61). Briefly, the stock solutions of the tested antifungal agent were prepared in RPMI 1640 broth medium and 200 µl were placed in the first well of the microplate (flat-bottom). Afterwards, serial twofold dilutions were performed, so that each well contains 100 µl of each solution.

The inoculum was prepared as previously described (section 3.2.7.1) and diluted so that after inoculation in the microplate, each well contained approximately 0.5 x 10^3 to 2.5 x 10^3 CFU/mL and the final volume was 200 µl, as demonstrated in Figure 13. The microplate was stored in a plastic bag and incubated at 37ºC for 48 h.

The MIC results were obtained using the previously described methodology in section 3.2.6.

The MBC results were reported as the lowest concentration producing a 100% reduction in yeast viable count in the wells, relative to the initial inoculum.

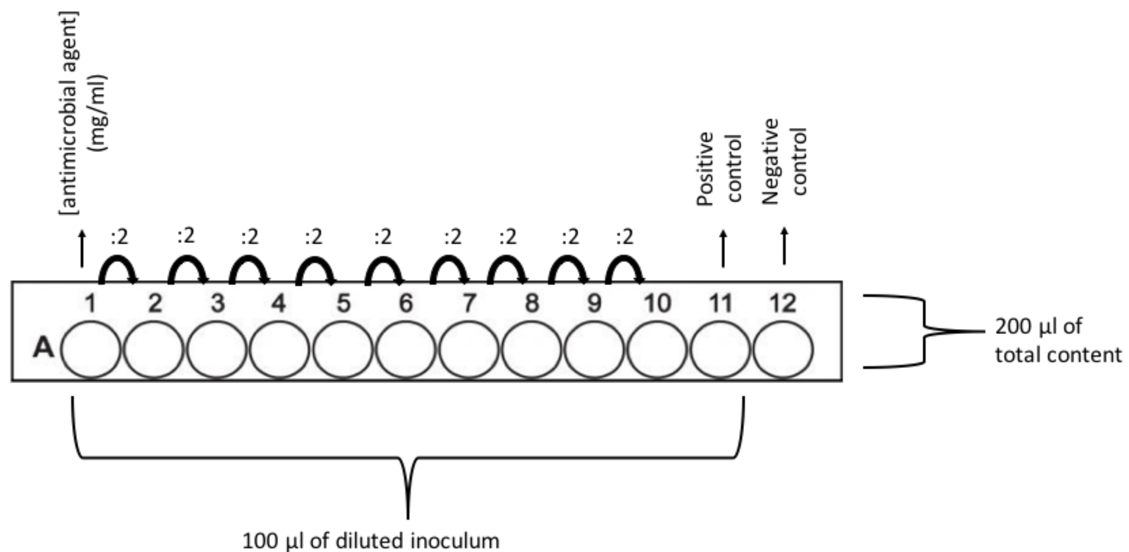

Figure 13: Schematic representation of the 96-well microplate using the broth microdilution method for yeast strains.

Chapter 4

4. Results and discussion

4.1. Lipids extraction and characterization

4.1.1. Waste-stream sample selection

The initial goal was to select the most promising sample that eventually could lead to a better lipid extraction and achieve superior antimicrobial results in the overall study.

After a compilation of information in the literature regarding the lipid composition of sardine, cod-fish and crustaceans shells, an analysis was performed to select the most promising sample. The analysis was performed considering the fatty acids in higher quantities to differentiate between samples, and not considering lipids in minor quantities. All information is summarized in Table 3.

The information in the literature has not showed considerable differences between fatty acids compositions of the evaluated samples. All of them are rich in EPA, DHA, palmitic acid, oleic acid and ω-3 PUFA, which are fatty acids that have previously been identified in other studies as having antimicrobial activity (section 1.3.3). However, the sardine has been described as having a higher total lipid content (5.08-24.8 g/100g) when compared with cod (0.30-4.3 g/100g), shrimp (0.70-2.40 g/100g) and crab (0.99-6.66 g/100g).

The information available in the literature has shown the sardine to be the most promising sample to be used in this study.

Table 3: Fatty acids found in higher amounts in sardine, cod, shrimp and crab with total lipid content ranges and references.

Common name	Fatty acids in higher quantities	Total lipid content ranges (g/100g)	References
Sardine	Myristic acid, palmitic acid, oleic acid, palmitoleic acid, EPA, DHA and ω-3 PUFA.	5.08-24.8	(62–65)

Cod	Palmitic acid, palmitoleic acid, oleic acid, gadoleic acid, stearic acid, EPA, DHA and ω-3 PUFA.	0.30-4.3	(62,65,66)
Shrimp	Palmitic acid, oleic acid, stearic acid arachidonic acid, EPA, DHA and ω-3 PUFA.	0.70-2.40	(65,67,68)
Crab	Palmitic acid, oleic acid, arachidonic acid, EPA, DHA and ω-3 PUFA.	0.99-6.66	(65,69,70)

4.1.2. Moisture content determination

The moisture content of the SS and FDSS samples was determined. Those results are presented in Table 4, they were used for the B&D and supercritical CO_2 sample characterization.

Table 4: Moisture content of the SS and FDSS samples.

Sample	Moisture content (%)
Steam-cooked sardine	67.50
Freeze-dried steam-cooked sardine	2.90

As expected, the moisture content of the FDSS has shown a minimal quantity of water in its composition because the sample has suffered a dehydration. In contrary, the SS sample presented more water in its composition, and so, in further lipid extractions processes a higher quantity of that sample will be necessary, for normalization of the extraction values of both samples to dry weight.

4.1.3. Bligh and Dyer method: total lipid extraction and purification

The B&D method was developed as a rapid method for isolating lipids from fish muscle, which makes this really popular method a good choice for the study. One reason for the popularity of this method is the combination of methanol and chloroform used to extract a broad range of lipid classes from a wide variety of sample matrices, allowing

the separation of all lipids from the samples, such as polar lipids, phospholipids and other lipids bound to the components of the cell membranes (71,53).

Seven B&D extractions were performed using the SS, and only 3 extractions using the FDSS, since the FDSS can produce more total lipid content after the extraction. As mentioned before, when these extractions are used for the SS samples they have less total lipid content extracted when compared to the FDSS samples, because 67.50% of their composition is water. For each extraction a yield was calculated using the equation from section 3.1.4, where in the sample weight value, a dry weight value was considered, calculated using the moisture content previously determined (section 4.1.2).

The total lipid content extracted from each extraction, the yield and average yield are presented in Table 5.

Table 5: Extracts obtained, yield and average yield from each sample after a B&D extraction.

Sample	Extract (g)	Yield ($g_{extract}/100\ g_{dry\ residue}$)	Average yield ($g_{extract}/100\ g_{dry\ residue}$)
SS	0.13	19.60	23.92
	0.14	20.73	
	0.15	22.46	
	0.18	27.76	
	0.16	24.39	
	0.17	25.81	
	0.18	26.70	
FDSS	0.55	28.09	28.93
	0.63	31.73	
	0.52	26.96	

The average yields were $23.92\ g_{extract}/100\ g_{dry\ residue}$ for SS and $28.93\ g_{extract}/100\ g_{dry\ residue}$ for FDSS. It makes sense that the FDSS extractions could produce more lipid content because, in contrary to the SS sample, the FDSS was dehydrated, which could imply a better extraction when samples are drier, without abundance of water to interfere. Either way, the results of both samples don’t diverge considerably. Despite that, both samples, excluding moisture, seemed to have approximately 60-70% of other components besides lipids in their compositions. Those components could be proteins or even ashes, as has been previously characterized in other studies using similar samples (63,72).

4.1.4.Supercritical CO_2 of lipids extraction

The supercritical CO_2 extraction method offers one less polluting application, in contrast to the conventional methods, which normally involve toxic and inflammable solvents, rendering the applicability of those conventional extracts in foodstuffs difficult. Furthermore, the CO_2 used in the method is easier to separate from the extract due to it being a gas at environmental pressure and temperatures (73,74).

Nine extractions were performed using the FDSS with various pressure and temperature conditions. The total lipid content extracted from each extraction, the yield and average yield are represented in Table 6.

Table 6: Extracts obtained, yield and average yield from FDSS sample after a supercritical CO_2 extraction at different pressures and temperatures (the yield considers the extract obtained during the extraction times and the retained extract obtained in the separators washed with acetone).

Extraction	Pressure (bar)	Temperature (ºC)	Extract (g)	Yield (g $_{extract}$/100 g $_{dry\ residue}$)
1	300	35	2.01	20.10
2		55	1.99	19.95
3		75	2.18	21.80
4	425	35	2.58	25.81
5		55	2.54	25.43
6		75	2.40	23.97
7	550	35	2.70	26.98
8		55	2.68	26.84
9		75	2.74	27.40

After the completion of 9 extractions with variations of temperature (35ºC, 55ºC and 75ºC) and pressure (300 bar, 435 bar and 550 bar) differences in yield results were observable in all extractions, but not considerably. The yield values range between 19.95 g $_{extract}$/100 g $_{dry\ residue}$ and 27.40 g $_{extract}$/100 g $_{dry\ residue}$, a difference of 7.90 g $_{extract}$/100 g $_{dry\ residue}$ from the worst to the best extraction performances.

It is noticeable that the extraction yield tends to increase when higher pressures are used, promoting an increase in solubility of the solutes. About the extractions made at constant 300 bar, the variations in temperature showed little difference in the final content extracted, with the extraction at 75ºC (extraction 3) promoting extractions values slightly higher, but with the extraction at 35ºC (extraction 1) promoting a better extraction curve at the initial stage, with an efficiency decrease in the final minutes (Figure 14).

Likewise, the extractions made at 425 bar had similar yields, apart from the temperature changes, with the extraction 6 (75ºC) having the lower curve of all of the extraction times considered. Once again, the extraction at 35ºC (extraction 4) started with a better performance but after 60 min the curve declined and the extraction at 55ºC (extraction 5) happened to have a better yield. The extraction at 75ºC (extraction 6) had the lower curve.

The best yield results were from the extractions at constant 550 bar, but in these conditions the higher temperature influenced a better curve (extraction 9) compared to the other temperatures.

Overall, the supercritical CO_2 extractions have shown to be a good alternative method of lipid extraction, even more if the conditions applied are the ones used in extractions 7, 8 or 9. Those 3 extractions have yields similar to the average yield of the conventional FDSS extraction. When compared to the average yield of the conventional SS extraction, the values are even superior.

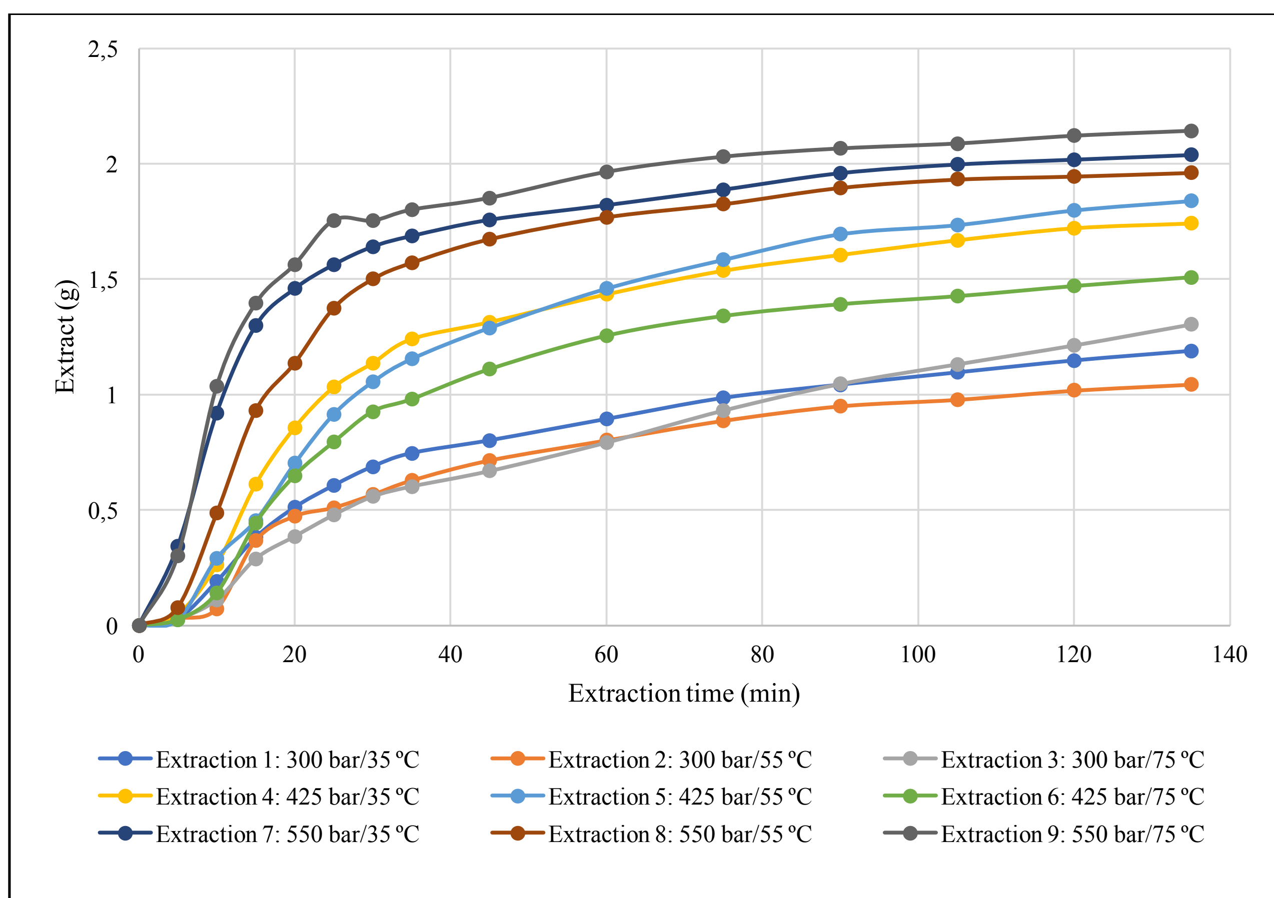

Figure 14: Supercritical extraction curves for the FDSS supercritical CO_2 extraction at different pressures and temperatures (the extraction values only take account the extract obtained during the extraction times and not the retained extracted obtained in the separators washed with acetone).

4.1.5. Lipid characterization (gas chromatography analysis)

The fatty acid composition of the lipids extracted from the conventional and supercritical CO_2 extractions are summarized in Table 7. It is noticeable that all the identified fatty acids are similar for all analysed samples. When compared to what was found in literature (section 4.1.1), the samples have a majority of palmitic acid (C16:0), oleic acid (C18:1 (n-9)), EPA (C20:5 (n-3)) and DHA (C22:6 (n-3)) in their composition, similar to what was found in the other studies.

In a minor abundance, myristic acid (C14:0), palmitoleic acid (C16:1 (n-7)), stearic acid (C18:0), cis-vaccenic acid (C18:1(n-7)), gadoleic acid (C20:1 (n-11)) and cetoleic acid (C22:1 (n-11)) were also identified.

It is noticeable that the lipids extracted by supercritical CO_2 extraction had lower DHA and EPA content compared to the solvent extraction, maybe due to the alteration in temperature and pressure that could influence the composition of these acids (53).

Table 7: Fatty acid composition of the sardine samples identified by gas chromatography analysis.

Sample	Fatty acid	C14:0 (Myristic acid)	C16:0 (Palmitic acid)	C16:1 (n-7) (Palmitoleic acid)	C18:0 (Stearic acid)	C18:1 (n-9) (Oleic acid)	C18:1 (n-7) (Cis-vaccenic acid)	C20:1 (n-11) (Gadoleic acid)	C20:5 (n-3) (EPA)	C22:1 (n-11) (Cetoleic acid)	C22:6 (n-3) (DHA)	ω-3 PUFA	ω-6 PUFA	SFA	MUFA	Total FA
B&D SS	**$mg_{FA}/g_{dry\ residue}$**	26.05	73.75	15.72	16.82	30.33	8.08	14.92	28.27	18.27	50.73	94.61	10.72	129.67	101.53	336.53
B&D FDSS	**$mg_{FA}/g_{dry\ residue}$**	15.82	49.01	10.13	9.64	23.21	3.85	11.33	22.35	9.27	32.28	67.57	8.59	83.08	63.91	223.14
300 bar | 35ºC	**$mg_{FA}/g_{dry\ residue}$**	7.95	27.45	5.14	5.53	11.74	2.80	5.01	6.46	4.87	8.71	21.62	3.59	46.00	32.58	103.79
300 bar | 55ºC	**$mg_{FA}/g_{dry\ residue}$**	9.28	24.46	4.77	4.50	8.88	2.37	4.41	5.18	4.37	7.25	17.60	2.65	42.50	28.87	91.62
300 bar | 75ºC	**$mg_{FA}/g_{dry\ residue}$**	10.62	30.88	5.82	5.83	12.34	2.79	4.99	6.61	4.63	9.07	22.29	3.52	52.94	35.45	114.19
425 bar | 35ºC	**$mg_{FA}/g_{dry\ residue}$**	14.17	40.01	7.59	7.54	15.19	3.73	6.97	9.78	6.83	15.24	34.13	4.65	69.28	46.29	154.36
425 bar | 55ºC	**$mg_{FA}/g_{dry\ residue}$**	16.82	45.45	8.64	7.80	15.82	3.99	6.96	7.93	7.64	12.40	29.23	4.42	77.48	49.84	160.97
425 bar | 75ºC	**$mg_{FA}/g_{dry\ residue}$**	11.47	33.21	6.25	6.51	13.03	3.28	6.11	8.00	8.43	12.92	28.67	3.78	57.27	43.37	133.09
550 bar | 35ºC	**$mg_{FA}/g_{dry\ residue}$**	14.23	42.51	8.39	9.03	18.63	4.47	7.62	12.76	10.25	19.64	43.68	6.03	74.09	57.06	180.86
550 bar | 55ºC	**$mg_{FA}/g_{dry\ residue}$**	16.89	49.16	9.53	8.77	17.48	4.61	7.66	8.06	8.09	10.99	28.69	4.68	82.47	54.90	170.74
550 bar | 75ºC	**$mg_{FA}/g_{dry\ residue}$**	18.32	52.55	9.94	9.66	19.74	4.70	1.53	9.74	8.58	13.17	33.32	5.77	89.49	53.09	181.67

ω-3 PUFA: omega-3 polyunsaturated fatty acids

ω-6 PUFA: omega-6 polyunsaturated fatty acids

SFA: saturated fatty acids

MUFA: monounsaturated fatty acids

Total FA: total fatty acids

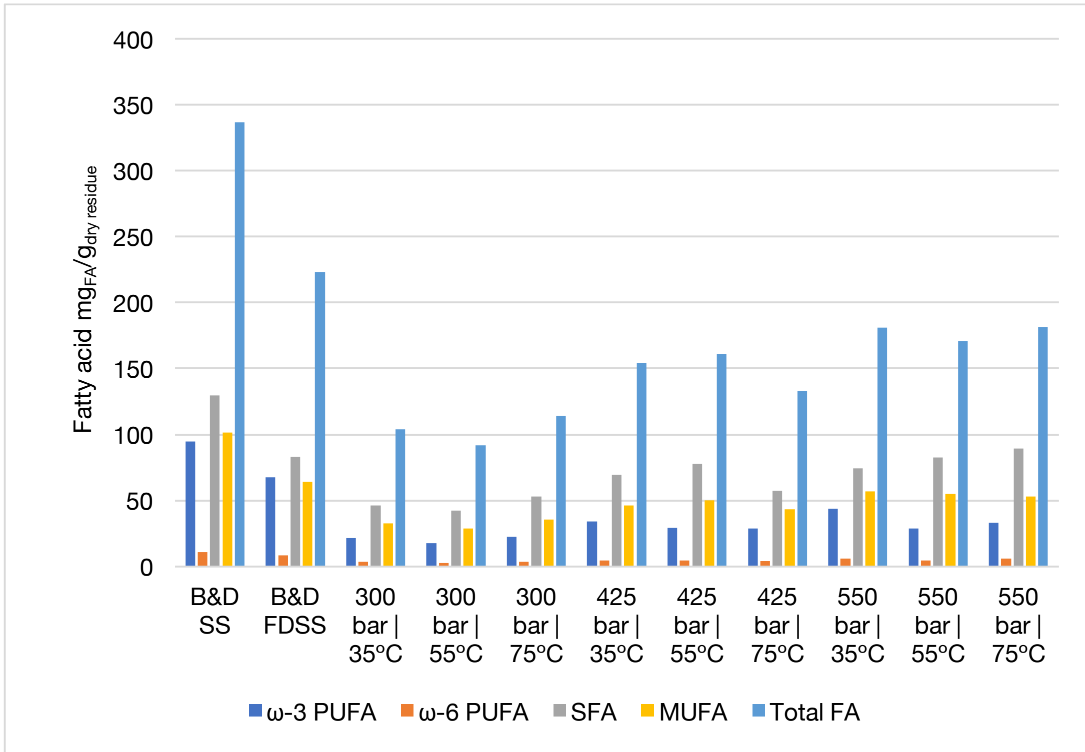

Figure 15: Content of ω-3 PUFA, ω-6 PUFA, SFA, MUFA and total FA from several extracts.

Analysing the results from Figure 15, a decrease of total FA in the supercritical method extracts is noticeable. Also, when higher pressures are applied in the supercritical extraction, the extracts tend to have more total FA in their composition. Once more a suggestion that the pressure of 550 bar is the better option for this method. Comparing both extracts from the B&D method, a better total FA content is observed when the original sample was SS. Which means if a dehydration is made in the sample before the extraction, some fatty acids can be lost in the extract.

Generally, all sardine extracts have more ω-3 PUFA in their composition rather than ω-6 PUFA, and since it was stated that ω-3 PUFA exhibit strong antimicrobial activity, these values are in favour of the study. Likewise, the content of MUFA in the extracts is rather less than the content of SFA.

The results of the analysis hypothetically indicate a better antimicrobial susceptibility to the B&D extracts than the supercritical ones. Still, all of them should be good options for the following part of this study.

4.2. Antimicrobial susceptibility testing

4.2.1. Well diffusion and disk diffusion methods

The evaluation of the antimicrobial activity of the lipids extracted from sardine waste streams started with the implementation of the well diffusion and disk diffusion methods. The disk diffusion method is a commonly used method for testing the susceptibility of certain fastidious bacterial pathogens to a determined compound, by observing the presence or absence of inhibition halos (75). Likewise, the well diffusion method is an adaptation of the disk diffusion method and follows the same principle.

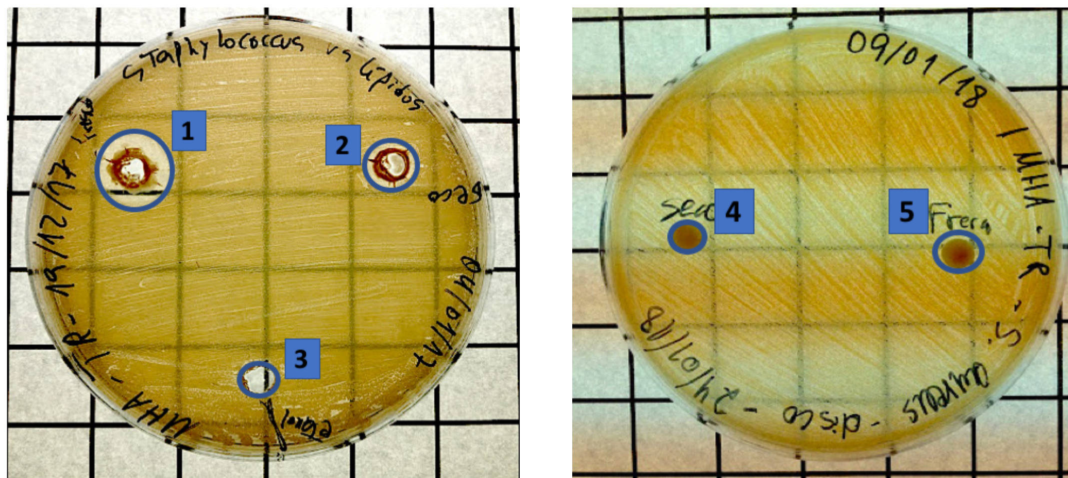

Figure 16: Well diffusion method in the left and disk diffusion method in the right against *S. aureus*. 1- SS_C; 2- $FDSS_C$; 3- Absolute ethanol (blank control); 4- $FDSS_C$; 5- SS_C.

In the well diffusion assay, the results showed an antimicrobial activity of the SS_C and $FDSS_C$ when determined against *S. aureus*, with inhibitory halos of 13 and 9 mm, respectively, as shown in Figure 16. The inhibitory halos were absent or unreadable when the antimicrobial agents were tested against *E. coli*, *P. aeruginosa* and *K. pneumoniae*, suggesting a better bacterial susceptibility against the gram-positive bacteria tested. This could be due to the outer membrane of gram-negative bacteria behaving as an entry barrier against fatty acids, while the cell wall of gram-positive may allow the partition of fatty acids into the inner membranes (11).

In the disk diffusion assay, the results showed an antimicrobial activity of the SS_C when determined against *S. aureus*, with an inhibitory halo of 6.5 mm. The antimicrobial activity of the $FDSS_C$ was absent when using this methodology, as shown in Figure 16.

The $FDSS_C$ extract is thicker than SS_C, and consequently the homogenization with the absolute ethanol was more difficult to accomplish. Maybe because the $FDSS_C$ was

more subjected to oxidation (a consequence from the dehydration), which could explain the lower antimicrobial activity observable in the $FDSS_C$ (72). Other reason for the lower activity could simply be the lower FA content from $FDSS_C$, when compared with SS_C (section 4.1.5).

Comparing these two methodologies, the well diffusion method seems to present wider inhibitory halos, so, in qualitative terms, it is more useful. The direct contact between the antimicrobial agent and the agar could justify the well diffusion having a better performance. This contrasts with the disk diffusion, where the antimicrobial agent is loaded on a disk and not directly in contact with the agar. It is not certain if the antimicrobial agent, when loaded onto the disks, would have a reduced diffusion in the agar. However, the disks retained a brown colour, which demonstrate that at least a fraction of the sample has not diffused to the agar. Additionally, in the well diffusion, the quantity of antimicrobial agent used is slightly higher (5 µl) than the quantity used in the disk diffusion, which could also justify the better performance of the well diffusion.

These two methodologies have shown that lipid extracts from the canned fish industry waste streams can present antimicrobial activity against at least a gram-positive bacteria (*S. aureus)*. On the other hand, since the extracts aren't so active against gram-negative microorganisms, further quantitative methodologies had to be performed.

4.2.2.Agar microdilution method

The agar microdilution was selected as a quantitative susceptibility testing method. This method allows for a quantitative assessment by determining the lowest concentration of the agent capable of inhibiting the growth of the tested microorganism (58). It allows a uniform and stable dispersion of the extract when incorporated in the agar medium and has the ability to form a visible bacterial growth spot onto the solid agar medium.

The test was performed in an initial concentration of 40 mg/ml for SS_C and $FDSS_C$, followed by two-fold dilutions (20, 10, 5, 2.5 and 1.25 mg/ml), applied against *S. aureus*, *E. coli*, *P. aeruginosa* and *K. pneumoniae* bacterial targets.

The results were difficult to interpret due to the nature of the lipids (Figure 17). When the more concentrated wells were observed with the naked eye, the bacterial growth was extremely difficult to distinguish from the lipid dispersion in the agar, which could be a result of the reddish colour and opacity of the lipids. It was suggested in other studies that this technique should be used only for pure substances (76,77).

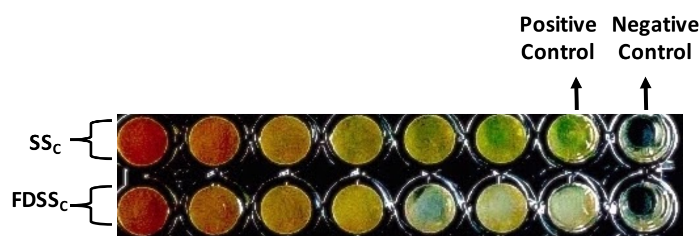

Figure 17: Example of an agar microdilution test. *P. aeruginosa*.

The MIC results were recorded as described in section 3.2.5, where a better antimicrobial activity from SS$_C$ was observed (Table 8). On the other hand, in 3 assays, the FDSS$_C$ results were more inconsistent, oscillating between 5, 10 and 20 mg/mL. Those results may result from the higher viscosity of the FDSS$_C$, in comparison to the SS$_C$, which makes it more difficult to dissolve in the agar. Similarly, as mentioned in disk and well diffusion methods, the lower activity could be from the lower FA content from FDSS$_C$, when compared with SS$_C$ (section 4.1.5).

Table 8: MICs (mg $_{extract}$/ml) of SS$_C$ and FDSS$_C$ against *S. aureus* using the agar microdilution method.

Extract	Median MIC (mg $_{extract}$/ml) (MIC $_{n=1}$/ MIC $_{n=2}$/ MIC $_{n=3}$)			
	S. aureus	*E. coli*	*P. aeruginosa*	*K. pneumoniae*
SS$_C$	**5** (5 / 5 / 10)	>20	>20	>20
FDSS$_C$	**10** (5 / 10 / 20)	>20	>20	>20

The only observable MIC results were from *S. aureus*, maybe because the MIC was lower than the other bacteria, therefore, the medium with a more diluted antimicrobial agent was more translucent, with a better readable bacterial growth, as it is represented in Figure 18. As so, this can mean that the other bacteria are more resistant to those antimicrobial agents, and so, another method had to be performed to verify this assumption.

In this susceptibility testing, the FDSS$_{SC}$ was still not used as an antimicrobial agent, since the method did not provide consistent results.

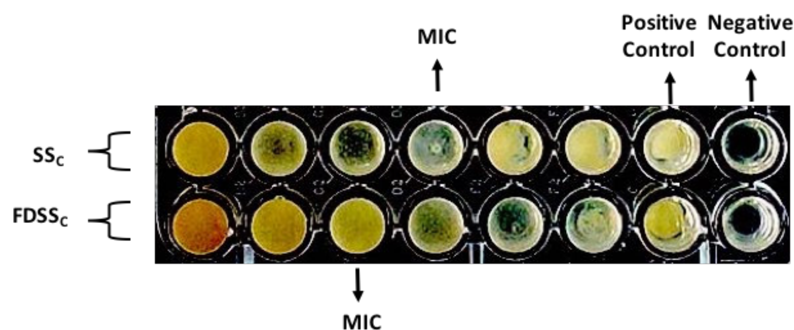

Figure 18: Example of an agar microdilution test. *S. aureus* (n=3).

4.2.3.Broth microdilution methods

The broth microdilution was selected as a quantitative susceptibility testing method. It is determined in a 96-well microplate, where bacteria are inoculated into liquid growth medium with several different concentrations of the antimicrobial agent. Similarly to the agar microdilution method, this methodology allows the quantitative assessment by establishing the lowest concentration of the agent capable of preventing the growth of the tested microorganism (58,78).

To screen the extract with the best activity, all the extracts from the CO_2 supercritical extractions were used. Their antimicrobial activities were determined against 2 bacterial target strains (*S. aureus* and *E. coli*) with the determination of the MIC values (Table 9, Figure 19) as described in section 3.2.6.

Table 9: MICs (mg $_{extract}$/ml) of all the $FDSS_{SC}$ extractions against *S. aureus* and *E. coli* where the broth microdilution method was used.

Extract	Pressure (bar)	Temperature (ºC)	Yield (mg $_{FA}$/g $_{dry\ residue}$)	Total FA mg $_{FA}$/g $_{dry\ residue}$	Median MIC (mg $_{extract}$/ml) (MIC $_{n=1}$/ MIC $_{n=2}$/ MIC $_{n=3}$)	
					S. aureus	*E. coli*
$FDSS_{SC}$(1)	300	35	201.00	103.79	**4.69** (9.38;4.69;2.34)	**75.00** (75.00;75.00;75.00)
$FDSS_{SC}$(2)		55	199.50	91.62	**4.69** (4.69;0.59;469)	**75.00** (75.00;75.00;75.00)
$FDSS_{SC}$(3)		75	218.00	114.19	**2.34** (2.34;2.34;2.34)	**75.00** (75.00;75.00;75.00)
$FDSS_{SC}$(4)	425	35	258.10	154.36	**4.69** (4.69;4.69;1.17)	**75.00** (75.00;75.00;75.00)
$FDSS_{SC}$(5)		55	254.30	160.97	**2.34** (2.34;2.34;2.34)	**75.00** (75.00;75.00;75.00)
$FDSS_{SC}$(6)		75	239.70	133.09	**2.34** (2.34;2.34;2.34)	**75.00** (75.00;75.00;75.00)
$FDSS_{SC}$(7)	550	35	269.80	180.86	**4.69** (4.69;4.69;2.34)	**75.00** (75.00;75.00;75.00)

$FDSS_{SC}$(8)	55	268.40	170.74	**2.34** (4.69;2.34;2.34)	**75.00** (75.00;75.00;75.00)
$FDSS_{SC}$(9)	75	274.00	181.67	**2.34** (9.38;2.34;1.17)	**75.00** (75.00;75.00;75.00)

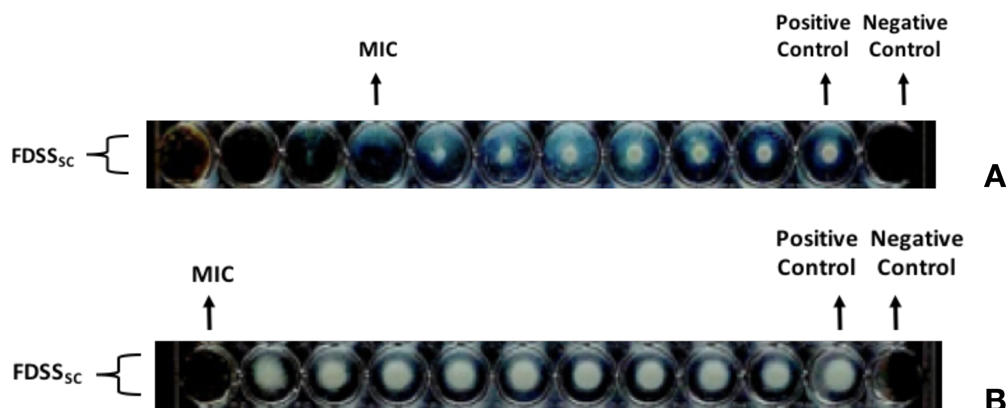

Figure 19: Examples of broth microdilution test. MIC observation using *S. aureus* (A) and *E. coli* (B).

Afterwards, one single $FDSS_{SC}$ extract was chosen. That extract should be the most profitable and effective in order to be select for the following analyses, which means, the extract that had the lowest MIC results and, at the same time, one proper yield and higher total FA content (Table 9). By observing the previous table, a pattern can be deduced from these extracts: extracts from higher temperatures (55ºC and 75ºC) produce better results, such as a MIC of 2.34 mg $_{extract}$/mL against *S. aureus*. This could be because some lipids or antimicrobial component can't be extracted efficiently at lower temperatures (35ºC).

Extracts 8 and 9 were the more promising ones. They had higher yields, higher FA contents and lower MICs. Since extract 8 was produced at 55ºC instead of 75ºC, that extract was chosen for the remaining tests. Considering extract 9 resulted from a higher temperature of extraction, it could have been subjected to an increased lipid degradation and alterations in composition.

Another feature was the constant and high MIC results when the extracted lipids were tested against *E. coli*. This shows that this extract is not as effective against these bacteria, maybe due to *E. coli* being a gram-negative bacterium and having a different cell wall structure.

The selected $FDSS_{SC}$ was tested along with the conventional extracts against 5 different microorganisms and the obtained values were compared. Each of the extracts was obtained according to different extraction conditions, so, the MICs were observed in order to evaluate if the extracts obtained by the supercritical method could be equally efficient in terms of antimicrobial activity as the ones obtained by conventional methods.

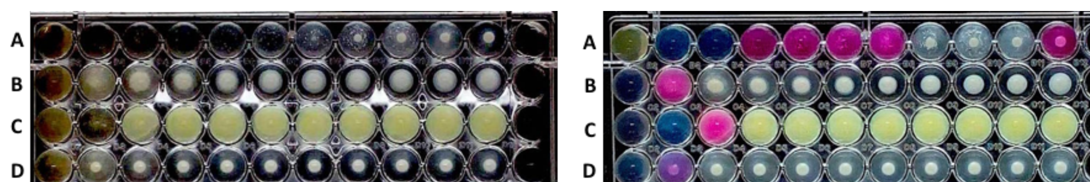

Figure 20: Examples of a broth microdilution test. The picture on the left is representative of result from where MIC values can be observed and the picture on the right shows the same plate after the addition of PrestoBlue reagent. The target bacteria were *S. aureus* (A), *E. coli* (B), *P. aeruginosa* (C), and *K. pneumonia* (D)

The MBC values were also determined (Table 11) as described in sections 3.2.6 and 3.2.7.2 in order to further characterize the extract. The MBC is a less common determination compared to the MIC but still interesting because, in some cases, killing bacteria could be preferable instead of inhibiting their growth.

Table 10: MICs (mg $_{extract}$/ml) of SS_C, $FDSS_C$ and $FDSS_{SC}$ (8) extractions against *S. aureus, E. coli, P. aeruginosa, K. pneumoniae* and *C. albicans* when used the broth microdilution method.

Extract	Median MIC (mg $_{extract}$/ml) (MIC $_{n=1}$/ MIC $_{n=2}$/ MIC $_{n=3}$)				
	S. aureus	*E. coli*	*P. aeruginosa*	*K. pneumoniae*	*C. albicans*
SS_C	**0.29** (0.29; 0.59; 0.29)	**18.75** (37.50;18.75;18.75)	**37.50** (37.50;37.50;37.50)	**18.75** (37.50;18.75;18.75)	**4.69** (4.69;9.38;4.69)
$FDSS_C$	**9.38** (9.38;18.75;4.69)	**75.00** (75.00;75.00;75.00)	**75.00** (75.00;75.00;75.00)	**75.00** (75.00;75.00;75.00)	**9.38** (9.38;18.75;9.38)
$FDSS_{SC}$ (8)	**2.34** (2.34;2.34; 9.38)	**75.00** (75.00;75.00;75.00)	**37.50** (37.50;75.00;37.50)	**75.00** (75.00;75.00;75.00)	**4.69** (4.69;4.69;4.69)

Table 11: MBCs (mg $_{extract}$/ml) of SS_C, $FDSS_C$ and $FDSS_{SC}$ (8) extractions against *S. aureus, E. coli, P. aeruginosa, K. pneumoniae* and *C. albicans* when used the broth microdilution method.

Extract	Median MBC (mg $_{extract}$/ml) (MBC $_{n=1}$/ MBC $_{n=2}$/ MBC $_{n=3}$)				
	S. aureus	*E. coli*	*P. aeruginosa*	*K. pneumoniae*	*C. albicans*
SS_C	**9.38** (18.75; 9.38; 9.38)	**37.50** (37.50;37.50;37.50)	**37.50** (37.50;37.50;37.50)	**37.50** (37.50;37.50;37.50)	**18.75** (18.75;18.75;18.75)
$FDSS_C$	**75.00** (75.00;75.00;75.00)	**75.00** (75.00;75.00;75.00)	**75.00** (75.00;75.00;75.00)	**75.00** (75.00;75.00;75.00)	**18.75** (18.75;37.5;18.75)
$FDSS_{SC}$ (8)	**37.50** (18.75;37.50;37.50)	**75.00** (75.00;75.00;75.00)	**37.50** (37.50;75.00;37.50)	**75.00** (75.00;75.00;75.00)	**18.75** (18.75;18.75;18.75)

Both $FDSS_{SC}$ and $FDSS_C$ extracts where freeze-dried before being extracted by two different methods. Therefore, when the sample used is freeze-dried, the supercritical method seems to be the best option to extract lipid components. Comparing MIC and MBC results of both samples (Table 10 and Table 11), the $FDSS_{SC}$ was more effective against *S. aureus*, *P. aeruginosa* and *C. albicans* than the $FDSS_C$.

Both SS_C and $FDSS_C$ extracts where extracted by the conventional B&D method. When comparing both samples, the SS_C produced lower MIC and MBC results against all tested microorganisms. Despite an increase in extraction yield of the B&D method for the freeze-dried samples, if the goal is to obtain a better antimicrobial agent, the freeze-drying process should be avoided.

All the extracts tested have an antimicrobial activity against all the selected microorganisms used in this study. Although, the antimicrobial activity was stronger against some of them. The SS_C, $FDSS_C$ and $FDSS_{SC}$ showed lower MIC values against *S. aureus*, the gram-positive bacteria, which means a higher antimicrobial activity. Conversely, the MIC values were higher against the gram-negative bacteria (*E. coli, P. aeruginosa, K. pneumoniae*), which corresponds to a lower antimicrobial activity. Once again, for this methodology, the extracts were more active against gram-positive bacteria than gram-negative bacteria. Likewise, the extracts also show a clear antimicrobial activity against *C. albicans*. This activity happened to be more effective against yeasts as opposed to gram-negative bacteria.

Overall, results show that the SS_C is the extract with the best antimicrobial activity, followed by $FDSS_{SC}$ and $FDSS_C$, respectively. It seems the B&D method produces the best antimicrobial extract, if the sample hasn't been previously freeze-dried. But, for the purpose of this study, if the goal is to get an extract free of toxic or inflammable solvents, with the possibility to be used as a natural preservative, the supercritical extract has shown to be a good alternative.

Chapter 5

5. Conclusion

This study evaluated the antimicrobial potential of lipids extracted from canned fish industry waste streams.

Initially, a comparison of the yields between the supercritical and the conventional extracts was made, in order to analyse the efficiency of the supercritical extractions regarding this waste stream matrix. The results show that the yield of the extracts obtained by the supercritical method, when compared to the yields obtained in the conventional method, are similar. In addition, despite the similar yields, a lipid characterization has shown that the supercritical method produces samples with lower total FA, when compared with the samples produced by B&D method. Despite that, the supercritical extraction has the advantage of producing extracts free of toxic solvents, suggesting a possible application in foodstuffs for the future.

The well and disk diffusion methods were used to screen for the antimicrobial potential of lipid extracts from sardine waste streams. This screening was further pursued using quantitative methodologies, such as the agar and broth microdilutions.

The obtained MIC and MBC results confirms that all tested samples have antimicrobial potential against *S. aureus*, *E. coli*, *K. pneumoniae, P. aeruginosa* and *C. albicans*. Regarding the lipids extracted by the supercritical method, this study suggests that they could be good for food applications, as natural preservatives. The extraction yields are similar to the conventional ones as is the lipid composition, even if the total FA content is slightly lower than the conventional extracts.

Overall, this study shows a good alternative use of fish wastes produced by the fish industry which are generally discarded. From these wastes, lipids with antimicrobial potential could be extracted, and then be reintroduced in foodstuffs, or even cosmetics, as a natural preservative.

Finally, the incorporation of the lipids extracted by supercritical method in real matrices, and study the results obtained, could be an interesting step to follow in the future.

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