Vanessa Andreia da Costa Rocha

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Master dissertation

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Supervisor: Doctor Maria do Pilar de Araújo Teixeira

Co-supervisor: Doctor Diana Alexandra Ferreira Rodrigues

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Abstract

Microbial contamination is a serious concern in areas of food processing because of its negative impact on public health. Numerous foodborne outbreaks occur every year due to various types of pathogenic microorganisms. The most common procedures to control these undesirable microorganisms include the use of disinfectants, however the disinfection procedures often demonstrate some inefficiency in the inactivation and removal of the same. Thus, the main objective of this study was the characterization of cells that survived to cleaning and disinfection in food processing areas of one meat retail facility. For that, samples were collected from meat processing surfaces of one meat retail facility in the center of Braga (Portugal), before and after the cleaning and disinfection procedures. Then the isolation of bacteria by selective media and the identification by 16S sequencing were performed. Finally, the isolates were phenotypically characterized in terms of biofilm formation ability, susceptibility of planktonic cells and biofilms to two disinfectants commonly used in the food industry (sodium hypochlorite (SH) and hydrogen peroxide (HP)), and susceptibility of planktonic cells to broad spectrum antibiotics (ampicillin (AMP) and rifampin (RIF)).

Listeria innocua, *Serratia* spp. and *Hafnia alvei* were the microorganisms identified in the samples collected before cleaning and disinfection procedures, while *Cellulosimicrobium* spp., *Serratia* spp., and *Enterobacter* spp. were identified in the samples collected after cleaning and disinfection procedures. These microorganisms can represent a risk for public health, since some species are pathogenic. The results demonstrated that planktonic cells of all isolates tested were more susceptible to HP compared to SH. Moreover, these isolates showed good biofilm formation capacity, with an increase of total biomass along the time and, as expected, less susceptibility to disinfectants compared to planktonic cells. After exposure of biofilms to HP and SH at concentrations higher than the recommended ones, biofilms were still able to survive which may contribute to bacterial resistance to these compounds, as well as cross-resistance to antibiotics.

In view of the results obtained in this study, it was concluded that cells that survive to cleaning and disinfection procedures represents a risk in terms of contamination due to biofilm formation ability, which can contribute to bacterial resistance to these compounds as well as cross-resistance to antibiotics.

Resumo

A contaminação microbiana constitui um grave problema em áreas de processamento de alimentos devido ao impacto negativo na saúde pública. Diversos surtos alimentares ocorrem todos os anos devido aos vários tipos de microrganismos patogénicos presentes. Os procedimentos mais comuns de controlo destes microrganismos indesejáveis incluem a aplicação de desinfetantes, contudo esses procedimentos de desinfeção demonstram, frequentemente, alguma ineficiência na inativação e eliminação dos mesmos. Assim, o principal objetivo deste estudo foi a caracterização das células que sobrevivem à limpeza e desinfeção em áreas de processamento de alimentos num talho. Para isso, recolheram-se amostras antes e após a limpeza e desinfeção das superfícies de processamento das carnes num estabelecimento no centro de Braga (Portugal) e, de seguida, foi realizado o isolamento de microrganismos por meios seletivos e identificados por sequenciação 16S. Finalmente, os isolados foram caracterizados fenotipicamente em termos da sua capacidade de formação de biofilme, suscetibilidade das células planctónicas e do biofilme a dois dos desinfetantes comumente utilizados na indústria alimentar (hipoclorito de sódio (HS) e peróxido de hidrogénio (PH)), a susceptibilidade de células planctónicas a dois antibióticos de largo espectro (rifampicina (RIF) e ampicilina (AMP)).

Listeria innocua, *Serratia* spp. and *Hafnia alvei* foram os microrganismos identificados nas amostras recolhidas antes dos processos de limpeza e desinfeção, enquanto que *Cellulosimicrobium* spp., *Serratia* spp., and *Enterobacter* spp. foram identificados nas amostras recolhidas após os processos de limpeza e desinfeção. Estes microrganismos podem representar um risco para a saúde pública devido a algumas espécies serem patogénicas. Os resultados obtidos mostraram que as células planctónicas foram mais suscetíveis ao peróxido de hidrogénio em comparação com o hipoclorito de sódio. Os isolados apresentaram boa capacidade de formação de biofilme, com um aumento de biomassa total ao longo do tempo e, como esperado, menor suscetibilidade aos desinfetantes em comparação com as células planctónicas. Após a exposição dos biofilmes ao peróxido de hidrogénio e ao hipoclorito de sódio, em concentrações mais elevadas do que as recomendadas, os biofilmes foram ainda capazes de sobreviver e podem contribuir para a resistência bacteriana a esses compostos, bem como uma resistência cruzada aos antibióticos.

Face aos resultados obtidos é possível concluir que as células que sobrevivem aos processos de limpeza e desinfeção representam um risco em termos de contaminação devido à capacidade de formação de biofilme, contribuindo para a resistência bacteriana a esses compostos bem como para a resistência cruzada aos antibióticos.

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Glossary of abbreviations

AMP   Ampicillin

CLSI   Clinical and Laboratory Standards Institute

CV   Crystal Violet

EU   European Union

EUCAST   European Committee on Antimicrobial Susceptibility Testing

EPS   Extracellular Polymeric Substance

HACCP   Hazard Analysis Critical Control Point

HP   Hydrogen Peroxide

ISO   International Organization for Standardization

LPS   Lipopolysaccharide

MIC   Minimum Inhibitory Concentration

MBEC   Minimum Biofilm Eradication Concentration

PBS   Phosphate-Buffered-Saline

PS   Peak Serum

RIF   Rifampicin

SH   Sodium Hypochlorite

SPSS   Statistical Package for the Social Sciences

TAE   Tris-acetate-EDTA

TSA   Tryptic Soy Agar

TSB   Tryptic Soy Broth

WHO   World Health Organization

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Chapter 1

Introduction

This chapter provides a general framing of this thesis, as well as a description of the objectives of the work. Finally, it is presented the outline of the dissertation, working as a guide line to the overall works presented in the further chapters.

1.1. Context

Develop products with quality and that do not harm the health of consumers is a challenge for industries, and the food industry has a big importance in relation to others mainly due to its impact in global public health. In this sector, apart the floor and windows, there are equipments that require an efficient cleaning due to the existence of microorganisms that survive to disinfection programs leading to biofilm formation, which constitutes a higher risk of food contamination. Throughout of the food chain, maintenance of hygienic conditions is a fundamental factor in the control of foodborne diseases. Microorganisms such as *Listeria monocytogenes*, *Escherichia coli* and *Salmonella* spp. represent the most common foodborne pathogens that cause diseases in humans. Products provided from retail facilities are fractionated and can be subjected to the risks of contamination. Various factors such as cross-contamination, poor hygiene in food preparation and inappropriate processing may allow the multiplication of microorganisms until they reach infectious doses. When this happens, the products that do not undergo heat treatment can cause foodborne diseases, with unpredictable implications for consumers. Inadequate cleaning and disinfection of food processing environments causes economic losses and represents a serious risk to public health. Foodborne pathogens present on food contact surfaces have ability to adhere and form biofilms on different surfaces, as shown by numerous studies. Also, it is very well documented the difficulty in eliminating adhered and biofilm forms of these microorganisms compared to planktonic cells.

Disinfection of surfaces is an important issue related with the acquisition of bacterial resistance to disinfectant agents and the possible relation between disinfectants and the resistance to antibiotics. Disinfectants and antibiotics can have similar modes of action and, thus, induce in similar ways the development of bacterial resistance.

1.2. Objectives

Given the context described above, the main aim of this work was the characterization of cells that survived to cleaning and disinfection in food processing areas of one meat retail facility in the center of Braga, Portugal. More specifically, the work was focused on the following goals:

- Phenotypical characterization of the isolates in terms of biofilm formation ability, susceptibility to disinfectants in planktonic cells and biofilm, and eventual cross-resistance to antibiotics.

1.3. Outline of the dissertation

This dissertation is divided in eight chapters. This first chapter consists on a brief introduction to the theme of this dissertation and defines the objectives proposed with this work.

On the second chapter it is presented the state of the art regarding microbial food contamination and cross-contamination, foodborne diseases, as well as a description of the related microorganisms. Several aspects related with microbial contamination of food contact surfaces and control of foodborne pathogens is also presented.

The third chapter describes some considerations of the methodology.

The fourth chapter presents the methods used for realization this thesis.

The fifth chapter shows the results regarding the identification of isolates collected before and after disinfection, biofilm formation ability of the bacterial isolates, the susceptibility of planktonic cells and biofilm to different disinfectants, and the eventual acquisition of cross-resistance to antibiotics.

The sixth chapter presents a discussion about the results.

The seventh chapter contains the conclusions of the work done and the proposals for future work.

Finally, the bibliography used for research is presented in the chapter 8.

Chapter 2

State of the art

This chapter encloses the literature review, presenting in the first sections a brief introduction to microbial food contamination and cross-contamination, foodborne diseases and pathogenic microorganisms associated. Also, adhesion and biofilm formation, different approaches to control foodborne microorganisms, and cross-resistance to antimicrobials are described.

2.1. Microbial food contamination and cross-contamination

Food contamination is a constant public concern. There are three main types of food contaminants: microbiological, chemical, and physical (Scott, 1996) but the biggest problem related with food is due to pathogenic microorganisms rather than chemical or physical contaminants. The consumption of food contaminated with particular microorganisms or microbial products can cause diseases, as food infections and food poisoning (Reij and Den Aantrekker, 2004). There is a relationship between certain types of food and certain pathogens, which results from the natural contamination of the different foods and the processing or cooking customarily applied (Varman and Evans, 1996). There are several factors responsible for the occurrence of foodborne diseases. Cross-contamination of food is one of the most important factors contributing to the increasing number of foodborne diseases. This term is frequently used to refer to, in a general way, direct or indirect transfer of bacteria/virus from a contaminated product to a product not contaminated. In addition, other terms have been used to refer to bacterial transfer, as recontamination (contaminating food after an inactivation process), poor hygiene of food handlers, and contaminated equipment (Rodríguez *et al.,* 2008). The risk of cross-contamination is associated with various stages of food preparation (Greig and Ravel, 2009). It has been observed that among the most common causes associated with cross-contamination of food are the inadequate cleaning of equipment and utensils, as well as poor personal hygiene (Rodríguez *et al.,* 2008). Food industries has taken measures to ensure food safety principles, as the use of effective programs of quality control, implementation of Hazard Analysis Critical Control Point (HACCP) programs, and the increasingly use of safe methods during processing, transportation, storage and distribution of food. However, it is very important the training of food handlers and education of consumers in order to avoid foodborne diseases (Havelaar *et al.,* 2010).

2.2. Foodborne diseases and associated pathogenic microorganisms

A diverse range of microorganisms can be present in food and food ingredients, which can include spoilage organisms as *Pseudomonas aeruginosa*, and also important pathogens as *E. coli*, *L. monocytogenes* and *Salmonella enterica*. These organisms are able to grow due to intrinsic (water activity and pH) and extrinsic (temperature, processing conditions and gaseous

atmosphere) properties of the food (Eviras, 2001). Table 2.1 shows the major foodborne pathogens and the main characteristics of the diseases they cause.

Table 2.1. Microorganisms responsible for common foodborne disease.
Adapted from: FDA, 2014 and Varnam and Evans, 1996.

Microorganism	Symptoms	Disease	Food sources
Salmonella spp.	Diarrhea, vomiting, fever and malaise, usually 12 to 16 hours after ingestion.	Infection	Contaminated eggs, poultry, unpasteurized milk or juice, cheese, contaminated raw fruits, vegetables.
Shigella spp.	Diarrhea with mucoid, bloody stools, 12 to 50 hours after ingestion.	Infection	Food or water contaminated with human fecal material, ready-to-eat foods touched by infected food workers (raw vegetables, salads, sandwiches).
Campylobacter jejuni	Prodromal fever and malaise, 2 to 11 days after ingestion followed by abdominal pain and profuse diarrhea.	Infection	Raw and undercooked poultry, unpasteurized milk, contaminated water.
Escherichia coli	Symptoms vary according to type of *E.coli* infection.	Infection	Water or food contaminated with human feces, undercooked beef, unpasteurized milk and juice, raw fruits and vegetables.
Listeria monocytogenes	Meningitis in neonates, abortion in pregnant females, septicaemia. Usually extended period between ingestion and appearance of symptomas.	Infection	Fresh cheeses, unpasteurized or inadequately pasteurized milk, ready-to-eat deli meats.
Staphylococcus aureus	Vomiting, abdominal pain and diarrhoea on some accasions, 2 to 6 hours after ingestion. Severe dehydration may result in collapse.	Intoxication	Unrefrigerated or improperly refrigerated meats, potato and egg salads, cream pastries.

Table 2.1. (Cont.) Microorganisms responsible for common foodborne disease.

Adapted from: FDA, 2014 and Varnam and Evans, 1996.

Microorganism	Symptoms	Disease	Food sources
Bacillus cereus	Two distinct syndromes: diarrhea and emetic.	Intoxication	Meats, stews, gravies, vanilla sauce.
Clostridium botulinum	Fatigue, lassitude, dizziness and effects on the central nervous system including speech difficulties and visual disturbances. Onset is 24 to 72 hours after ingestion.	Intoxication	Inadequately processed, home-canned foods, sausages, seafood products, chopped bottled garlic, honey.

Foodborne diseases represent a global threat to human health. Most of these diseases are associated with pathogenic microorganisms and/or their toxins, while other causes, such as parasites and chemical substances naturally present in some foods, are also responsible (Newell *et al.,* 2010). According to the World Health Organization (WHO) a foodborne disease is often of toxic or infectious nature, and is caused by pathogenic microorganisms that come in contact with human body through ingestion of contaminated food or water (Balbani and Butugan 2001). In the year 2013, most of the reported outbreaks in European Union (EU) remain to be caused by *Salmonella*, followed by viruses, bacterial toxins, and *Campylobacter* (Figure 2.1).

In 2013, 5196 foodborne outbreaks were reported in EU causing a high number of human cases and hospitalizations. According to European Food Safety Authority (EFSA) and European Centre for Disease Prevention and Control (ECDC), the three main food vehicles in the reported foodborne outbreaks were eggs and egg products (18.5%), followed by others foodstuffs (15.4%) and finally mixed food (10.7%). The category 'Household/domestic kitchen' (38.5 %) was the most commonly reported setting, followed by 'Restaurant, cafe, pub, bar, hotel' (22.2 %). Apart from restaurants and households, the next most common settings in strong-evidence outbreaks were 'Other settings' (8.6 %) and 'School, kindergarten' (8.3 %) (EFSA, 2015).
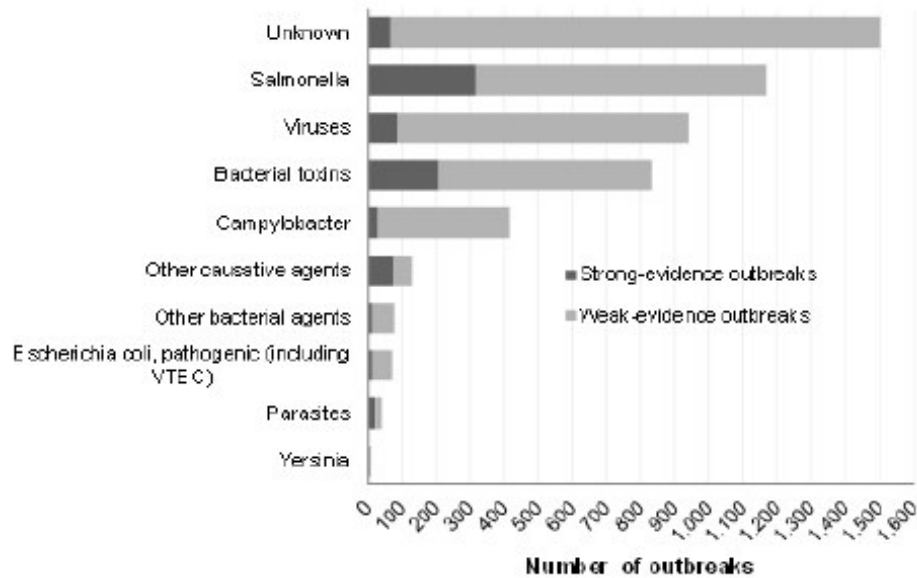
Figure 2.1. Foodborne outbreaks in Europe Union, 2013. Bacterial toxins include toxins produced by Bacillus, Clostridium and Staphylococcus. Foodborne viruses include calicivirus, hepatitis A virus, Flavivirus, Rotavirus and other unspecified viruses. Other causative agents include mushroom toxins, marine biotoxins, histamine, mycotoxins and escolar fish (wax esters). Parasites include primarily Trichinella, but also Cryptosporidium, Giardia and other unspecified parasites. Other bacterial agents include Listeria, Brucella, Shigella, Vibrio and other unspecified bacterial agents. In this figure, the category 'Escherichia coli, pathogenic (including VTEC)' also includes one strong-evidence outbreak due to pathogenic E. coli other than VTEC. Adapted from: EFSA, 2015.

2.3. Microbial contamination of food contact surfaces

Adhered cells and biofilm show increased resistance against stress factors commonly used in the decontamination of food contact surfaces and, due to this fact, the adherence and biofilm formation of bacteria on these surfaces have implications on hygiene (Aarnisalo *et al.,* 2000). The existence of pathogenic bacteria on food and food contact surfaces increases the food safety risk (Shi and Zhu, 2009). Pathogenic microorganisms that cause diseases associated with biofilms have been related with the presence of *L. monocytogenes*, *Yersinia enterocolitica*, *Campylobacter jejuni*, *Salmonella* spp., *Staphylococcus* spp*.* and *E. coli O157:H7* (Aarnela *et al.,*

2007; Dykes *et al.,* 2003; Sharma and Anand, 2002; Waak *et al.,* 2002) thus, it is important to know the mechanisms involved in bacterial adhesion and biofilm formation.

2.3.1. Bacterial adhesion

Biofilm development is subsequent to adhesion of microorganisms to surfaces. Both processes are very complex and are affected by various factors. To explain the adherence process and biofilm formation on food contact surfaces several mechanisms have been suggested. Microbial adhesion corresponds to the first stages of biofilm formation and can be divided into reversible and irreversible adhesion. In the initial phase, the surface is conditioned by the presence of food residues and, then, planktonic cells are transported from the bulk liquid to the surface, reversibly attaching to the surface. At this stage, the interactions involved in bacterial adhesion to materials' surface can be classified as nonspecific or specific (Busscher, 1987). The nonspecific interactions comprehend physicochemical interactions between bacterial cell wall and materials' surface. These interactions involve Van der Waals forces, electrostatic interactions and hydrophobic effects. The specific interactions are those in which the adhesion becomes irreversible. In irreversible adhesion, chemical reactions between the cells that remain immobilized and the surface may occur, determining firmer adhesion of bacteria to the surface by the bridging function of microbial surface polymeric structures (Figure 2.2) (Dunne, 2002).
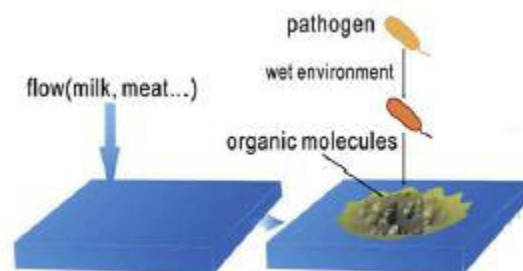
Figure 2.2. Mechanisms of bacterial adhesion. Initially, the surface is conditioned by presence of food waste. Then transport of planktonic cells from the bulk liquid to the surface, where adsorption of cells at the surface is initiate and starting of EPS formation and production of cell-cell signaling molecules. Last step is irreversible adsorption of cells. Adapted from: Shi and Zhu, 2009.

Microbial adhesion can be influenced by several factors such as properties of the bulk media, properties of the surface, and properties of the microorganisms (Schryver *et al.,* 2009). The properties of the bulk media that mostly influence the adhesion process include the presence of conditioning substances or antimicrobial compounds, pH, temperature, flow velocity, exposure time, microorganism's concentration, surface tension and ionic strength. For example, it was showed that maximum adhesion to stainless steel surfaces at 30 ^{0}C occurred at pH 7 for *L. monocytogenes* and pH 8-9 for *Y. enterocolitia* (Herald and Zoottola, 1988a, 1988b). In a laboratory study it was observed that an increase in nutrient concentration correlated with an increase in the number of attached bacterial cells (Cowan *et al.,* 1991). The physicochemical and morphological properties of the surface also contribute to the effectiveness of microbial adhesion as, for example, the van der Waals forces or the repulsive electrostatic forces, which strongly depend on surface charge and hydrophobicity (Kokare *et al.,* 2009). Also, chemical composition, porosity and roughness of the surface determine the higher or lower affinity of microorganisms to the substratum. Physicochemical properties of microorganisms' surface are also responsible for the adhesion process. Other characteristics of microorganisms that are also known for their important role in the adhesion process include their ability to produce extracellular polymeric substances (EPS) and the presence of extracellular appendages (Watnick and Kolter, 2000). The surfaces of several bacterial cells have negative charge, and this varies with growth environments. Due to electrostatic repulsive forces, the negative charge of the cell surface is adverse to bacterial adhesion, which maintains the cells at a short distance from the surface. Hydrophobicity plays an important role in the reduction of the repulsive forces of interaction between two surfaces and, for this reason, the existence of fimbriae, flagella and LPS, with an hydrophobic behavior, leads to an easier attachment of the cells to the surfaces (Shi and Zhu, 2009).

2.3.2. Biofilm formation

Several areas are affected by biofilm formation, and food environment is of special importance since biofilms act as a source of contamination that may lead to food contamination (Maukonen *et al.,* 2003). Biofilm is a well-organized community of microorganisms capable of grow on biotic (living tissue or cells) as well as abiotic surfaces (metal, concrete, biomedical implants, etc.). Genetic studies showed that biofilms are formed through multiple steps. These

steps require intracellular signaling, involving cell–surface and cell–cell interactions which determine structure, function and composition of biofilm. Moreover, a different set of genes is transcribed compared with planktonic cells and, thus, biofilm formation can be considered a developmental process, since it shares some of the features of other bacterial developmental processes (Watnick and Kolter, 2000; Wong and O'Toole, 2011). Biofilm formation occurs step by step, such as: (1) initial attachment, that may be mediated by fimbriae, pili, flagella, and EPS that act to form a bridge between bacteria and the conditioning film; (2) irreversible attachment; (3) early development of biofilm architecture; (4) maturation; and (5) dispersion (Figure 2.3) (Srey *et al.,* 2013).
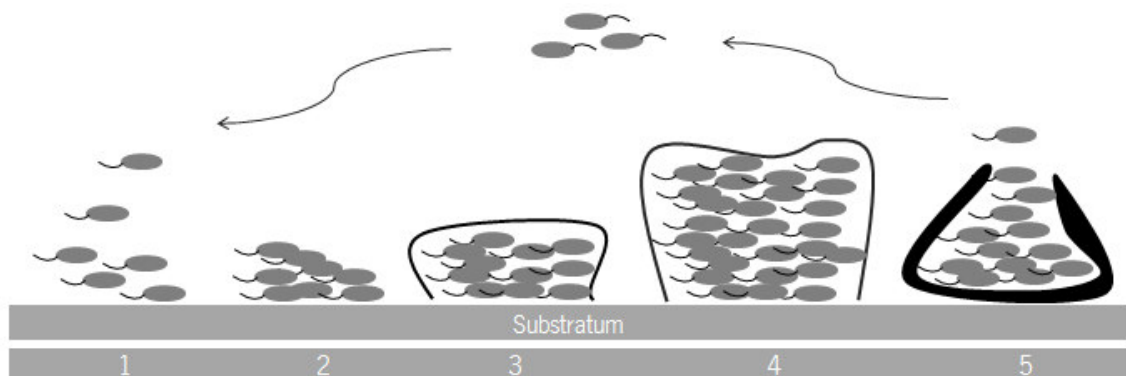
Figure 2.3. Representation of the five main stages of biofilm formation. Stage 1 - Initial and reversible attachment; Stage 2 - Irreversible attachment; Stage 3 - Development of biofilm architecture; Stage 4 - Biofilm maturation; Stage 5 - Dispersion of biofilm cells.

Initial (stage 1) and irreversible attachment (stage 2) occur between cell-surface (explain in section 2.3.1.). Division and growth of microorganisms as well as greater production and excretion of EPS, leads to the development of biofilm architecture (stage 3) (Srey *et al.,* 2013). Then, the development of an organized and defined structure, with quorum sensing playing an important role, contributes to biofilm maturation (stage 4). Quorum sensing is a process by which bacteria sense and respond to their own population density or changes in their environment and is related with cell-to-cell communication, being an important factor in biofilm regulation. In addition, quorum sensing is related with the expression of exopolysaccharide biosynthesis genes (Davey and O'Toole, 2000) and it regulates colonization and virulence (Walters and Sperandio, 2006). Finally (stage 5), release of the cells occurs due to endogenous enzymatic degradation, release of EPS, movement of fluid or mechanical shock (Srey *et al.,* 2013), and thus allows motile cells to colonize others surfaces. Shedding of planktonic cells is part of the biofilm cycle,

being important in the dissemination of the infection in the host or contamination in the food processing plant (Stoodley *et al.,* 2001).

Biofilms in nature may exist in single or multiple species communities. By forming biofilms on surfaces, the microorganisms are protected against dehydration, biocides and other environmental threats, and due to this fact biofilms can be seen as a survival strategy for microorganisms (Costerton *et al.,* 1995). A Biofilm consists of microcolonies of distinct or of the same species of microbial cells, embed themselves in a slimy matrix composed of extracellular polymeric substances (EPS) (Donlan and Costerton, 2002). These substances are considered compounds to determinate the physicochemical properties of biofilms, and consist on polysaccharides, proteins, nucleic acids, and lipids. EPS promote a matrix that allows the cells to stand firm compared with planktonic cells (Kokare *et al.,* 2009). Moreover, the spatial localization of cells within biofilm matrix may be responsible for different behaviors and expression patterns (Bridier *et al.,* 2011), and even a development of a dormant state (Monds and O'Toole, 2009). EPS are responsible for the morphology and internal structure of biofilms, allowing the functional and structural integrity of the biofilm (Flemming *et al.,* 2003).

2.4. Control of foodborne pathogens

The objective of microbial control includes biofilm and adhered cells removal to prevent spoilage of products and to ensure that quality specifications of the products are met. The most important means to maintain an efficient microbial control include: reducing the microbial load from outside sources to the process; efficient control of growth at microbiologically vulnerable area (area with bigger risk of bacterial growth), as for example, cracks in table; and an adequate cleaning and disinfection of the process lines (Wirtanen *et al.,* 2000). There are evidences that the biofilm mode of life leads to increased resistance to antimicrobial products. There are researches that showed that biofilms are more resistant to antimicrobials compared to planktonic cells, and this makes their elimination from food processing facilities a great and very important challenge (Simões *et al.,* 2009).

2.4.1. Cleaning and disinfection

Disinfection means the use of disinfectants to kill microorganisms, aiming to reduce the population of viable cells after cleaning, and prevent an eventual microbial growth and biofilm formation on surfaces before production restart (Bremer *et al.,* 2002). In food industry, disinfection is influenced by biological factors, pH, exposure concentration, surface characteristics, contact time, temperature, and chemical and physical properties of contaminating substances that may be present (Schmidt, 2003). Also, the design and type of surfaces used can lead to the efficacy, or not, of the cleaning and disinfection processes. The effect of these processes is limited by the ability of bacteria to attach to surfaces and, eventually, to form biofilms (Carballo and Araújo, 2012). Cleaning does not allow the total removal of bacteria from the surfaces, leading to possible re-attach and formation of biofilm (Srey *et al.,* 2013). Thus, the application of cleaning and disinfection procedures is essential for maintaining health and safety in food industries. There are three types of cleaning (physical, chemical, and microbiological) essential in food processing plants. Physical cleaning means that there is no visible foreign matter, waste on the equipment surfaces. Chemical cleaning surfaces are those on which there are no undesirable chemical residues, while microbiological cleaning surfaces imply the elimination of spoilage microbes and pathogens (Gould, 1994).

2.4.2. Disinfectants in the industry

Biocides are used to control unwanted organisms that are harmful to human or animal health, or that cause damage to human activities. Biocides include disinfectants which are products used for a thorough combined cleaning and disinfection, being essential in the food industry hygiene to control pathogenic and spoilage microorganisms (Holah, 2000). In this way, the use of disinfectants can also enhance the shelf life of the products, and reduce the risks of foodborne diseases (Wirtanen, 2003). The mode of action and disinfectant efficacy against different microorganisms depends on the chemical nature, thus disinfectants can be classified according to their chemical nature and activity (Morello *et al.,* 1998). In general, the disinfectants initially binds to targets within the cell wall to disrupt the latter's integrity and then penetrates the cell wall and interacts with cytoplasmic constituents (Maillard, 2002). A summary of

disinfectants used in the industry, as well as their targets and mechanism of action, is presented in Table 2.2.

Table 2.2. Mechanisms of antibacterial action of some disinfectants used in food industry. Adapted from: Sheldon, 2005.

Class	Antimicrobial Agent	Antimicrobial target	Mechanism(s) of action
Alcohols	Ethanol Isopropanol	Bacterial membrane	Denaturation of proteins; inhibition of DNA, RNA, protein and peptidoglycan synthesis.
Aldehydes	Glutaraldeyde Formaldehyde	Cell envelope (cell wall, outer membrane) and cross-linking of macromolecules	Cross-linking of proteins, RNA and DNA; inhibition of cellular metabolism and replication.
Bisphenols	Triclosan	Essential enzymes and cell wall	Binding to enoyl-acyl carrier protein reductase, causing inhibition of fatty acid biosynthesis and precipitating cell wall proteins.
Halogen releasing agents	Iodine and chlorine compounds	DNA and amino groups in proteins	Inhibition of DNA synthesis; disrupt; oxidative phosphorylation and membrane-associated activities.
Peroxygens	Hydrogen peroxide Peracetic acid (PAA)	DNA and protein thiol groups	Hydrogen peroxide produces hydroxyl free radicals that function as oxidants, which react with lipids, proteins, and DNA, thus increasing cell permeability; PAA causes disruption of thiol groups in proteins and enzymes.
Phenols	Lysol Staphene Amphyl	Cytoplasmic membrane	Rupture of cell membranes and denaturation of cellular constituent.
Quaternary ammonium compounds	Cetrimide Benzalkonium chloride	Cytoplasmic membrane	Damage of cell wall and cytoplasmic membrane mediated by binding to phospholipids, resulting in loss of structural integrity of the cytoplasmic membrane, leakage of intracellular components and cell lysis.

Chlorine compounds, peroxygen compounds, quaternary ammonium compounds (QACs) and bis-phenols are among the types of disinfectant more commonly used in food processing areas and, as the two first were used in this study, these chemicals agents will be addressed here.

Chlorine Compounds – Sodium Hypochlorite

Chlorine is one of the most commonly used sanitizer in the food industry, on processing and handling applications (Schmidt, 2003). Chlorine compounds are available in various forms that include: liquid chlorine, hypochlorites, inorganic chloramines, and organic chloramines. Chlorine compounds are broad spectrum germicides that have as target the microbial membrane, inhibiting cellular enzymes involved in glucose metabolism; have a lethal effect on DNA, and oxidize cellular protein (Dychdala, 2001). This compound has activity at low temperature, is relatively cheap, and leaves minimal residue or film on surfaces. The activity of chlorine is affected by factors such as pH, temperature, and organic load (Huss, 2003). However, chlorine is less affected by water hardness when compared to other sanitizers (especially the quaternary ammonium compounds). Chlorine is a disinfectant that presents disadvantages as be corrosive to many metal surfaces (especially at higher temperatures) (Schmidt, 2003) and deteriorate fabrics; in high concentrations irritates the mucus membranes, eyes and skin (Fukuzaki, 2006). However, also presents advantages as the efficiency at low concentrations for disinfecting objects, and its low cost, which are advantageous characteristics to be used on a wide scale in food industries. Finally, chlorine is also effective against fungi, bacteria, and algae but is not effective against spores (Kennedy *et al.,* 2000).

Peroxides – Hydrogen Peroxide

Peroxides contain at least one pair of covalently bonded oxygen atoms (-O-O-) and one of the oxygen atoms is loosely bound in the molecule and is readily detached as freely active oxygen. Peroxides can be divided into two groups: the inorganic group (includes hydrogen peroxide), and the organic group (includes peroxyacetic acid). HP has been widely used in the medical field and has become commonly used as a sanitizer in food industry for disinfection, sterilization, and antisepsis (Schmidt, 2003). The HP agent has as primary mode of action that

creates an oxidizing environment and generates a single or superoxide oxygen ($O_2 \bullet$). HP is fairly broad spectrum with a slightly higher activity against gram-negative than gram-positive organisms (Schmidt, 2003). HP, which chemical formula is H_2O_2, acts as an oxidant by producing hydroxyl free radicals ($\bullet$ OH) that attack essential cell components, including lipids, proteins, and DNA (McDonnell and Russell, 1999). This disinfectant is a clear, colorless liquid, and is commercially available in a variety of concentrations, ranging from 3 to 90%. It is also considered to be environmentally friendly, since it can rapidly degrade into the innocuous products water and oxygen (McDonnell and Russell, 1999). The consequences of using high concentrations of HP (5% and above) include eye and skin irritation, thus high concentrations should be handled with care (Schmidt, 2003).

2.4.3. Cellular and biofilm resistance

Resistance is defined as the insusceptibility of a microorganism to a particular treatment under a particular set of conditions (Gilbert and McBain 2003). Resistance is an important term regarding disinfection in food industry. If a microorganism (or species) survives or grows in a higher concentration of disinfectant than another microorganism (or species), it is said to have higher resistance. Within a species, strains that survive to (or are not inhibited by) a concentration of disinfectant that kills (or inhibits) the majority of the strains of that species will be termed resistant. Strains with intermediate resistance will be termed tolerant (Langsrud *et al.,* 2003). Among several studies, industrial disinfectants including quaternary ammonium compounds, sodium hypochlorite, alcohols, chlorinated compounds, and other oxidizing agents such as peracetic acid, ozone and peroxide derivatives have been tested in several microorganisms (Ibusqyuiza *et al.,* 2011; Yan *et al.,* 2013). Typically there are three types of bacterial resistance described: natural or intrinsic resistance, acquired resistance, and resistance by adaptation (McDonnell and Russell, 1999). The intrinsic microbial resistance is a natural property of a microorganism and is frequently associated to cellular impermeability imparted by the outer layers of a bacterial cell that limit the uptake of antimicrobial agents, although active efflux pumps are also an important transposon process. Efflux pumps are common membrane components in all cell types, from prokaryotes to eukaryotes. These components aim at conferring to bacteria a common and basic mechanism of resistance by extruding toxic molecules (Bambeke *et al.,* 2003). Moreover, reduced susceptibility of microorganisms to

antimicrobial agents may be acquired through mutation, or by the acquisition of a plasmid or transposon (Gilbert and McBain, 2003).

It has been demonstrated that the survival and persistence of microorganisms in food matrices and food contact surfaces is problematic, since microorganisms have resisted to disinfectants (Burgess *et al.,* 2014). In particular, biofilms exhibit a pattern of high resistance towards biocidal agents when compared with the planktonic counterparts (Wong *et al.,* 2010). Reports showed that bacteria in biofilms can respond to antibiotic treatment by increasing the synthesis of EPS, which contribute to the matrix of the biofilm (Sailer et al., 2003; Bagge et al., 2004). When biofilms are exposed to disinfectants, reaction-diffusion limited penetration might result in only low levels of the disinfectant reaching the deeper regions of biofilms (Szomolay *et al.,* 2005). Thus, the sheltered cells are then able to enter in an adapted resistant state if the local time scale for adaptation is faster than that of disinfection, and this mechanism is not available to a planktonic population (Szomolay et al., 2005). Other defenses, between the most common in biofilms, are based on a high population density, as well as less defined phenotypic changes. These phenotypic changes can be caused by nutrient gradients and toxins within the biofilm (Champman, 2003). Persister cells also are resistant to disinfectants and antibiotics. These cells are small subpopulations of bacteria that become tolerant to lethal concentrations of disinfectants and antibiotics without any specific resistance mechanisms. Usually, these cells comprise about 1% in the stationary state and in biofilms, due to a state of dormancy (Lewis, 2008). Biofilms that contain them, exhibit tolerance and, thus, cells do not grow in the presence of disinfectants and antibiotics but also do not die.

2.4.4. Cross-resistance to antibiotics

Cross-resistance is another problem related with bacterial resistance. This term means that a microorganism that is resistant to a biocidal agent may also acquire resistance to other antimicrobials. This fact can be due to a pre-exposure or adaptation to a biocidal agent that can affect the bacterial susceptibility to other different desinfectants or to antibiotic, leading to similar resistance responses by bacteria. Cross-resistance may occur if two antibacterial agents (1) use the same pathway to reach the target (e.g. porins), (2) have a similar mechanism of action (e.g. inhibition of protein synthesis), or (3) are affected by the same resistance mechanisms (e.g. reduction in permeability). Alternation between two disinfectants is commonly used to avoid

resistant strains in food production environments (Gilbert and McBain, 2003). Although disinfectants and antibiotics present several distinct aspects, there are also similarities in the mode of action of these two kinds of biocidal compounds. Thus, between the similarities, these may be: the uptake through bacterial envelope by passive diffusion; the effect on the membrane integrity and morphology; and the effect on diverse key steps of bacterial metabolism (SCENIHR, 2009). Concerning antibiotics, the main factors related with the increase of resistance are related with the misuse and overuse of these compounds, misdiagnoses, bacteria lacking the target structure of a given antibiotic, and the widespread use and abuse of poorly controlled antibiotics given to cattle as prophylaxis, growth promoters or treatment, as well as in the meat and aquaculture industries (Adetunji and Isola, 2011). Other hypothesis may be related with a possible sub-lethal exposure to disinfectants that are common in food industry, which can induce an increased tolerance to multiple antibiotics (Condell *et al.,* 2012).

Chapter 3

Considerations of the methodology

This chapter describes the theoretical foundations of the methodology used to perform the work presented and others methods that will be possible of to apply.

3.1. Surfaces sampling methods

Microbiological sampling methods including swabs, agar contact plates, wipes, tapes, hygiene monitors, dust and bulk sampling, as well as microscopy of the surface are employed to assess the cleanliness of surfaces. Traditional swabs can be made from a wooden or plastic shaft with cotton, rayon, Dacron$^{®}$, or alginate fibers, spun to form a bud at one end. In this work was used the swab made from plastic shaft with cotton. The sterile swab is put over the surface to be tested. Bacteria are removed and then transferred directly to a transport media that permits the transfer of swabbed organisms to the laboratory without loss in viability. However with the type of swab used, there is a wide variation in the choice of wetting solution or diluent. Some formulations, for example, aid the recovery of stressed bacteria, whilst others include agents capable of neutralizing the effects of any residual detergents and/or disinfectants that may be picked up by the swab during sampling. It is imperative that the characteristics of the wetting solution do not alter, either qualitatively or quantitatively, the microbial population between sampling and enumeration (Moore and Griffith, 2007).

3.2. Isolation Methods

Enrichment

The enrichement step is applied when pathogenic microorganisms are present in small numbers. Enriched media is a media with specific and known qualities that favors the growth of a particular microorganism while inhibiting the growth of others (Varman and Evans, 1996). The selective agents frequently contained in enrichment broths are acriflavine, nalidixic acid, potassium tellurite and antibiotics, as novobiocin (Gasanov *et al.,* 2005), depending on each media for each microorganism. There are some variety in terms of enrichment media for *Listeria* spp. such as Listeria Enrichment Broth Base, Listeria Fraser Broth, and Listeria Enrichment Broth, Modified and for *E. coli* as E.E. Broth (Enterobacteriaceae Enrichment Broth), and TSB Broth with Novobiocincin (Modified Tryptic Soy Broth with Novobiocin). For identification of *L. monocytogenes* was used the Listeria Enrichment Broth Base media and for *E. coli* was utilized the E. E. Broth media. The enrichment procedure includes a period of incubation in a selective

media to suppress the competitive flora and enable the multiplication of the target organism for subsequent isolation or detection by a variety of techniques (Jasson *et al.,* 2010).

Selective plating

Enrichment methods are followed by the isolation of the target microorganism on specific plate media (Figure 3.1). Selective media allows the growth of certain type of organisms, while inhibiting the growth of other organisms (Gasanov *et al.,* 2005). Examples of these type of media are Listeria PALCAM Agar and Listeria Oxford Agar Base for *Listeria* spp. For *E. coli* there is E. coli Direct Agar. For isolation of *L. monocytogenes* was used Listeria Oxford Agar Base and for *E. coli* was CHROMagar™ E.coli. Nowadays, this task has been facilitated by the introduction of chromogenic and fluorogenic media for the detection. A wide range of chromogenic (colour reaction) and fluorogenic (fluorescent reaction) substrates are available, and these compounds are applied in several commercial systems and media. The target population is characterized by enzyme systems that metabolize the substrate (sugar or amino acid) that leads to the release of chromogen/fluorogen, resulting in a colour change in the media and/or fluorescence under long wave UV light (Perry and Freydiere, 2007). As example, it is known that *L. monocytogenes* and *L. ivanovii* produce the enzyme phosphatidylinositol-specific phospholipase C (PIPL-C) (Coffey *et al.,* 1996), which activity is measured on chromogenic media. There are however chromogenic agars used in others bacteria, as is the case of some STEC (Shiga toxin-producing *Escherichia coli*) strains that are sensitive for sorbitol-fermenting (Boer, 2000).

Enrichment in selective media
Listeria Enrichment Broth Base (30 °C, 48 h)
E.E. Broth (37 °C, 24 h)

Plating in selective media
Oxford Listeria Agar Base (37 °C, 96 h)
CHROMagar™ E.coli (37 °C, 24 h)
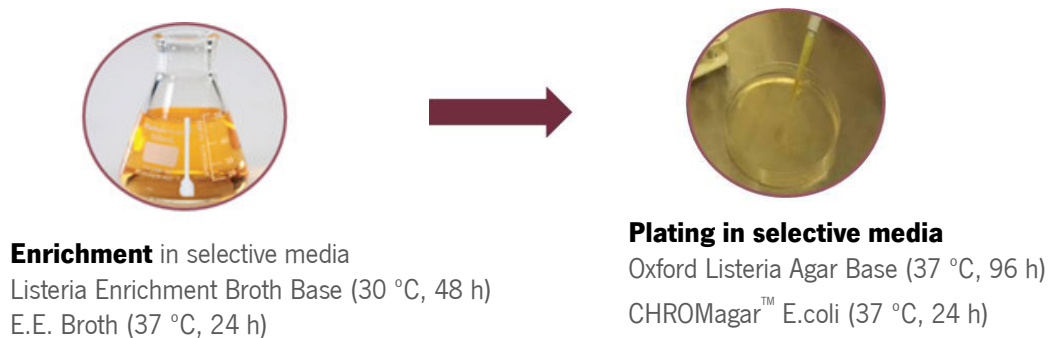
Figure 3.1. Isolation procedure: enrichment and plating in selective media.

3.3. Rapid methods for identification of isolates

Rapid methods are used for the detection and identification of microbial pathogenic agents, and include nucleic acid-based methods, immunological-based methods, oligonucleotide DNA microarray, and biossensors-based methods (Law *et al.,* 2015).

Polymerase chain reaction (PCR)

PCR is a method which operates by amplifying a specific target DNA sequence, and involves a cyclic three steps process. The choice of specific primers is made in order to amply the interest region. Primers are short oligonucleotides, usually 20-30 nucleotides in length, whose sequence matches the end of the region of interest. For this work were chosen universal primers (27F and 338R). Universal primers are complementary to nucleotide sequences that are very common in a particular set of DNA molecules and cloning vectors. Thus, they are able to bind to a wide variety of DNA templates (Jill and Claridge, 2004). During each cycle, the double-stranded DNA is denatured into single-stranded DNA at high temperature (step 1). Two single-stranded synthetic oligonucleotides or specific primers (forward and reverse primer) will anneal to the DNA strands (step 2). Then, the primers complementary to the single-stranded DNA are extended due to the presence of deoxyribonucleotides and a thermostable DNA polymerase, whose process is polymerization (step 3). In subsequent cycles, primers will bind to both the original DNA and the newly synthesize DNA resulting in an exponential increase in the number of copies. The size of the PCR product is usually detected by electrophoresis gel (Ikeda *et al.,* 2007).

This method has advantages over culture and other standard methods including specificity, sensitivity, rapidity, accuracy and capacity to detect small amounts of target nucleic acid in a sample (Ikeda *et al.,* 2007). It has been used in the detection of distinct foodborne pathogens like *L. monocytogenes, E. coli* O157:H7, *S. aureus, C. jejuni, Salmonella* spp. and *Shigella* spp. (Lee *et al.,* 2008; Alves *et al.,* 2012; Chiang *et al.,* 2012; Zhou *et al.,* 2013). PCR is also used for toxins detection by amplification of specific genes that encode bacterial toxins. This has been performed with several bacterial species, as *Vibrio cholera, B. cereus, E. coli,* and *S. aureus* (Planche *et al.,* 2008).

16S rRNA Gene Sequencing

16S rRNA gene sequencing involves the amplification of a phylogenetically informative target as the 16S rRNA gene (Bosshard *et al.,* 2003). Several primers that recognize 16S ribosomal (rDNA) sequences (conserved in a wide variety of bacteria) are used to amplify regions of interest (Kolbert and Persing, 1999). From the sequence determination and from the comparative database searches, it can be assigned a group of bacteria to the unknown isolate (Bosshard *et al.,* 2003). This technique traces phylogenetic relationships between bacteria and identifies bacteria from various sources, such as environmental or clinical specimens. Identification based on the 16S rRNA sequence is of interest because ribosomal SSU (small subunit) exists universally among bacteria and includes regions with species specific variability (Mignard and Flandrois, 2006). This technology is used today in clinical laboratories to provide genus and species identification for isolates that do not fit any recognized biochemical profiles, for strains generating only a "low likelihood" or "acceptable" identification according to commercial systems, or for taxa that are rarely associated with human infectious diseases (Janda and Abbott, 2007).

3.4. Biofilm formation

Currently there are a variety of systems for examining biofilm formation and several devices available for the formation of bacterial biofilms. These in vitro systems are usually divided into flow (Modified Robbins Device, and flow cell biofilm system) and static models (microtiter plate, Calgary Biofilm Device, perfused biofilm fermentor, and Constant Depth Film Fermentors). Static models are preferable due to the facility to handle and versatility, allowing to study the effect of different conditions of biofilm formation as well as different behaviors of these bacterial communities (Abdallah *et al.,* 2014).

The microtiter plate assay was developed by Christensen, and is the most widely used method to assess the early stages of biofilm formation in abiotic surfaces (Christensen *et al.,* 1985), providing a large number of parallel and miniaturized reactors in small scale with the same conditions of space and fluid dynamics (Kumar *et al.,* 2004). A microtiter plate may have 6, 24, 96, 384 or even 1536 sample wells arranged in a rectangular matrix, but in laboratory the most used is the 96-well format (Coenye and Nelis, 2010; Kumar *et al.,* 2004). The use of this

method has the advantage of being easily combine with compatible side equipment, such as micropipettes, pipetting robots and microplate's readers (Duetz, 2007). Also, the advantage of a regular renewal of liquid phase allows avoiding the limitation of nutrients and accumulation of potentially toxic metabolites, and due to its low cost, flexibility and speed, this technique allows the processing of multiple samples simultaneously with simplicity. Furthermore, it is in agreement with the requirement of common laboratory equipment to use smaller quantities of reagents and culture media (Machado *et al.,* 2012). Microtiter plates can be used for a wide variety of biofilm analysis, including assays for biomass quantification (Lopes *et al.,* 2010), assessment of biofilm metabolic activity (Lopes *et al.,* 2010), enumeration of biofilm cells (Lopes *et al.,* 2010), quantification of biofilm matrix (Peeters *et al.,* 2008), and susceptibility testing (Sandberg *et al.,* 2008). However, this system does have some drawbacks such as: its static nature, that results in possible nutrient limitation and the inability to easily generate mature biofilms (Merritt *et al.,* 2005); the operator-dependent nature of its procedures, for example washing steps, that can cause a poor reproducibility even in intra-laboratory experiments; and its configuration that hinders the observation of the biofilm structure by direct microscopy (Azevedo *et al.,* 2009).

3.5. Quantification of biofilm biomass

Biofilm biomass can be measured by distinct methods, such as microscopy, molecular probes, biochemical analysis of biomass components, and, the most usual, staining of biofilms with specific compounds and subsequent determination of optical density (Stepanovic *et al.,* 2000; Li *et al.,* 2003; Peeters *et al.,* 2008; Azevedo *et al.,* 2009).

Crystal violet (CV) staining was first described by Christensen and has been one of the most expedites methods to determinate biofilm biomass (Christensen *et al.,* 1985). In this staining assay, the dye binds to negatively charged surface molecules and polysaccharides located in the extracellular matrix staining also both living and dead cells (Li *et al.,* 2003), allowing the determination of biofilm biomass without disrupting the biofilm. Once total biomass (cells and matrix) is stained in purple, the dye can be easily dissolved in acetic acid (Stepanovic *et al.,* 2000) and, finally, the absorbance is read at 570 nm. However, problems are frequent when using this method, due to the existence of clear fails in the reproducibility of results, and the requirement of successive washing steps, which may lead to loss of part of the biomass

present (Peeters *et al.,* 2008). Although of the disadvantages of this technique, the CV method is a straightforward, quick, and low cost technique to indirect quantification of microbial adhesion and amount of biofilm formed on inert surfaces by a broad range of microorganisms (Stepanovic *et al.,* 2000; O'Toole, 2011),

3.6. Microbial susceptibility testing

For the assessment of microbial susceptibility to disinfectants and antibiotics, different methods may be used, as disk diffusion and broth dilution. These assays are standard procedures to quantify planktonic cells' susceptibility to biocidal or antimicrobial agents, but they are not directly applicable to biofilms. With the broth dilution method it is evaluated the ability of bacteria to grow in a range of concentrations of a given biocidal/antimicrobial agent. Briefly, dilutions of biocidal/antimicrobial compounds solutions are prepared in a liquid bacterial growth media, which is then inoculated with the standardized cell suspension. Microtiter plates are then incubated overnight, at the incubation conditions recommended for the bacteria under study, and then the plates are examined for visible evidence of bacterial growth in the form of turbidity. The biocidal activity of a compound can be quantified by determining the minimum concentration of the compound capable of inhibiting the visible growth of a microorganism, a value called Minimum Inhibitory Concentration (MIC) (mg/l or μg/ml) (Andrews, 2001). Posteriorly, MICs values may be translated into clinical categories, namely sensitive (S), intermediary (I) or resistant (R). Concerning antibiotics, these clinical categories and correlated MICs are provided by several committees, including the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (EUCAST, 2014).

Several techniques are also available to evaluate susceptibility of biofilms, such as Biofilm Eradication Surface Test (BEST) Assay™ (Harding *et al.,* 2011) and BioTimer Assay (De Giusti *et al.,* 2011). Calgary Biofilm Device is usually used to determinate Minimum Biofilm Eradication Concentration (MBEC) which is the lowest biocidal concentration that eradicates the biofilm (Ceri *et al.,* 1999). However, susceptibility assay of biofilms on microtiter plates is also a method used. This technique consists in, after biocidal exposure, the biocidal compound is removed from the wells and biofilms are then scraped thoroughly. Posteriorly, samples correspondent to each concentration of biocidal agent are plated on solid media and incubated from a specific period of time. In order to assess MBEC, the presence of colonies is evaluated

and MBEC value is determined, as the lowest concentration of antibiotic that prevented bacterial growth (Mataraci and Dosler, 2012).

Chapter 4

Materials and methods

This chapter describes the general methodology used to perform the work presented.

4.1. Sampling

Samples were collected from one meat retail facility in center of Braga, Portugal. The sampling procedure followed the standard ISO method 18593:2004. Contact surfaces swabbed were cutting boards and the mincer (Figure 4.1) due to a higher probability of contamination. For each one of these surfaces, triplicate samples were collected before and after cleaning and disinfection procedure. After collecting samples, these were transported in a refrigerated box to the laboratory, where they were processed.

Enrichment in selective media

Platting in selective media

Direct platting in selective media

Collection of samples from meat retail facilities
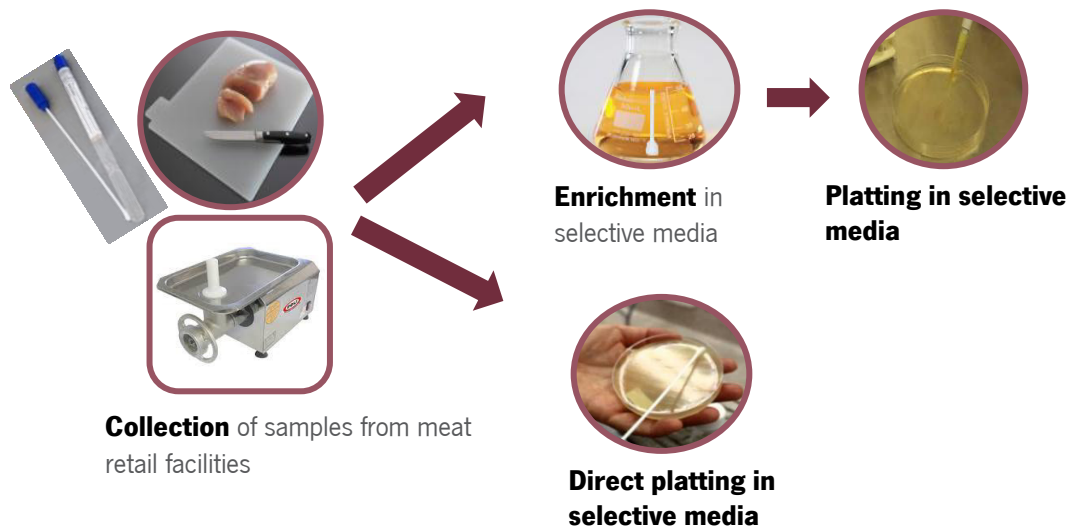
Figure 4.1. Sampling scheme used in the present work (cutting boards and mincer).

4.2. Isolation of microorganisms

Listeria monocytogenes: Some swabs were directly placed into plates containing solid selective media (Oxford Listeria Agar Base, Liofilchem) with supplement (Listeria Oxford Supplement, Liofilchem) and incubated at 37 ^{0}C for 96 h. The other swabs were placed into 40 ml containing selective enrichment broth media (Listeria Enrichment Broth Base, Liofilchem) with supplement (Listeria for Enrichment Supplement, Liofilchem) and incubated at 30 ^{0}C for 48 h. After growth in selective enrichment broth media, 100 µL of broth was inoculated into plates containing selective solid media and incubated at 37 ^{0}C for 96 h. These steps were performed for samples collected before and after disinfection in both surfaces analyzed. For the selective media it would be expected to have brown-gray colonies with black center and black halo correspondents to *L. monocytogenes*.

Escherichia coli: Some swabs were directly placed into plates containing solid selective media (CHROMagar™ E.coli, Liofilchem) and incubated at 37 °C for 48 h. The others swabs were placed into 40 ml containing selective enrichment broth media (E.E Broth, Liofilchem) and incubated at 30 °C for 24 h. After growth in selective enrichment broth media, 100 µL of broth was inoculated into plates containing selective solid media and incubated at 37 °C for 48 h. These steps were performed for samples collected before and after disinfection in both surfaces analyzed. For the selective media it would be expected to have blues colonies correspondent to *E. coli* and colorless correspondent to other Gram-negative bacteria.

4.3. Identification by 16S sequencing

The colonies grown in selective media (Oxford Listeria Agar Base, and CHROMagar™ E.coli) were picked to microcentrifuge tubes containing 10 µL of sterile water. PCR reactions were performed using the Kapa HiFi DNA Polymerase (Kapa Biosystems), with the master mix prepared according to manufacturer's instructions, using the universal 16S primers 27F (AGA GTT TGA TCM TGG CTC AG) and 338R (TGC TGC CTC CCG TAG GAG T). PCR was performed by an initial denaturation for 10 s at 95 °C, and 15-30 cycles of: denaturation for 20 s at 98 °C; annealing for 15 s at 50-75 °C; extension for 40-60 s/kb at 72°C; and final extension for 5 min/kb at 72 °C. PCR results were run on a 1% agarose gel stained with SYBR Safe (Fisher Scientific). Samples were stained with loading buffer (6x NZYDNA loading die, NZYTech) and the gel was run on 1xTAE (1mM EDTA, pH 8, e 0.5M Tris-acetate) at 90V for 35 min. The 50 bp DNA ladder (New England Biolabs) was used. Then, the DNA fragments were visualized using the ChemiDoc XRS transilluminator (Bio RAD). The bands observed were cut from the gel and the DNA was extracted using the Nucleospin Gel and PCR Clean-up (Macherey-Nagel) following the manufacturer's instructions. After clean-up, the concentration and quality of the DNA was determined using Nanodrop 1000. Finally, samples were prepared for sequencing by StabVida following the company's recommendations (10 µL of DNA at ≥ 20 ng/ µL with 3 µL of primer at 10 µM). Sequencing results were analyzed using the SnapGene software.

4.4. Microorganisms and growth conditions

The isolated strains were stored at - 80 ^{0}C in tryptic soy broth (TSB, Liofilchem) containing 20% glycerol. In order to prepare the bacterial suspension, TSB and tryptic soy agar (TSA, Liofilchem) were prepared according to the manufacturer's instructions. All bacterial cells were inoculated into 40 ml of TSB from TSA plates and grown for 18 ±2 h at 37 ^{0}C, in a horizontal shaker under agitation at 120 rpm (Shaker & Incubator, NB-205Q, N-Biotek). After incubation, cells were harvested by centrifugation at 7830 rpm for 5 min at 4 °C (5430 R Centrifuge, Eppendorf) and washed twice with 0.9% sodium chloride (NaCl) sterile solution (Panreac Química, Espanha). Cells were resuspended in 0.9% NaCl, and the suspension was adjusted to a concentration of 1x10^{9} CFU/mL before being used in the subsequent assays, which corresponds to an optical density at 640 nm of ≈ 0.3, as confirmed by colony forming units (CFU's) count after plating serial dilutions on TSA.

4.5. Evaluation of planktonic cells susceptibility to chemical disinfectants and antibiotics

The chosen disinfectants represent different chemical agents that are commonly used in the food industry, which also allows studying the effect of disinfectants with different mechanisms of interaction with the bacterial cells. Thus, in this study, two different disinfectants were tested: SH 1.5% with available chlorine and HP 50% wt/v solution in water (both from Sigma-Aldrich).

Concerning the antibiotics, the choice fell on those of wide spectrum such as rifampicin (AppliChem) and ampicillin (Sigma-Aldrich), which are effective against a variety of microorganisms.

4.5.1. Chemical disinfectants and antibiotics preparation

For disinfectants, working solutions were prepared fresh in TSB, immediately before application, and maximum concentrations tested were 15000 μg/mL for HP, and 1600 μg/mL for SH. The stock solutions of rifampicin and ampicillin were prepared at a 5120 μg/mL concentration. According to EUCAST recommendations, methanol and PBS (0.1M Phosphate-Buffered-Saline, pH 8) were used as solvent for rifampicin and ampicillin, respectively. Both

antimicrobial agents were diluted in TSB for a maximum concentration of 32 μg/mL for rifampicin, and 128 μg/mL for ampicillin, also according to EUCAST recommendations.

4.5.2. Determination of Minimum Inhibitory Concentration (MIC)

The Minimum Inhibitory Concentration of the selected antibiotics and disinfectants against the strains under study was determined by microbroth dilution, according to the Clinical and Laboratory Standards Institute (CLSI). MIC determination was performed by serial two-fold dilution method for both disinfectants and antibiotics, using 96-well tissue culture plates. The disinfectants and antibiotics were first diluted in TSB to the highest concentration, and then serial two-fold dilutions were made along the plate. At the same time, it was always performed the correspondent controls with no addition of antibiotic or disinfectant solution, in order to support bacterial growth (positive control). The final cell concentration used was approximately 5×10^5 cells/ml. After addition of the cell suspension (100 μL) to the wells already containing the antibiotic and disinfectant solutions (100 μL), the culture plates were incubated at 37 °C, in an orbital shaker at 120 rpm, for 16-20 h. MIC was then determined visually as the lowest concentration required to inhibit bacterial growth, and confirmed by optical density reading at OD_{640}nm. All these experiments were performed in triplicate in at least three independent assays.

4.6. Biofilm formation

In order to obtain a cell concentration of 1×10^7 CFU/mL on the well (concentration to initiate biofilm formation), the initial cell suspensions (prepared as described above) were diluted in 0.9% NaCl. Biofilm formation was performed on regular microtiter, and each assay was performed in triplicate. Briefly, in each well of 96-wells flat-bottom polystyrene microtiter plates (Orange Scientific) the bacterial inoculum (10 μL) was added to fresh TSB media (240 μL), so that a concentration of 1×10^7 CFU/mL in a final volume of 250 μL was obtained. Culture plates were incubated at 37 °C in an orbital shaker at 120 rpm during three days. During this period, at each 24 ± 2 h of incubation, 200 μL of liquid phase were removed and an equal volume of fresh TSB media were added to the wells.

4.7. Evaluation of biofilm formation ability by Cristal Violet assay

Quantification of biofilm production in 96-wells flat-bottom polystyrene microtiter plates was based on the previously described method (Stepanovic *et al.,* 2000) with some modifications. In each 24 h of biofilm formation, the media was removed from the wells and biofilms were washed once with 0.9% NaCl (250 μL) in order to remove free cells. In order to fix the biofilms, 250 μl of 100% methanol were added to each well and left there for 15 min. After removing the methanol, culture plates were allowed to dry at room temperature until they were completely dehydrated. The fixed biofilm on each well was stained with 250 μl of 1% (V/V) CV solution, for 5 min, and then washed once with 0.9% NaCl (250 μL). Once again, culture plates were allowed to dry to ensure that there are no traces of liquid in the wells. At last, in order to solubilize the CV bound to the biofilms, 250 μL of 33% (V/V) acetic acid were added to each well, and the optical density was measured at 570 nm in a microtiter plate reader. All these experiments were performed in triplicate, in at least three independent assays.

4.8. Evaluation of biofilm susceptibility to chemical disinfectants

4.8.1. Disinfectants and Neutralizer Preparation

To evaluate the biofilm susceptibility to chemical disinfectants, SH and HP were used, both with a final concentration corresponding at 10xMIC. Working solutions were always fresh, prepared immediately before application. To inactivate disinfectants after biofilms challenge, a universal neutralizer was used, composed by 0.5 g/L of L-histidine (Sigma Aldrich), 0.5 g/L of L-cysteine (Sigma Aldrich) and 1 g/L of reduced glutathione (Sigma Aldrich) dissolved in double distilled water. Aliquots of 1 mL each were prepared and preserved at -20 °C. For each disinfection challenge, a fresh solution of media TSB + neutralizer was prepared by adding 1 volume of universal neutralizer per 40 volumes of recovery media.

4.8.2. Minimum Biofilm Eradication Concentration assay

In order to evaluate the susceptibility to disinfectants, Minimum Biofilm Eradication Concentration (MBEC) (Ceri *et al.,* 1999) was determined by using biofilms formed as described above but with an incubation period of only 24 hours. After incubation, biofilms were washed once with 0.9% NaCl in order to remove free cells. Thereafter, 11 different concentrations of disinfectant solution prepared in TSB were added to the wells and left there for a total of 5 min. contact with the biofilm. After this period, culture media was discarded, wells were washed once with 250 µL of 0.9% NaCl, and 250 µL of TSB + neutralizer solution were added. Biofilms were then scraped from the wells with the aid of a sterile micropipette tip, and the resultant bacterial suspensions were serially diluted in 0.9% NaCl for subsequent platting on TSA. After 24 h incubation at 37 °C, TSA plates were observed for evaluation of MBEC - the minimum concentration at which no cell growth was observed.

4.9. Statistical Analysis

Statistical analysis was performed using the statistical program SPSS (Statistical Package for the Social Sciences). Results were compared using the non-parametric Mann Whitney U-test for not normal distributions, and one-way analysis of variance (ANOVA) for normal distributions by applying Tukey's test at a 95% confidence level.

Chapter 5

Results

This chapter comprises the evaluation of biofilms susceptibility to two disinfectants (HP and SH) as well as planktonic cells susceptibility to some disinfectants and two antibiotics (AMP and RIF). Biofilm formation ability is also presented.

5.1. Isolates collected before cleaning and disinfection

5.1.1. Isolation of bacteria by liquid enrichment and solid selective media

In this study, sampling was the first step. The samples were collected by swabbing, and then isolation and identification methods were applied. The incubation time for the media Listeria Enrichment Broth Base, E. E Broth, Oxford Listeria Agar Base and CHROMagarTM™ E.Coli were 48 h, 24 h, 96 h and 24 h respectively. The abbreviations used to label the swab were according to the number of the swab, microorganism to be isolated, and media. The results concerning microbial growth in the different media used are presented in Table 5.1.

Table 5.1. Microbial growth of samples collected before disinfection on liquid enrichment media (Listeria Enrichment Broth Base, or E.E Broth), and on solid selective media (Oxford Listeria Agar Base, or CHROMagarTM™ E.coli).

	Enrichment media	Solid selective media, after enrichment		Solid selective media, without enrichment
C1listL	G	N.G	C1listS	N.G
C2listL	G	G	C2listS	N.G
C3listL	G	G	C3listS	N.G
M1listL	N.G	N.G	M1listS	N.G
M2listL	N.G	N.G	M2listS	N.G
M3listL	G	N.G	M3listS	N.G
C1e.coliL	N.G	N.G	C1e.coliS	G
C2e.coliL	G	G	C2e.coliS	G
C3e.coliL	N.G	N.G	C3e.coliS	G
M1e.coliL	N.G	N.G	M1e.coliS	N.G
M2e.coliL	N.G	N.G	M2e.coliS	G
M3e.coliL	G	G	M3e.coliS	N.G

G – Growth; N.G – No growth; C - Cutting boards; M – Mincer; e.coli – *Escherichia coli*; list – *Listeria*

For the samples collected on the cutting boards (C) and directly inoculated in liquid enrichment media (Listeria Enrichment Broth Base), it was observed growth in all the samples within the time recommended by the manufactures of the culture media (48 h). For the samples collected on the mincer (M) and directly inoculated in liquid enrichment media (Listeria

Enrichment Broth Base, or E.E Broth), it was observed growth just in one sample. After enrichment and platting on solid selective media relative to *Listeria*, it was observed growth of some samples, but they required a longer period of time (96 h) than the recommended one (48 h). When platting directly in selective media, the majority of the samples grown on CHROMagar™ E. coli and these grown was relative to isolates collected from cutting boards, no growth was observed in Oxford Listeria Agar Base. The samples that grown in selective media Oxford Listeria Agar Base presented gray colonies with black center and black halo, while the colonies that grown in CHROMagar™ E.coli media were colorless (Figure 5.1).
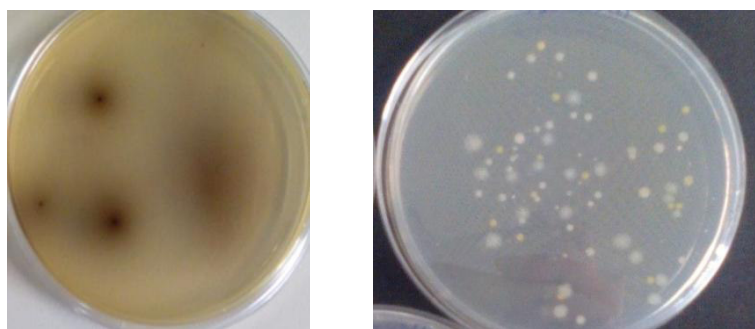
Figure 5.1. Aspect of the colonies grown (samples collected before cleaning and disinfection) using Oxford Listeria Agar Base and CHROMagar™ E.coli media.

5.1.2. Identification of isolates by 16S sequencing

Some of the isolates (C2listL, C3listL, C2e.coliS and C3e.coliS) collected before disinfection were identified by 16S sequencing. The choice these isolates was performed since they correspond to equipment where there was the majority of the growth. The results showed the presence of *Listeria innocua* (C2listL), *Serratia* spp. (C3listL), *Pseudomonas* spp. (C2e.coliS) and *Hafnia alvei* (C3e.coliS). *Pseudomonas* spp. was not included in this work due to time limitations.

5.1.3. Evaluation of susceptibility to disinfectants and antibiotics

Planktonic cells of the isolates *Listeria innocua*, *Serratia* spp., and *Hafnia alvei* were exposed to disinfectants to determine their susceptibility to HP and SH, and also to antibiotics AMP and RIF. Determination of the MIC was performed through the microdilution method, as

described above. MIC results obtained for the different disinfectants and antibiotics are presented in Table 5.2 and Table 5.3, respectively.

Table 5.2. MIC values obtained with the isolates collected before disinfection, and recommended concentrations of each disinfectant (µg/mL).

Microorganism	HP	SH
Listeria innocua	58.59 – 117.18	400 – 800
Serratia spp.	58.59 – 117.18	400 – 800
Hafnia alvei	58.59 – 117.18	400 – 800
Recommended concentration in food contact surfaces	200 – 300	200

Based on the MIC values obtained, it was not observed variability between the identified isolates. For all these tested isolates, the MIC values of disinfectants SH and HP were equal between them. However, it was observed a higher susceptibility to HP comparatively to SH, when comparing the absolute values of MIC. The MIC values of HP were within the recommended concentration for food surfaces disinfection procedures, while the MIC values for SH were above of the recommended concentration.

Table 5.3. MIC values of each antibiotic obtained with the isolates collected before disinfection (µg/mL).

Microorganism	AMP	RIF
Listeria innocua	4 – 8	8 – 16
Serratia spp.	4 – 8	8 – 16
Hafnia alvei	4 – 8	16 – 32

After exposure to AMP, it was observed the same value of MIC for all isolates tested and below of the maximum concentration used (128 µg/ml). This did not happen regarding RIF, since different MIC values of this antibiotic were observed between the different microorganisms. All bacteria tested revealed a higher susceptibility to AMP comparing with RIF. For the isolate *Hafnia alvei* the MIC value was equal to maximum concentration used.

5.1.4. Evaluation of biofilm formation ability

The colorimetric assay involving crystal violet staining of biofilms was used for quantification of biofilm formation by the bacteria isolated before disinfection. The results obtained are presented in Figure 5.2.
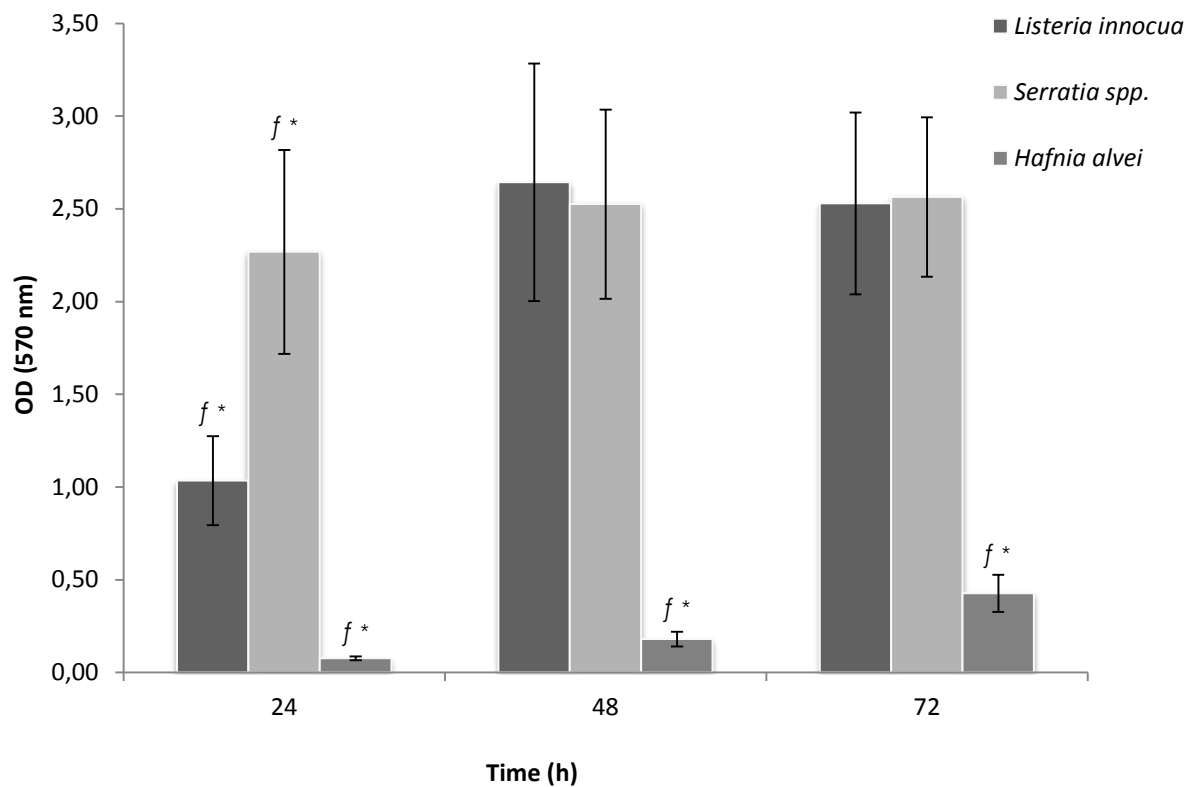
Figure 5.2. Evaluation of biofilm formation ability of the isolates (collected before cleaning and disinfection) by violet crystal staining. Each bar represents average CV–OD$_{570}$ values and standard errors. Symbols indicate statistically different values (p<0.05) between different growth periods for the same bacteria (*), and between different bacteria considering the same growth period (f).

In some of the presented cases in the previous figure (*Serratia* spp. and *Listeria innocua*), the amount of total biomass was similar for some conditions tested (48 h and 72 h), both within the same strain and between strains. All strains presented a lower amount of biofilm biomass after 24 h incubation, comparing with biofilms formed after 48 h and 72 h. Also concerning the 24 h incubation period, it was observed significant differences between all the microorganisms tested (p $\leq$ 0.05). Finally, between the three days of incubation period it was observed statistical differences of biomass of *Hafnia alvei* biofilms.

5.1.5. Minimum Biofilm Eradication Concentration

In this study, biofilms were also exposed to the same disinfectants tested on planktonic cells (HP and SH) to determine their susceptibility, as describe above (section 4.8.2). The MEBC value of each disinfectant tested is presented in Table 5.4.

Table 5.4 MBEC value obtained with the isolates collected before disinfection, and recommended concentration of each disinfectant (µg/mL).

Microorganism	HP	SH
Listeria innocua	>1171.8	>8000
Serratia spp.	>1171.8	>8000
Hafnia alvei	>1171.8	>8000
Recommended concentration in food contact surfaces	200 – 300	200

After analysis of the table above, it was observed that all isolates presented MBEC values higher than the maximum concentration used in this study, this is, 10xMIC (1171.8 µg/ml for HP, and 8000 µg/ml for SH), and also much higher than the recommended concentrations for disinfection of food contact surfaces.

5.2. Isolates collected after cleaning and disinfection

The daily cleaning and disinfection of meat retail facility under study is performed with manual washing, using some cleaning products, including leach.

5.2.1. Isolation of bacteria by liquid enrichment and solid selective media

The samples collected by swabbing after disinfection of the meat retail facility were isolated and identified by the methods described above. The incubation time for the media Listeria Enrichment Broth Base, E. E Broth, Oxford Listeria Agar Base and CHROMagar™ E.Coli were 48 h, 24 h, 96 h and 24 h respectively. The abbreviations used to label the swab were

according to the number of the swab, microorganism to be isolated, and media. The results concerning the microbial growth on the different media used are presented in Table 5.5.

Table 5.5. Microbial growth of samples collected after disinfection on liquid enrichment media (Listeria Enrichment Broth Base, or E.E Broth), and on solid selective media (Oxford Listeria Agar Base, or CHROMagar™ E.coli).

	Enrichment media	Solid selective media, after enrichment		Solid selective media, without enrichment
C1listL	G	G	C1listS	G
C2listL	G	G	C2listS	G
C3listL	G	G	C3listS	G
M1listL	G	G	M1listS	N.G
M2listL	G	G	M2listS	N.G
M3listL	G	N.G	M3listS	N.G
C1e.coliL	G	G	C1e.coliS	G
C2e.coliL	G	G	C2e.coliS	G
C3e.coliL	G	G	C3e.coliS	G
M1e.coliL	G	G	M1e.coliS	G
M2e.coliL	G	G	M2e.coliS	G
M3e.coliL	G	G	M3e.coliS	G

G – Growth; N.G – No growth; C - Cutting boards; M – Mincer; e.coli – *Escherichia coli*; list – *Listeria*; S - Solid; L – Liquid

All samples correspondent to cutting board (C) and mincer, when directly inoculated in the liquid enrichment media (Listeria Enrichment Broth Base or E.E Broth), have grown within the period of time recommended by the manufacture of the culture media (48 h and 24 h, respectively). After enrichment, all samples grown on the solid selective media correspondent to *Listeria* (except one), but after an incubation period superior (96 h) to the recommended one (48 h). The samples correspondent to *E. coli* grown within the period of time recommended by the manufacture of the culture media (24 h). When samples were platted directly on CHROMagar™ E.coli media, all of them have grown, and different colonies were observed: some were colorless and others were blue; while on Oxford Listeria Agar Base media only half of the samples have grown and presented gray colonies with black center and black halo (Figure 5.3).
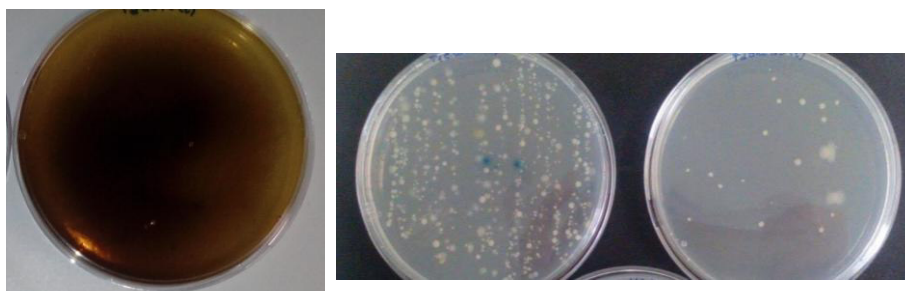
Figure 5.3. Aspect of the colonies grown (samples collected after cleaning and disinfection) using Oxford Listeria Agar Base and CHROMagar™ E.coli media.

5.2.2. Identification of isolates by 16S sequencing

The sample (M2e.coliL, M3e.coliL, C1e.coliS and C3listS) were chosen since they had the same aspect indicated by manufactures of the culture media and due to presence of much quantity of bacteria in the plate. Bacteria isolated after disinfection were also identified by 16S sequencing, and revealed the presence of the following microorganisms: *Cellulosimicrobium* spp. (C3listS), *Enterobacter* spp. (C1e.coliS) and *Serratia* spp. (M2e.coliL). It was not possible to identify the sample M3e.coliL, probably due to a great quantity of template DNA that may have inhibited the PCR reaction. Another possibility is the production of a protease by the cells which can have inactivate enzyme involved in the PCR reaction. Also, although universal primers can be used for all bacteria this can be more difficult for some bacteria than for others. For example, certain bacteria have different nucleotides in the area where the primer binds, making it more difficult to achieve PCR amplification. Thus, it is necessary to optimize PCR conditions for the bacteria.

5.2.3. Evaluation of susceptibility to disinfectants and antibiotics

Planktonic cells of *Cellulosimicrobium* spp., *Enterobacter* spp., and *Serratia* spp. were exposed to disinfectants (HP and SH) and to antibiotics (AMP and RIF) in order to determine the MIC values of such biocidal compounds, through the methodology described anteriorly. The results obtained after exposure to disinfectants and to antibiotics are presented in Table 5.6 and Table 5.7, respectively.

Table 5.6. MIC values obtained with the isolates collected after disinfection, and recommended concentrations of each disinfectant (µg/mL).

Microorganism	HP	SH
Enterobacter spp.	117.18 – 234.4	800 – 1600
Cellulosimicrobium spp.	117.18 – 234.4	800 – 1600
Serratia spp.	29.30 – 58.59	200 – 400
Recommended concentration in food contact surfaces	200 – 300	200

The MIC values obtained for these microorganisms were similar between them concerning the same disinfectant, with exception of *Serratia* spp. Based on the absolute values of concentration, it is possible to say that the isolates tested were less susceptible to SH than to HP. The MIC values observed for HP were within the range of recommended concentrations for disinfection of food surfaces, while most of the MIC values of SH were above the recommended concentration.

Table 5.7. MIC values each of antibiotic obtained with the isolates collected after disinfection (µg/mL).

Microorganism	AMP	RIF
Enterobacter spp.	1 – 2	16 – 32
Cellulosimicrobium	4 – 8	8 –16
Serratia spp.	4 –8	8 – 16

The analysis of the MIC values obtained showed that *Enterobacter* spp. was more susceptible to AMP than *Cellulosimicrobium* spp. and *Serratia* spp., but was more resistant to RIF. For AMP, the isolates present MIC values below the maximum concentration used in this work (128 µg/ml), and it was found a MIC value correspondent to the maximum concentration (32 µg/ml) for RIF relative to isolate *Enterobacter* spp.

5.2.4. Evaluation of biofilm formation ability

The colorimetric assay involving crystal violet staining of biofilms was used for quantification of biofilm formation by the bacteria isolated after disinfection. The results obtained are represented in Figure 5.4.
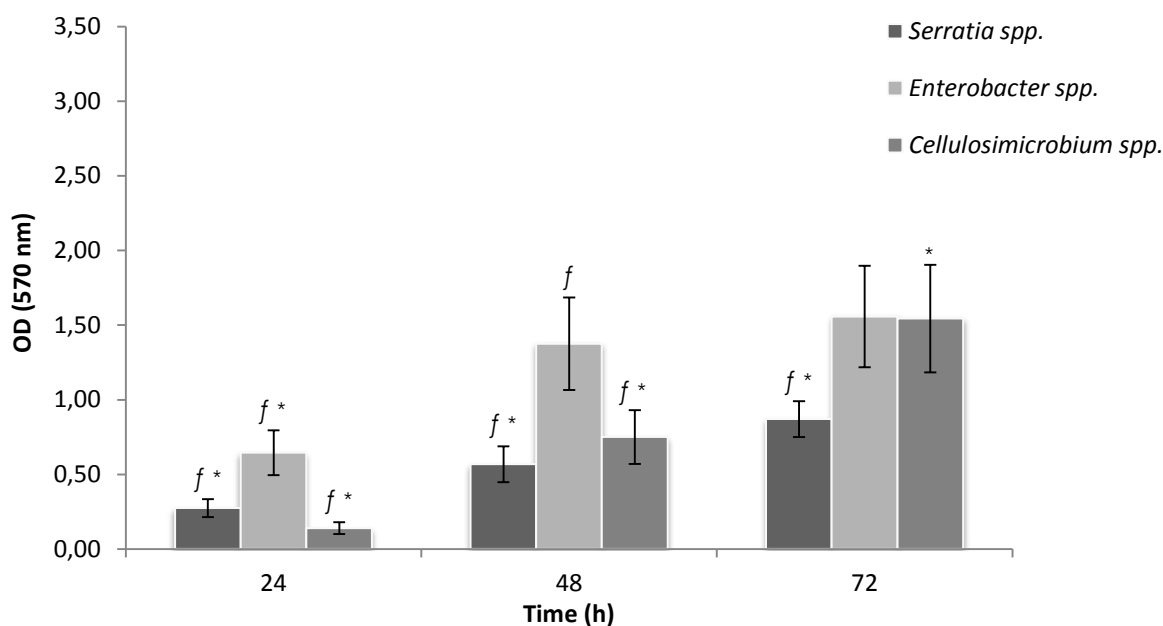
Figure 5.4. Evaluation of biofilm formation ability of the isolates (collected after cleaning and disinfection) by violet crystal staining. Each bar represents average CV–OD$_{570}$ values and standard errors. Symbols indicate statistically different values (p<0.05) between different growth periods for the same bacteria (*), and between bacteria considering the same growth period (f).

In the previous picture it is possible to observe significant differences in the biofilm formation ability along the incubation period, with exception of *Enterobacter* spp. that presented similar CV-OD values between 48 h and 72 h. Although *Enterobater* spp. presented a higher value of total biomass than the other bacteria after 24 h and 48 h of incubation, after 72 h its amount of total biomass was similar to that of *Cellulosimicrobium* spp.

5.2.5. Minimum Biofilm Eradication Concentration

In this study, biofilms were also exposed to the same disinfectants tested on planktonic cells (HP and SH) to determine their susceptibility, as describe above (section 4.1). The MEBC value of each disinfectant tested is present in Table 5.8.

Table 5.8. MBEC value obtained with the isolates collected after disinfection, and recommended concentration of each disinfectant (µg/mL).

Microorganism	HP	SH
Enterobacter spp.	>2344	>16000
Cellulosimicrobium spp.	>2344	>16000
Serratia spp.	>585.9	>4000
Recommended concentration in food contact surfaces	200 – 300	200

The isolates *Enterobacter* spp., *Cellulosimicrobium* spp., *and Serratia* spp. presented a MBEC value higher than 10xMIC (585.9 µg/ml and 2344 µg/ml for HP; and 16000 µg/ml for SH). All the MBEC values were much higher than the recommended concentrations for disinfection of food contact surfaces.

Chapter 6

Discussion

This chapter presents a discussion concerning the results of isolates collected before and after disinfection.

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Biofilm and planktonic cells have phenotypical and physical differences, and one of them is the resistance to disinfectants. Cleaning and disinfection procedures are used in medical, industrial and domestic environments to control the contamination of surfaces. Although these disinfectants treatments eliminate most surface contamination, some microorganisms may survive and, thus, lead to problems in terms of public health (Bridier *et al.,* 2011). On the other hand, bacteria resistant to antibiotics can be also a threat to public health. In this context, the present work had as aim the characterization in terms of biofilm formation ability, susceptibility to disinfectants and antibiotics of bacterial isolates collected from meat processing surfaces of a meat retail facility in the center of Braga, Portugal. It was evaluated the susceptibility of planktonic cells, of the isolates collected before and after cleaning and disinfection, to two antibiotics and two disinfectants. It was also assessed the capacity of biofilm formation of the isolates, and the susceptibility of those biofilms to two disinfectants. With the purpose of developing an appropriated and structured evaluation of the results obtained in this research, two sections concerning biofilms and planktonic cells characterization, in terms of susceptibility to disinfectants and antibiotics, as well as biofilm formation ability of the isolates collected before and after disinfection, will be analyzed separately. Following, a general analysis will be performed based on the disinfection procedures that are used in the food industry and their potential impact on human health.

Concerning the growth of the microorganisms in the media - collected before and after cleaning and disinfection - the results showed that the growth was higher in the samples collected on the cutting boards. This may be due to the existence of grooves on the cutting boards which facilitates the growth of microorganism. After cleaning and disinfection it was observed more bacterial growth of the samples which was not expected. This can be due to the selective pressure that disinfectants exert on bacteria. Probably, disinfectants eliminated those bacteria that grew before disinfection, which allowed the growth of the bacteria that initially did not grow. In the samples C1listL, M3listL (collected before cleaning and disinfection) and M3listL (collected after cleaning and disinfection) was observed growth in the enrichment step but was not growth after to put in selective media (Oxford Listeria Agar Base).

This can be due to presence of antimicrobials agents in the supplement added to selective media. The results of the 16S sequencing revealed that it was not possible to isolate *Listeria monocytogenes* and *Escherichia coli*. The presence of the different isolates - *Enterobacter* spp., *Hafnia alvei* and *Serratia* spp. - in media for *E. coli* is due to the fact that E. E. Broth is a

media for selectively enriching and detecting *Enterobacteriaceae*, particularly from foods and, as these belong to the same family of *E. coli,* they suffered the step of enrichment. CHROMagar™ E. Coli media is recommended for detection and enumeration of *E. coli,* allowing also the growth of other Gram-negative bacteria as referred in datasheet. *L. innocua* has grown in media recommended to *L. monocytogenes* because it belongs to the same family and, being one of the species of *Listeria* spp., has very similar characteristics. Although *Serratia* spp. is a Gram-negative bacteria and has grown in media of *E. coli* (as explained above), it also grown in media of *Listeria.* One hypothesis can be due to the meat extract contained in the media that can have favors the growth of *Serratia* spp. and *Cellulosimicrobium* spp. This media also contains esculin and ammonium ferric citrate that allows a presumptive identification of the black colonies. In fact, *Listeria* species hydrolize esculine to glucose and esculetin, which react with the ferric ions in the media. However, the *Serratia* spp. also hydrolyzes esculin forming esculitine and glucose (Edberg *et al.,* 1977). All these isolates can be found in meat or surfaces in contact with meat. The microbiological quality of meat depends on some factors, such as the physiological status of the animal at slaughter, the spread of contamination during slaughter and processing, the temperature, and the conditions of storage and distribution (Nychas *et al.,* 2008). Thus, one possible hypothesis for the presence of these microorganisms in the meat is due some contamination that occurred between slaughter and distribution.

Enterobacter is a genus that belongs to the *Enterobacteriaceae* family*,* and corresponds to Gram-negative and nonspore-forming bacteria (Davin-Regli and Pagès, 2015). *Enterobacter* species grow well, aerobically and anaerobically, at temperatures between 20 and 37 °C on general laboratory media at neutral pH (Cooney *et al.,* 2014). *Enterobacter* species are common causes of nosocomial infections in humans (Stock and Wiedemann, 2002). Finally, they can be found in a high variety of food, such as animal feed (dried pellets), eggs, fish, pork and water (Cooney *et al.,* 2014). *L. innocua* is characterized as Gram-positive bacteria of the genus *Listeria.* This specie is generally considered non-pathogenic and is able to survive in various extreme conditions such as, high pH, high and low temperatures, and high salt concentration (Favaro *et al.,* 2014). *L. innocua* can be found in meat, liver, heart, and kidney (Dorcheh *et al.,* 2013). *Hafnia alvei* is a Gram-negative of the *Enterobacteriaceae* family, and is mobile by flagella, with peritrichous arrangement rod-shaped and facultative anaerobic bacillus (Stock *et al.,* 2005), with optimal growth temperature between 30–37°C (Padilla *et al.,* 2015). This microorganism can be found in soil, sewage, freshwater, and a number of foods as meat and milk products (Stock *et al.,*

2005). *Hafnia* species are considered opportunistic pathogens especially in immunocompromised individuals, and are rarely associated to human disease (Baillie and Rise, 2014). The genus *Cellulosimicrobium* is considered Gram-positive, rod-shaped, nonmotile, and comprises three species designated as *C. cellulans*, *C. funkei*, and *C. terreum* (Sharma *et al.*, 2014), being these species opportunistic pathogens (Herrmann *et al.*, 2015).

Nowadays, disinfectants are commonly applied in food industries but little information is available on the molecular mechanisms displayed by microorganisms in response to disinfectants. Planktonic cells of the bacteria isolated before and after disinfection were more susceptible to HP (with MIC values between 58.59 – 117.18 µg/mL and 29.30 – 234.4 µg/mL respectively) than to SH (with MIC values between 400 – 800 µg/mL and 200 – 1600 µg/mL, respectively). The MIC values of HP concerning isolates collected before disinfection were much lower than the in-use recommended concentration (200 – 300 µg/mL) (Table 5.2). These results show that HP is a strong disinfecting agent due to its antioxidant properties. Hydrogen peroxide is a biocidal agent that acts as disinfectant due to the production of hydroxyl free radicals, which act as oxidants and react with cells components such as lipids, proteins and DNA, increasing cells permeability (Kahnert *et al.*, 2005). Despite being used against most bacteria, fungi and viruses, and being used as a strong oxidizing agent, the MIC values of SH concerning isolates collected before disinfection were higher than the in-use recommended concentration (200 µg/mL). This is a chlorine compound used as disinfectant, which bactericidal effect is based in the penetration and reactive action on essential enzymes inside the cells (Lomander *et al.*, 2004).

In food processing environment there are certain surfaces and areas where cleaning is difficult to accomplish, such as bends in pipes of machines and cracks in surfaces, and thus in these locations the main danger of cross-contamination of pathogens may be from biofilms cells existent. Biofilms from bacteria isolated before and after cleaning and disinfection (Table 5.4 and Table 5.8, respectively) were more resistant than planktonic cells. Peroxides-based disinfectants have been reported to be effective for removal bacterial biofilms, and are widely used in the food industry (Trachoo and Frank, 2002). However, no biofilms eradication was achieved by this disinfectant, with MBEC values higher than 10xMIC that also happed with SH. Making an overall appreciation of the results obtained with the two different disinfectants used in this study, it is noticed that, although there was not a defined MBEC value, it was always correspondent to a much higher value than the in-use recommended concentration for food surfaces' disinfection procedures. This fact may be due to the interaction between antimicrobial agents and biofilm

matrix, namely extracellular polymeric substances. The multiple layers of cells and EPS may constitute a complex and compact structure that may act as a permeability barrier which is difficult to penetrate and reach internal layers reducing uptake of the disinfectants and their efficacy (Bridier *et al.,* 2011). Also, it may be due to genetic changes in cells inside the biofilm (Donlan and Costerton, 2002). Gene transfer are involved in microbial adaptation to the environment through the exchange of genetic sequences including plasmids, transposons or integrons that confer specific phenotypic traits on cells such as, virulence expression and antimicrobial resistance (Bridier *et al.,* 2011). Phenotypic adaptations of biofilm cells can also explain the resistance to disinfectants. This may be due to sub-lethal concentrations of these compounds during disinfection process and/or to the reaction-diffusion limited penetration of disinfectants into a biofilm. These can lead to low levels of exposure of specific regions of the biofilm to the agents. These cells will therefore develop adaptive responses to sub-lethal concentrations of the disinfectant (Arce *et al.,* 2013). The growth of a biofilm is associated with physiological adaptations of cells that may lead to an increase in resistance to disinfectants. These phenotypic adaptations result from the expression of specific genes in response to their direct conditions of the microenvironment (Bridier *et al.,* 2011). Finally, in monoculture biofilms can exist a population diversity. Cells on upper layers of biofilm have more access to nutrients and oxygen becoming more active, while cells with less access to these compounds become dormant and resistant to disinfectants (Donlan and Costerton, 2002).

Regarding the results obtained for the antibiotics tested, the isolates were analyzed through the breakpoints according to the EUCAST. The MIC values of *Hafnia alvei*, *Serratia* spp. and *Enterobacter* spp. were compared with the MIC breakpoint relative to AMP (S ≤ 8 R > 8) of *Enterobacteriaceae*, since these bacteria belong to family *Enterobacteriaceae*. *Cellulosimicrobium* spp. and *L. innocua* were compared with the MIC breakpoint relative to AMP of Gram-positive anaerobes (S ≤ 4 R > 8), because these microorganisms have characteristics such as to be Gram-positive and anaerobe facultative bacteria. The isolates collected before cleaning and disinfection procedures and identified as *Serratia* spp. and *Hafnia alvei* were susceptible, with a MIC of 4 – 8 μg/mL each. *L. innocua* presented intermediate susceptibility to AMP, with a MIC of 4 – 8 μg/mL. For the isolates collected after cleaning and disinfection procedures, *Cellulosimicrobium* spp. showed intermediate susceptibility to AMP, with a MIC of 4 – 8 μg/mL. *Enterobacter* spp. was susceptible (MIC of 1 – 2 μg/mL) and *Serratia* spp. showed the same susceptibility above mentioned. According to EUCAST, RIF is not recommended for susceptibility

testing for some *Enterobacteriaceae* and Gram-positives anaerobes bacteria due to existence of several bacteria resistant to antibiotic. RIF inactivates bacterial RNA polymerase (RNAP). It has activity against a wide range of microorganisms such as mycobacteria (ex. *Mycobacterium tuberculosis*). This compound is highly active against Gram-positive bacteria, but is less active against Gram-negative organisms (Corley and Stephen, 2008). The MIC values between Gram-positive and Gram-negative microorganisms showed that Gram-positive bacteria were more susceptible than Gram-negative bacteria. It has been suggested that other antibiotics can be combined with rifampicin to avoid the appearance of antimicrobial resistance (Forrest and Tamura, 2010). *Cellulosimecrobium* spp. and *L. innocua* present intermediate susceptibility to RIF and can become resistant. Antimicrobials are delivered to animals for a variety of reasons, including disease treatment, prevention, control, and growth promotion/feed efficiency. However, the resistance of some isolates can be explained by the use excessive of antimicrobial agents, leading to the risk of spreading resistant bacteria via the food chain and wastewater (Messaoudi *et al.,* 2009).

By using the microtiter plate method, it was possible to find out that all isolates identified had the capacity to produce biofilm with increase of biofilm total biomass along the time. The capability of the isolates to form biofilm allows its persistence in the food processing environment. Subsequently, such persisting cells may unknowingly get added in the food. Therefore, understanding the biofilm-forming capabilities of these microorganisms is of great interest. The data obtained in this study is very important, because it shows that bacterial isolates from the cutting board and mincer can produce biofilm, which may cause the persistence of these microorganisms during meat processing and, consequently, lead to higher risks of food contamination.

In summary, it was presented an overview of the impact that disinfecting agents commonly used in food processing facilities may have on biofilm and planktonic cells. The phenotypic characterization of the isolates, performed in this study, demonstrated that the isolates have capacity to form biofilm and have resistance to disinfectants.

Chapter 7

Conclusions and future work

In this chapter, the major conclusions of the present thesis are addressed. Suggestions for future work are also proposed.

7.1. Conclusions

The aim of the present thesis was to characterize bacteria present in surfaces of one meat retail, particularly regarding biofilm formation ability and susceptibility to disinfectants and antibiotics.

The collected and identified isolates can be pathogenic microorganisms depending of the specie which lead to a potential risk for public health. All microorganisms of this study showed ability to form biofilm, which allows microorganisms to persist in the environments, and it also leads to an increased resistance to cleaning and disinfection procedures. The isolates collected before and after cleaning and disinfection procedures were more susceptible to HP than SH, presenting MIC values much lower than the in-use recommended concentration. Moreover, biofilms were less susceptible to oxidizing agents comparing with planktonic cells. In view of these conclusions, and transposing to the reality of meat processing areas, the application of SH and HP on these areas can be a matter of concern, since in this study they did not eradicate biofilms at concentrations above the recommended for use in food industries. Hence, even when the maximum concentration allowed is applied, those cells may, in fact, being exposed to a sub lethal concentration and, thus, can be triggered to develop resistance against the other disinfectants as well as to antibiotics.

Finally, this work allows concluding that it is important to assess and understand the phenotypic characteristics of planktonic cells and biofilms after exposure to chemical treatment since, besides enabling access to the mechanisms involved in biocidal resistance, this approach may allow the development of additional treatments that do not lead to cross resistance.

7.2. Future Work

To complete this study, some of the suggestions that should be considered for future work are:

To analyze the other isolates that was not possible to identify, and for that it should be interesting to use other identification techniques.

Other enrichment and selective media could be used to indicate the presence, or not, of other pathogenic microorganisms also usually found in meat, such as *Campylobacter jejuni*, *Salmonella* spp., *Clostridium perfringens*, *Staphylococcus aureus* and *Bacillus cereus*.

To have a more complete study about antibiotics, more compounds should be tested and should also be interesting to evaluate combinations between these agents. As antibiotics, more disinfectants used in the food industry should be tested. The disinfectants and antibiotics anteriorly tested and the new ones that can be tested, should be assayed in biofilm cells, in order to compare the influence of different lifestyles in the susceptibility to antibiotics.

Since biofilms can also be found as mixed microbial cultures, and as is known in this work that several microorganisms types were found, it would be very interesting to study the general response to different disinfectants challenges by mixed biofilms composed of different organisms isolated from food processing environments, as well as the effects of such challenges on each of the bacterial species involved.

It is also known that biofilm communities acquire resistance mechanisms to disinfectants, thus it would be important to investigate other methods for biofilm control, for example, modification of food processing surfaces.

Chapter 8

Bibliography

In this chapter is present the bibliography used throughout this work.

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