FOOD SAFETY AND QUALITY ENGINEERING MSC THESIS

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RESISTANCE OF FOODBORNE MICROORGANISMS AGAINST DISINFECTANTS

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1. INTRODUCTION AND OBJECTIVES

1.1 Statement of the Topic

The increasing concern over food safety has led to an emphasis on the control of microbial contamination. Microorganisms present in food can pose significant health risks when consumed, leading to foodborne illnesses. One critical aspect of this control is the efficacy of disinfectants in eliminating or reducing microbial contamination. This study focuses on the resistance of microorganisms isolated from food against disinfectants, specifically sodium hypochlorite.

1.2 Relevance of the Topic

Understanding the resistance of microorganisms to disinfectants is crucial for ensuring the effectiveness of disinfection practices in food processing and handling. Resistance to disinfectants can compromise food safety measures, leading to potential outbreaks of foodborne diseases. This topic is particularly relevant due to the increasing reports of microorganisms resistant to disinfectants, which can survive and proliferate even after disinfection procedures.

The choice of sodium hypochlorite as the disinfectant of interest, arises from its widespread use in food industry sanitation due to its broad-spectrum antimicrobial activity. However, there is a growing concern regarding the emergence of resistance against sodium hypochlorite among foodborne microorganisms. Addressing this issue is vital to maintain and improve food safety standards.

1.3 Objectives of the Study

The primary objectives of this study are:

- To isolate and characterize microorganisms from raw milk.
- To assess the resistance of these isolated microorganisms in addition to microorganisms isolated from another two food sources (red pepper and pork meat), against sodium hypochlorite using agar disk diffusion tests.
- To determine the minimum inhibitory concentration (MIC) of sodium hypochlorite against the isolated microorganisms using broth microdilution.
- To evaluate the biofilm formation capability in case of six of the isolated microorganisms.
- To assess the biofilm resistance to sodium hypochlorite.

1.4 Research Questions

To achieve the objectives outlined above, this thesis aims to answer the following research questions:

- What types of microorganisms can be isolated from the selected food sources?
- How resistant are these isolated microorganisms to sodium hypochlorite?
- What are the MIC values of sodium hypochlorite against the isolated microorganisms?
- Do the isolated microorganisms exhibit biofilm formation? If so, are they resistant to sodium hypochlorite?

1.5 Structure of the Thesis

The thesis is structured as follows:

Chapter 2: Literature Review - Provides an overview of existing literature on microbial resistance to disinfectants.

Chapter 3: Materials and Methods - Details the methods used for microbial isolation and characterization, disinfectant susceptibility testing, MIC determination, and biofilm formation evaluation.

Chapter 4: Results and evaluation - Presents the findings from the tests and discusses the implications of the results, compares them with existing literature, and provides insights into the significance of the findings in the context of food safety.

Chapter 5: Conclusion and suggestions - Summarizes the main findings of the study, draws conclusions, and offers recommendations for future research and practical applications.

Chapter 6: Conclusion - Provides a complete overview of the work in the order in which it follows the logic of the thesis.

By addressing these aspects, this thesis aims to contribute to the understanding of disinfectant resistance in foodborne microorganisms and its implications for food safety.

2. LITERATURE REVIEW

Over the time, the food production system has evolved significantly, with increased complexity in operations; processes have become more mechanized, foods are now more processed, and the production volumes have increased, at the same time, the growing trend of healthy eating and minimal processed foods has forced manufacturers to minimize the use of chemical preservatives. All these factors lead to a rising demand for strict sanitation programs within the food industry to ensure safety and prolong shelf-life, making necessary the use of chemical disinfectants to sanitize surfaces such as pipes, tanks, stirring blades, etc. and sometimes also the food container itself, stopping the growth of microorganisms, preventing foodborne illnesses, and extending product viability (Langsrud et al., 2003; Ramírez, 2019). With biological emergencies becoming more frequent, effective disinfection procedures and suitable disinfectants are essential for controlling pathogens and protecting public health (Kuti and Papp, 2020).

It can be seen from the many foodborne outbreaks happening all around the world, that it is imperative to implement preventive measures and programs, such as Good Manufacturing Practice (GMP), Hazard Analysis and Critical Control Points (HACCP) plans among others, to guarantee food safety and also for establishing a unified framework for maintaining hygiene throughout the food production process. Numerous factors contribute to the rise in foodborne diseases, including shifts in dietary patterns and consumer preferences, increased global travel, microbial adaptation to environments and development of antimicrobial resistance, as well as insufficient sanitation (Schirone et al., 2019).

It is of extreme importance to raise awareness of the seriousness of diseases caused by foodborne pathogens. These pathogens are capable of synthesizing toxins that induce severe illness and mortality. Millions of cases appear globally, triggering outbreaks that spread even among countries and the statistics on the severity of the crisis show that millions of annual deaths can be attributed to foodborne microorganisms (Heredia and García, 2018).

Due to the relationship between food industry and human health, disinfectants must be recognized as safe for human health ensuring that they are not irritating, toxic or corrosive. The efficiency of disinfectants is influenced by factors such as concentration, time of contact, form of application.

According to their chemical structure, their mechanism of action against microorganisms is different. Alcohols, phenols, quaternary ammonium compounds, glutaraldehyde, iodine compounds, chlorine species, ozone, among others, are the most used chemical disinfectants in the food industry (Korukluoğlu and Gülgör, 2016).

Studies indicate that some microorganisms are showing disinfectant resistance in food environments, and this is important because their survivance can cause spoilage in foods and other problems in food processing, but more importantly, those that are pathogens represent a risk to consumer's health. The potential combined resistance to disinfectants and other antimicrobials is a worrying issue for the future not only for food industry but for public health (Langsrud et al., 2003).

2.1. Disinfectants

A disinfectant is a substance that can eliminate virus, infectious fungi, and bacteria on non-living surfaces, however it may be not effective against bacterial spores. Disinfection is a milder process compared to sterilization (Marriott and Gravani, 2006).

The most used disinfectants in the food industry are, among others, acids, alcohols, aldehydes, alkalis, chlorine compounds, peroxygen compounds, phenols, and quaternary ammonium compounds (QAC). Based on the works of Maillard (2013) and McDonnell (2020), the mechanism of action of these types of disinfectants was summarized in Table 1.

Table 1. Mechanism of action of different disinfectants.

Disinfectant class	Mechanism of action
Acids	- Alters pH
Alcohols	- Precipitates proteins - Denatures lipids
Aldehydes	- Denatures proteins - Alkylates nucleic acids
Alkalis	- Alters pH
Chlorine compounds	- Denatures proteins - Disrupts cell membrane
Peroxygen compounds	- Denatures proteins and lipids
Phenols	- Disrupts cell wall - Denatures proteins
QAC	- Binds phospholipids of cell membrane - Denatures proteins

As it can be observed, according to the disinfectant composition, the mechanism of action is directed to different targets, hence, their efficacy against different microorganisms varies (Dvorak, 2008).

Disinfectant effectiveness depends on the following factors, it is important to find a suitable parameter depending on the type of disinfectant; concentration of disinfectant, temperature, pH, humidity, water hardness, contact time, type and number of microorganism present, and organic and inorganic interfering substances in the environment (Makhoahle and Gaseitsiwe, 2022).

2.1.2. Sodium hypochlorite

Among the chlorine compounds, hypochlorites are the most used disinfectants. Their use ranges from water and wastewater systems to food and nonfood contact surfaces, as well as postharvest disinfectant for fruits (Mishra et al., 2018).

Sodium hypochlorite (NaOCl), provides strong antimicrobial effectiveness, having the potential to kill bacteria, viruses, fungi, mycobacteria, and spores. Together with its affordability, and low toxicity to humans, makes it a very valuable disinfectant (Artasensi et al., 2021).

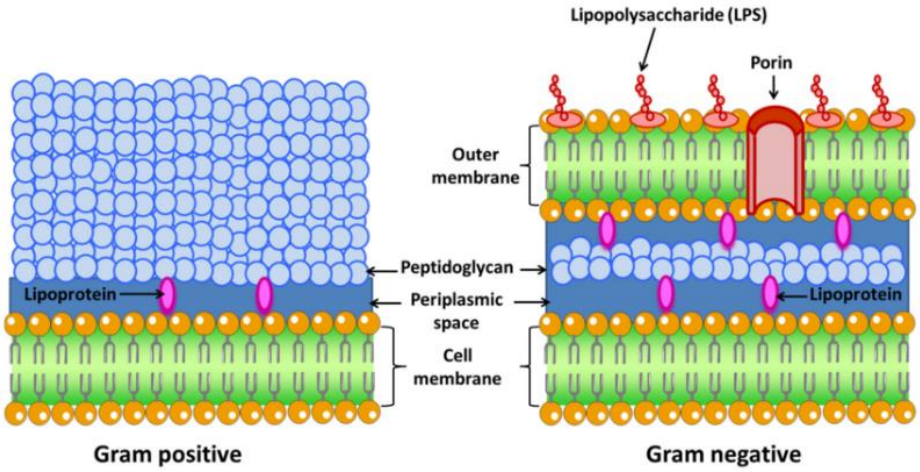
Hypochlorous acid is the active component in sodium hypochlorite which is produced through the hydrolysis of the hypochlorite ion. It acts as a potent oxidizing agent, damaging cell membranes, proteins, and nucleic acids upon contact. Its action is represented with a reaction: $NaOCl + H_2O \rightarrow HOCl + NaOH$ (Mishra et al., 2018; DeQueiroz, 2004).

NaOCl target three main areas within bacterial cells: the cell wall, cell membrane, and cytoplasm.

The cell wall consists of a porous peptidoglycan network. As shown in Fig. 1, Gram-negative bacteria have a thin peptidoglycan cell wall, surrounded by an outer membrane that contains lipopolysaccharide. In contrast, Gram-positive bacteria do not have an outer membrane but possess a porous peptidoglycan layer that is significantly thicker than that of Gram-negative bacteria. This structure permits the diffusion of particles smaller than 2 nm and is permeable to oxidative species like hydroxyl radicals, which can easily traverse it. Sodium hypochlorite, with its strong oxidizing properties, targets and degrades peptidoglycan, weakening the bacterial cell wall, leading to damage and eventual lysis of the cell. Furthermore, large protein structures called porins, embedded in the outer membrane of Gram-negative bacteria, can also act as channels for NaOCl. This

combination of factors makes the cell wall susceptible to oxidative damage and disruption by NaOCl, affecting both its assembly and structure, and compromising the integrity of the cell membrane. Nevertheless, Gram-negative bacteria, with their unique cell wall structure and outer envelope, generally exhibit greater resistance to antimicrobials compared to Gram-positive bacteria (DeQueiroz, 2004; Silhavy et al., 2010; Ersoy et al., 2019)

Fig. 1. Comparison of cell wall of Gram-negative and Gram-positive bacteria (Jiménez-Jiménez, et al., 2022)



Sodium hypochlorite act by oxidizing the lipid components of bacterial cell membranes, particularly targeting their high content of unsaturated lipids. This oxidation process is known as lipid peroxidation and can lead to mutual cross-linking and polymerization of membrane components, altering the lipid content of the cell membrane compromising its integrity and function, also damaging membrane-bound proteins, resulting in increased permeability and leakage of cellular contents, which contributes to the cell's eventual death (Ersoy et al., 2019; Jancic and Stosic, 2014).

Finally, the cytoplasm is one of the main targets of sodium hypochlorite penetrating bacterial cells and interacting with various cellular components, including proteins, nucleic acids, and other molecules. This interaction can lead to denaturation or damage of these cellular components, disrupting essential cellular processes and contributing to cell death.

Among the cellular components susceptible to rapid reaction with hypochlorous acid are purine and pyrimidine bases, amines, amino acids, and sulfhydryl groups. The oxidation of these

components by HOCl results in the loss of their physiological functions. HOCl has been found to disrupt oxidative phosphorylation and metabolic pathways involved in ATP utilization or generation. Additionally, HOCl can cause DNA damage through the formation of chlorinated derivatives of nucleotide bases. Furthermore, HOCl and hypochlorite ions can induce amino acid degradation and hydrolysis, releasing chlorine. This leads to the formation of chloramines that interfere with cellular metabolism and enzymatic activity, further compromising the cell's functionality and contributing to its death (Fukuzaki, 2006; Lipus et al., 2019).

Sodium hypochlorite has demonstrated effectiveness against a variety of microorganisms. Research indicates that sodium hypochlorite can suppress relevant pathogenic bacteria such as *Escherichia coli* O157:H7, *Salmonella* Typhimurium DT104, and *Staphylococcus aureus* (Kondo et al., 2006). Additionally, studies have shown that sodium hypochlorite is effective against biofilms of *Enterococcus faecalis* and *Candida albicans* (Gomez et al., 2020), and it has been found to have higher bactericidal efficacy against biofilms of *Staphylococcus aureus* and *Pseudomonas aeruginosa* compared to quaternary ammonium chloride disinfectants (Lineback et al., 2018).

2.2. Foodborne microorganisms

Foodborne microorganisms comprise bacteria, viruses, fungi, and parasites. They can be spoilage microorganisms or pathogens. Pathogens are mainly bacteria, viruses, and parasites. Is not common to find yeast that are pathogenic, and moulds are considered a toxicological risk due to their mycotoxins produced. Pathogens contaminate food at various stages of production and distribution, leading to foodborne illnesses. Certain bacteria and fungi can produce toxins, which are heat-resistant, meaning they cannot be eliminated through standard food preparation methods like cooking or frying, complicating food safety control measures (Martinović et al., 2016; Vaskoska, 2022).

On the other hand, spoilage microorganisms cause changes in sensory characteristics of foods through metabolic processes making them not suitable for consumption, although they may be safe to eat and not cause illness because there are no pathogens or toxins present (Rawat, 2015).

2.2.1. Raw milk microbiota

Milk is a perfect medium for the growth of microorganisms due to its nutritional value as it contains proteins, lipids, carbohydrates, vitamins, minerals, and amino acids, and it also has an ideal pH and water activity. Therefore, raw milk may contain many types of microorganisms with different characteristics in terms of classification, morphology, and physiology. Pathogenic and spoilage bacteria can be found in raw milk and can be mesophilic, psychrophilic, or thermophilic (Özer and Akdemir-Evrendilek, 2015).

The most predominant bacteria in raw milk are lactic acid bacteria, a group of bacteria that includes *Lactococcus, Lactobacillus, Leuconostoc, Streptococcus*, *Enterococcus* spp. among others. These bacteria have a significant role on food technology and have benefits for the gastrointestinal health. Psychrotrophic microorganisms including *Pseudomonas* and *Acinetobacter* spp. proliferate during cold storage and are also an important group of milk's composition; together with thermophilic species that includes *Alcaligenes, Microbacterium*, and spore-formers *Bacillus* and *Clostridium*, are responsible for spoilage. On the other hand, strains present in milk such as *Listeria* spp., *Staphylococcus* spp., some serotypes of *Escherichia coli*, and *Campylobacter* spp. are pathogens and cause illness if consumed (Quigley et al., 2013; Rawat, 2015).

Microorganisms can contaminate milk through various sources, including contact with the animal's teat and feces, as well as environmental conditions such as feed, air, and water. Additionally, interactions with farm and milking equipment, along with inadequate farm or personnel hygiene, can impact the microbial quality of milk (Quigley et al., 2013).

2.2.2. Microbiota of vegetables

Raw vegetables have been recognized as potential carriers for transmitting bacterial, viral, and parasitic pathogens. Contamination can occur at various stages, including harvesting, post-harvest handling, and distribution. Due to their high water content, neutral pH, and nutrient-rich composition, raw vegetables can support the growth of a wide range of microorganisms. Additionally, fruits, vegetables, and agricultural soils may naturally contain contaminants like mycotoxins (Feroz and Noor, 2019; Smith, 2023).

Vegetables can be affected by various microorganisms, including bacteria and fungi. Common bacterial species responsible for vegetable spoilage include *Pseudomonas* spp., *Erwinia carotovora*, and *Xanthomonas campestris*, while among fungi are *Aspergillus, Alternaria, Fusarium, Penicillium,* and *Rhizopus* spp. (Ava and Noor, 2022).

On the other hand, *E. coli* O157:H7, Enterotoxigenic *E. coli, Listeria monocytogenes*, and *Bacillus cereus* have been associated with outbreaks of gastroenteritis resulting from contaminated fresh vegetables. Additionally, consumption of foods like raw salad vegetables, which often undergo minimal or no heat treatment, can lead to *Shigella*-related infections. *Shigella* genus comprises four species - *S. dysenteriae*, *S. boydii, S. sonnei,* and *S. flexneri* - all of which are pathogenic to humans. Several significant shigellosis outbreaks have been attributed to the consumption of contaminated raw vegetables (Beuchat, 1996).

2.2.3. Microbiota of pork meat

Pork offers a nutrient-rich and high-moisture environment that supports the growth of various microorganisms, which can be introduced during the cutting process, either from personnel, equipment, or the slaughter environment (Zwirzitz et al., 2020).

Pathogens such as *Salmonella* spp., *Listeria monocytogenes*, *Campylobacter* spp., *Staphylococcus aureus*, *Toxoplasma gondii*, and *Trichinella spiralis* are associated with pigs or pork products, making pork a potential source of foodborne illnesses (Baer et al., 2013).

Moreover, depending on storage conditions, specific microorganisms like *Pseudomonas fragi*, *Pseudomonas putida*, lactic acid bacteria such as *Lactobacillus sakei* and *Leuconostoc mesenteroides*, *Enterobacteriaceae, Brochothrix thermosphacta*, and *Clostridium* spp. are known to be the primary agents responsible for meat spoilage (Papadopoulou et al., 2020).

2.3. Disinfectant resistance

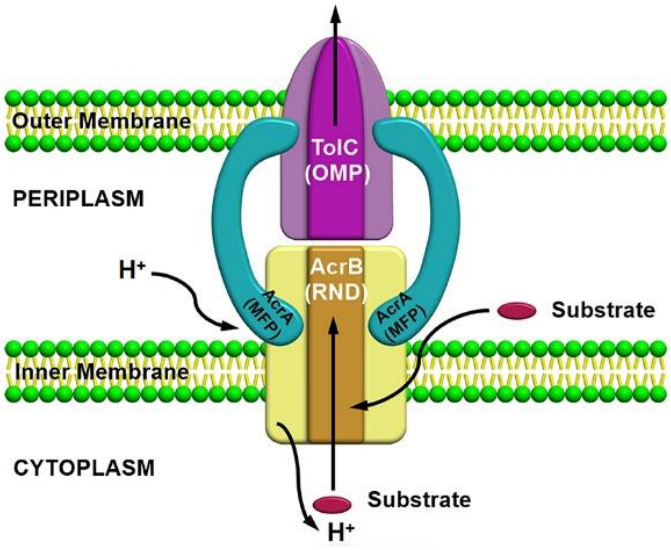
Disinfectant resistance refers to the reduced susceptibility of a strain to a disinfectant, with increases in minimum inhibitory concentration (MIC) or minimum bactericidal concentration (MBC) values observed (van Dijk et al., 2022). Microorganisms employ various strategies to

counteract the effects of disinfectants, including efflux pumps, enzymatic degradation, forming biofilms, among others, and can be intrinsic (through naturally occurring properties of the cell) or acquired (from mutations or horizontal gene transfer) (Bragg et al., 2018).

2.3.1. Efflux pumps

Efflux pumps are proteins found in the bacterial plasma membrane. Their role is to detect harmful substances that have penetrated the cell wall and are present in the periplasm or cytoplasm. As shown in Fig. 2, these pumps expel these substances into the external environment before they can harm the cell. Additionally, they can also identify and remove toxic by-products produced during the bacterium's metabolism, functioning as excretory mechanisms (Amaral et al., 2014).

Fig. 2. Efflux pump mechanism (Chetri, 2023)



The overexpression of efflux pumps, which may be leaded by the exposure to sublethal concentrations of disinfectants, can increase the MIC, resulting in resistance (Gnanadhas et al., 2012). According to Blanco et al. (2016), this overexpression has been shown to reduce the efficiency of different disinfectants, including hydrogen peroxide, benzalkonium chloride, chloroxylenol, QACs, and iodine compounds.

2.3.2. Enzymatic degradation

Another strategy includes enzymatically breaking down or inactivating disinfectants, especially when the concentrations of agents like formaldehyde, QACs, among others are lower than typically used. When bacteria are exposed to MIC of disinfectants, they increase the production of neutralizing enzymes essential for disinfectant biodegradation. Examples of the neutralization of disinfectants have been studied on hydrogen peroxide where *kat*A catalase gene and peroxidase enzymes present in certain microorganisms neutralize it; and in the case of aldehydes, the inactivation of formaldehyde occurs with the help of the enzymes Gfa, AdhC and EstD, which catalyze consecutive stages of formaldehyde oxidation to carbon dioxide. The transmissible plasmid *Adh*C gene encodes a glutathione-dependent formaldehyde dehydrogenase that causes the inactivation (Rozman et al., 2021).

2.3.3. Biofilms

Biofilms are clusters of microorganisms embedded by a matrix of extracellular polymeric substances (EPS) they produce. They can attach to both living and inert surfaces. Among non-living surfaces, materials like stainless steel, rubber, and polyurethane have been found to facilitate the adherence and growth of pathogenic biofilms, making them a significant challenge in the food industry. Unlike the more commonly studied planktonic bacteria, biofilms represent the predominant form of bacterial growth. It is estimated that 80% of human infections may originate from biofilms, as they can increase resistance to disinfectants by up to 1000 times. When these biofilms detach from equipment, they contaminate food, posing both spoilage and food safety risks (Kiskó and Szabó-Szabó, 2011; Cadena et al., 2019; Vaskoska, 2022).

Research indicates that the longer a biofilm remains attached, the more resistant the microorganisms become, affecting the ease of disinfection. Preventing biofilm formation is then less difficult than removing it (Pap and Kiskó, 2008). Biocides and disinfectants are commonly used to eliminate and avoid biofilm formation. The EPS matrix surrounding the biofilm, needs to be destroyed to allow the disinfectant to reach the viable cells, additional vigorous mechanical action, like scrubbing may be required in some cases, but with precaution, as some cleaning tools can be abrasive and cause cracks on surfaces, which can further promote biofilm formation (Kiskó and Szabó-Szabó, 2011).

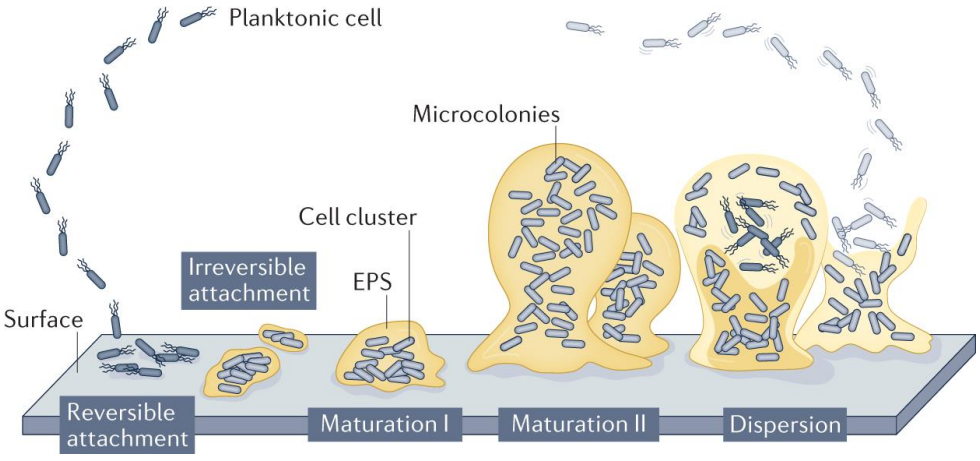
Although the ability of a microorganism to form biofilms may be inherent in some microorganisms, studies with *Pseudomonas aeruginosa* (Tashiro et al., 2014), *Staphylococcus aureus* (Cincarova, 2016), some strains of *Vibrio* (Mougin, 2024), and *Staphylococcus epidermis* (Milisavljevic et al., 2008) have shown that exposure to sub-lethal concentrations of disinfectants can promote biofilm formation.

2.3.3.1. Biofilm formation

Formation of biofilms initiates with single cells attaching to surfaces, a process divided into two stages of attachment: reversible, involving weak bonds like Van der Waals and electrostatic forces, and irreversible, with stronger bonds like dipole-dipole, ion-ion, and covalent bonds (Korukluoğlu and Gülgör, 2016).

As shown in Fig. 3., after irreversible adhesion, biofilm maturation starts by cells growing and dividing using nutrients from the forming biofilm and its environment, forming microcolonies. These microcolonies grow and merge to create a cell layer on the top. During this phase, the attached cells produce additional EPS that aid in attachment and protect the cell matrix from environmental changes. With time, some bacteria detach to colonize new areas and ensure survival, starting the biofilm formation process again (Myszka and Czaczyk 2011).

Fig. 3. Steps of biofilm formation (Sauer et al., 2022).



Since the food industry comprises many different sectors, the most relevant biofilm-forming microorganisms are summarized in Table 2.

Table 2. Most common pathogenic biofilms in the food industry (Carrascosa et al., 2021).

Pathogen	Contaminated Food
Bacillus cereus	dairy products, rice, vegetables, meat
Campylobacter jejuni	animals, poultry, unpasteurised milk
Escherichia coli	raw milk, fresh meat, fruits and vegetables
Listeria monocytogenes	dairy products, meat, ready-to-eat products, fruit, soft cheeses, ice cream, unpasteurised milk, candied apples, frozen vegetables, poultry
Salmonella Enterica	Poultry meat, bovine, ovine, porcine, fish
Staphylococcus aureus	meat products, poultry, egg products, dairy products, salads, bakery products, especially cream-filled pastries and cakes, and sandwich fillings
Pseudomonas spp.	fruits, vegetables, meat surfaces and low-acid dairy products
Geobacillus stearothermophilus	dried dairy products
Anoxybacillus flavithermus	dried milk powder
Pectinatus spp.	beer and brewery environment

2.3.3.2. Mechanisms of resistance in biofilms

The main mechanism involved in the disinfectant resistance of biofilms is the limited penetration of disinfectants into biofilms; disinfectants need to reach a sufficient concentration at the target site to effectively deactivate microorganisms. Yet, the matrix of cells and extracellular polymeric substances in biofilms form a barrier that can delay the antimicrobial diffusion, or even inactivating it (Giaouris et al., 2020).

Another explanation of disinfectant resistance of biofilms is the changes in biofilm cell physiology; due to the differences between planktonic and surface-attached bacteria, physiological changes occur after attachment. For instance, genes responsible for EPS production are often up-regulated in the cells attached to the surface. Additionally, bacterial membranes, acting as the primary defense

against disinfectants, can lead to modifications in the fatty acid composition, which can increase membrane rigidity, potentially limiting disinfectant penetration into the lipid bilayers (Yuan et al., 2020).

Furthermore, in food processing settings, due to the variety of microorganisms that can be found, the biofilm structure can be constituted of multiple species, interacting in various ways, forming a complex and dynamic network, influencing biofilm structure, and contributing to specific functions, such as disinfectant resistance. Typically, multi-species biofilms show greater resistance to disinfectants compared to their single-species counterparts (Yuan et al., 2019).

Lastly, persistent cells; microbial complexes produce persisters, cells that do not grow but also are not killed in the presence of antimicrobials, hence show tolerance to them. The mechanism of tolerance of persistent cells was first reported in 1944, and their understanding remains unclear. Persisters are fast spreading cells ensuring survival of the population when exposed to harmful conditions; studies have shown that persistent cells do not show any mutation, and when they are reinoculated, the new population formed contains sensitive and tolerant cells (Lewis, 2005).

According to Lewis (2005), proteins of persistent cells could shut down the cell's antimicrobial targets, turning it into a dormant state, hence developing tolerance.

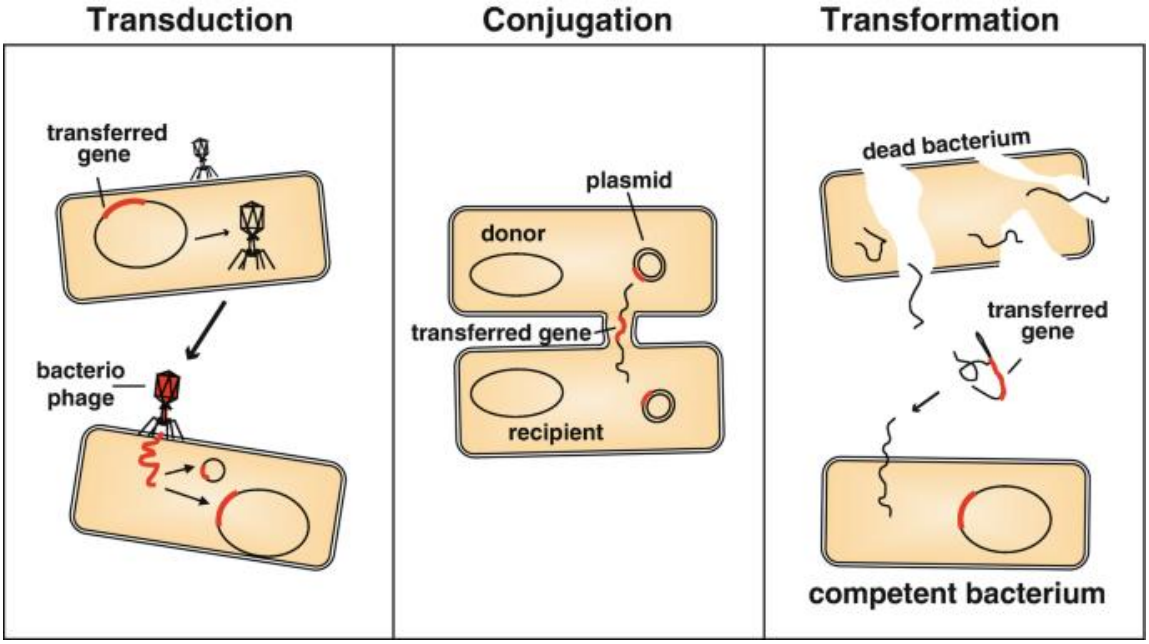
2.3.4. Horizontal gene transfer

Contrary to intrinsic resistance; acquired resistance arises from the effective transfer of mobile genetic elements that can contain one or multiple resistance genes. These antimicrobial resistance genes can be components of integrons, transposons, and insertion sequences, capable of transferring to plasmids or gene cassettes within cells. They can self-transfer between bacteria, expanding the presence of antimicrobial resistance genes within a bacterial community; these genes can express for efflux pumps, antimicrobial-degrading enzymes, among others (Mc Carlie et al., 2020).

The three main pathways of horizontal gene transfer (HGT) are conjugation, transduction, and transformation (Fig. 4). Conjugation is the predominant mechanism of HGT in bacteria, involving direct contact between cells for DNA transfer. In transformation, competent recipients take up short fragments of DNA. Transduction transfers DNA through bacteriophages. All these mechanisms can

significantly contribute to the rapid evolution of bacteria, enhancing their fitness, particularly aiding their survival in the presence of antimicrobials (Vinayamohan et al., 2022).

Fig. 4. Mechanisms of horizontal gene transfer (Blokesch, 2015).



Studies have explored the transfer of plasmids carrying disinfectant resistance genes in both *Escherichia coli* and *Staphylococcal* species by conjugation, in the latter, *qac* genes, which encode efflux pumps, are primarily transmitted by plasmids. While the conjugative transfer of disinfectant resistance genes has been well-documented, it remains unclear whether these genes can be transferred by transduction or transformation. Additionally, there is limited information available on the mobile genetic elements responsible for encoding disinfectant resistance (Mc Carlie et al., 2020).

Studies indicate that biofilms might be an ideal setting for the exchange of genetic material, facilitating the spread of disinfectant resistance genes within the population. Factors such as high cell density, the presence of EPS, the release of significant amounts of DNA and nutrient conditions within biofilms can enhance conjugation processes due to its potential to form multi-species biofilms (Bridier et al., 2011).

2.3.4.1. Cross resistance

Resistance to disinfectants becomes more alarming when it leads to increased resistance to other antimicrobial agents. Cross-resistance can appear when two antimicrobials share the same pathway to reach their target, have similar mechanisms of action, or if both are susceptible to the same resistant mechanisms. In the food industry it is common to switch between two disinfectants with the belief that it will prevent resistance. Yet, in reality, similar disinfectants are frequently used, leading to the possibility that microorganisms resistant to one disinfectant may also develop resistance to the other (Langsrud et al., 2003).

Another concern is the potential for cross-resistance between disinfectants and antibiotics. Mutations arising from the selective pressure of antimicrobial agents can lead to the upregulation of efflux pumps with broad specificity or alterations in membrane permeability, resulting in resistance and cross resistance. Furthermore, horizontal gene transfer mechanisms, such as conjugation, can facilitate the transfer of genetic elements encoding these efflux pumps or plasmids that had been associated with disinfectant resistance. This transfer can confer cross-resistance to both antibiotics and disinfectants (Langsrud et al., 2003; Mc Carlie et al., 2020).

2.4. Methods of assessing disinfectant resistance

In order to observe microbial adaptation and resistance to antimicrobial agents in detail, some microbiological assays used in the evaluation of disinfectant activity, such as the agar disk diffusion assay, MIC determination by broth microdilutions, or biofilm assays can be used.

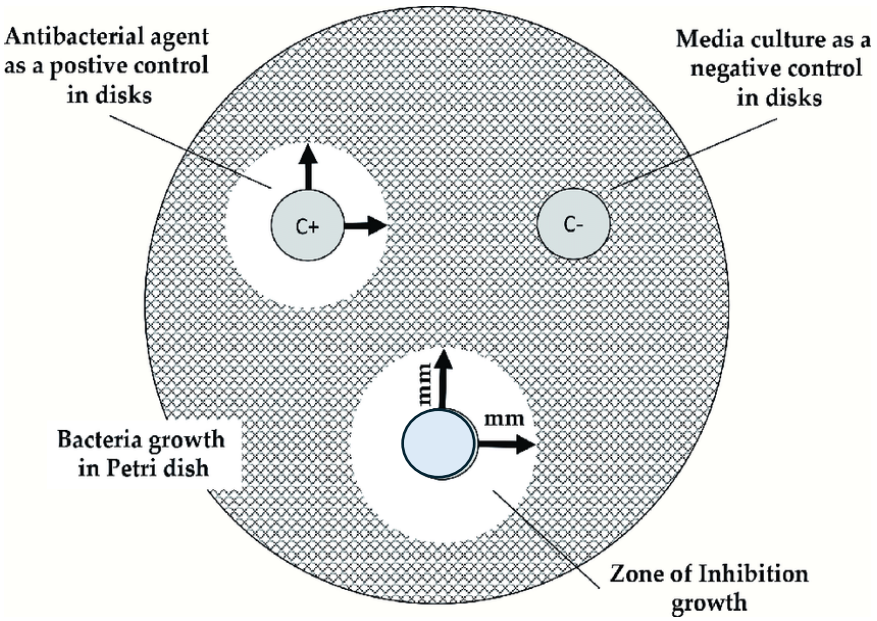
2.4.1. Agar disk diffusion test

Disk diffusion is a well-established method for testing antimicrobial susceptibility and remains popular in routine clinical laboratories. It is effective for testing most bacterial pathogens. This method is versatile in testing various antimicrobial agents and does not require any specialized equipment (EUCAST, 2024).

The first step of this method is the preparation of Petri dishes with the correspondent agar; then, the strain to be tested has to be made in a suspension in saline to the density of a 0.5 McFarland

turbidity standard, to then be inoculated in agar plates. The so-called “15-15-15 minute” rule has to be considered; using the microbial suspension within 15 minutes of preparation, applying the 6 mm paper disks with the antimicrobial on the surface within 15 minutes of inoculation, and incubate within 15 minutes of disks placement. After incubation, inhibition zones surrounding the disk as shown in Fig. 5, represent the inhibition of growth of the microorganism, which can be measured in millimeters in order to obtain valuable information from those values (EUCAST, 2024).

Fig. 5. Agar disk diffusion test (Vega-Jiménez et al., 2019).



EUCAST (European Committee on Antimicrobial Susceptibility Testing) and CLSI (Clinical and Laboratory Standards Institute), are organizations that provide two of the most commonly used methodologies for Antimicrobial Susceptibility Testing. One of the many useful tools they present is a set of equivalences of inhibition zone diameter in millimeters for specific strains and specific antimicrobial agents (including only antibiotics), for the evaluation of its resistance or susceptibility through stablished breakpoints (Wiegand et al., 2008).

2.4.2. MIC determination by broth microdilutions

The technique of broth microdilution is employed for assessing the in vitro efficacy of an antimicrobial agent against a microorganism and it is useful for determine the minimum inhibitory concentration (Leber and Burnham, 2022).

Broth dilution involves a liquid growth medium with increasing concentrations of the antimicrobial agent, usually in a two-fold dilution series. A specific number of bacterial cells are added to this medium. When performed in microtiter plates with ≤ 500 microliters per well, it is referred to as microdilution. After incubation, growth of the microorganism is indicated by turbidity. The MIC is the lowest concentration of the antimicrobial agent that inhibits visible growth of the microorganism under set conditions (Wiegand et al., 2008).

Automated photometers play a vital role in improving the efficiency and precision of broth microdilution assays for antimicrobial susceptibility testing. These automated systems allow for quick dispensing of antimicrobial agents into microplates, automated readings of absorbance, and data analysis. This greatly enhances the speed and consistency of testing (Smith and Kirby, 2016).

The MIC value doesn't indicate whether the antimicrobial agent acts as a bactericidal (killing) or bacteriostatic (inhibiting growth) agent. Even if no growth is visible in the well, tube, or agar plate at the MIC concentration, there might still be live cells if the drug has a bacteriostatic effect on the tested bacteria (Wiegand et al., 2008).

2.4.3. Biofilm assays

Biofilm-embedded bacteria can be genetically susceptible to disinfectants when studied in planktonic state but may show resistance at the phenotypic level in a biofilm state, making their susceptibility unpredictable. Various methods for testing biofilm susceptibility have been proposed, none have accurately represented the in vivo biofilm environment (Thieme et al., 2021).

For testing disinfectant resistance in biofilms, it is necessary to compare the quantity of cells before and after treatment. There are many approaches to quantitatively characterize biofilms; direct methods allow for the counting of cultivable cells and include techniques like plate counts, cell counts by microscopic observation, Coulter cell counting, flow cytometry, and fluorescence microscopy. Indirect methods, on the other hand, involve assessing parameters such as dry mass,

total organic carbon, ATP bioluminescence, total protein, among others. Many of these methods, require homogenisation of the biofilm to release the cells into a liquid medium before analysis (Wilson et al., 2017).

The most commonly used method to assess biofilm cell viability is by counting colony forming units (CFU) on agar plates. Utilizing dilution series for cell quantification, this method can be easily accessible in microbiology laboratories. Yet, it has notable limitations, one of them is that the detached live cells may not accurately represent the original biofilm population. Another drawback is that some biofilm cells might be viable but non-culturable (VBNC), not being able to detect through the CFU method. On the other hand, flow cytometry, combined with specific fluorophores, offers a rapid and precise way to assess biofilm cell viability. Although it is a more costly method, flow cytometry addresses the limitations of CFU counting by distinguishing between total, dead, and VBNC cells (Azeredo et al., 2016).

Once the biofilm is quantitatively characterized, the same selected direct or indirect quantification method should be used after the biofilm is treated with the disinfectant in order to assess its resistance (Cruz et al., 2018).

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Culture media

3.1.1.1. Plate Count Agar (PCA)

For the preparation of PCA, 5 grams of peptone (Biolab), 1 gram of glucose (Biolab), 15 grams of bacteriological agar (Biolab) and 2.5 grams of yeast extract, were weighted into a media bottle. 1000 ml of distilled water was added to the mixture and autoclaved at 121°C for 15 minutes.

3.1.1.2. De Man Rogosa and Sharpe agar (MRS)

For the preparation of MRS, 63 grams of MRS Agar (Biolab) was weighted into a media bottle, 10 ml of MRS supplement (Biolab) and 1000 ml of distilled water were added, and the mixture was autoclaved at 121°C for 15 minutes.

3.1.1.3. Cetrimide agar

46.7 grams of Cetrimide agar (Sigma-Aldrich) was suspended in 1000 ml of distilled water and 10 ml of glycerol (Sigma-Aldrich) was added, and the mixture was autoclaved at 121°C for 15 minutes.

3.1.1.4. ChromoBio® Coliform agar

30 grams of ChromoBio® Coliform agar (Biolab) was suspended in 1000 ml of distilled water and sterilised at 100 °C (in water bath) for 30 minutes.

3.1.1.5. Tryptone Soy Agar (TSA)

30 grams of Tryptone Soy Broth (TSB) (Biolab) and 15 grams of bacteriological agar (Biolab) was weighted into a media bottle and 1000 ml of distilled water were added. The mixture was autoclaved at 121°C for 15 minutes.

3.1.1.6. Tryptone Soy Broth (TSB)

30 grams of TSB (Biolab) was suspended in 1000 ml of distilled water and autoclaved at 121°C for 15 minutes.

3.1.1.7. Diluents

For serial dilutions, test tubes with 9 ml of diluent were used. The diluent solution was prepared by weighting 8.5 grams of NaCl and 1 gram of peptone into a media bottle. 1000 ml of distilled water were added to the mixture. Test tubes were filled with 9ml of the solution and autoclaved at 121°C for 15 minutes.

3.1.2. Samples

100 ml of raw milk was taken from a local farm, from which 1 ml was used for the preparation of serial dilutions: 1:10, 1:100, 1:1000, 1:10000.

3.1.3. Disinfectant

The disinfectant selected for research was Flóraszept (Unilever Magyarország Kft.) disinfectant liquid detergent (Active compound: 1.35g sodium hypochlorite in 100g of product)

3.1.4. Stainless steel slides

For the evaluation of biofilm formation and the effect of the disinfectant on biofilm, sterile stainless steel slides with dimensions of 2.5 cm x 7.5 cm (18.75 cm^2) were used.

3.2. Methods

3.2.1. Isolation of microorganisms

The isolation of foodborne microorganisms for their subsequent evaluation of their resistance to disinfectants was performed from three different food sources: Raw milk, vegetables (red pepper), and raw pork meat. Since this work was made in the frame of a project, each food source was assigned to a different student. The methodology described below corresponds to raw milk.

Different agars were prepared to detect different groups of microorganism: PCA for total plate count, MRS for *Lactobacillus* species, Cetrimide for *Pseudomonas* spp., and Chromocult for coliforms.

From the raw milk, the original sample (1:1) and the dilutions 1:100 and 1:10000 were chosen for plating.

The culture method selected was spread plate. 0.1 milliliter of each selected dilution was added to the petri dishes corresponding to the respective agars: PCA, MRS, Cetrimide and Chromocult. The

inoculum was spread over the surface evenly and incubated for 4 days at 30°C in the case of PCA, Cetrimide and MRS, the latter in anaerobic conditions, while for Chromocult an incubation temperature of 37°C was used.

3.2.2. Identification of isolates

After incubation, the plates were observed, colony count was carried out and a differentiation of colonies according to their morphology, color and texture was done, selecting and enumerating all the various types of colonies, getting a total of 25 colonies, naming them as RM# (Raw Milk) (Annex 1.1).

Once all the apparently different colonies were selected, pure cultures of each were made on TSA plates by streak plating, incubating them at 30°C for 24 hours to be analyzed in their early stationary phase.

The fresh 24-hour cultures were grouped according to the similarity of their macroscopic characteristics. From each of these different groups, one isolate was studied through different experiments:

- Simple staining with crystal violet for its microscopic examination,
- catalase test,
- oxidase test and
- KOH test for Gram determination (Annex 1.2). KOH test is based on the property of Gram negative bacteria of release cellular DNA when their cell walls are lysed, producing a viscous mixture. If a string is formed when applying KOH to the strain, the bacteria is Gram negative.

Once the analyses were performed, the isolates were transferred to new TSA plates by streak plating.

To the identification of the microorganisms, MALDI TOF-MS was used. Matrix-Assisted Laser Desorption/Ionization - Time Of Flight, is an analytical technique in which samples are ionized by mixing them in a metal plate with an organic compound solution called matrix; a laser irradiates the sample, and the matrix absorbs the laser's energy, producing charged molecules which are measured by the TOF detector by their mass-to-charge ratio. In the case of microbiology, the

proteins of the microorganisms are the ones analyzed and compared with a database to match it with the corresponding microorganism. Isolates were prepared 24 hours before the analysis.

Once obtained the identification results from MALDI TOF, an evaluation was done by comparing the organism suggested by the equipment as the best match, with their score value, being a high-confidence identification those score values within the range of 2.00 - 3.00. Based on this, 8 isolates were selected for the investigation (Annex 1.3). The sum of the microorganisms obtained from pepper (17) and pork meat (5) gave a total of 30 isolates to investigate for disinfectant resistance (Annex 1.3.1).

3.2.3. Preparation of stock cultures from all identified isolates

The preparation of stock cultures was done using TSA agar slants. Additionally, the isolates were stored at -80°C in Eppendorf tubes with TSB and glycerol, used as a cryoprotectant agent to preserve them long-term.

3.2.4. Determination of MIC values of disinfectant on isolates

3.2.4.1. Agar disk diffusion test

The first experiment carried out consisted of testing inhibitory efficacy of the disinfectant against the isolates by using agar disk diffusion test. Suspensions with optical density (OD) 0.5 were made from each microorganism in tubes with diluents to have an approximate bacterial cell count of 10^8 cells/ml. In Petri dishes with TSA, a lawn of bacteria was cultured for each of the isolates. This lawn was created by adding 1 ml of suspension and rotating the plate until a thin layer was distributed homogeneously on all the surface of the agar, the excess was pipetted out. Once the surfaces of the Petri dishes were dry, three filter paper disks (5 mm diameter) were placed on the top, to which 10 microliters of disinfectant were subsequently added to each one. The concentration of the disinfectant used in this first test was 3.2% (0.32 ml Flóraszept, 9.68 ml sterile distilled water) as suggested on the producer's label. The plates were incubated at 30°C for 48 hours.

The test was repeated, increasing the concentration 10 times; 32% (3.2 ml Flóraszept, 6.8 ml sterile distilled water). After the incubation, the diameter of inhibition was measured for each disk of each isolate.

Six microorganisms (PM1, PM5, P16, P28, RM4 and RM12) that showed the best inhibition were selected for further investigation, analyzing their Minimum Inhibitory Concentration (MIC) value

by agar disk diffusion at different concentrations of disinfectant. In this test, four filter paper disks were placed on top to which 10 microliters of disinfectant in different concentrations (64%, 32%, 16% and 8%) were added to each. The plates were incubated at 30°C for 48 hours and the inhibition diameter corresponding to each concentration was measured for each microorganism.

3.2.4.2. Broth microdilution assay

The six microorganisms that showed the best inhibition in the first agar disk diffusion test were tested by broth microdilution using Multiskan Ascent microplate photometer (Thermo Fisher Scientific). The MIC value was studied making a microdilution set of samples with different concentrations of disinfectant in each well of the microplate for turbidity measurement; Multiskan Ascent has a software that creates curves indicating the tendency of growth specific to each well, making it easy to identify at which concentration it inhibits the microorganism and how the growth of the microorganism behaves with different concentrations of disinfectant. In this way, a ~10^7 cell/ml suspension of each isolate was made and added to the wells of the microtiter plate (30 microliters), together with two times concentrated TSB (135 microliters), and the disinfectant (135 microliters) in the different concentrations: 64%, 32%, 16% and 8%. The sample arrangement design was done adding also wells for Blind samples (same mixture but using distilled water instead of microbial suspension), and Control samples (mixture without disinfectant) as can be seen in Fig. 6a. The composition of each sample is described in Fig. 6b.

Fig. 6a. Model of microtiter plate placement of samples.

	1	2	3	4	5	6	7	8	9	10	11	12
a	S1+F(64)	S1+F(64)	S1+F(64)	S2+F(64)	S2+F(64)	S2+F(64)	B F(64)	S1	S2			
b	S1+F(32)	S1+F(32)	S1+F(32)	S2+F(32)	S2+F(32)	S2+F(32)	B F(32)	S1	S2			
c	S1+F(16)	S1+F(16)	S1+F(16)	S2+F(16)	S2+F(16)	S2+F(16)	B F(16)	S1	S2			
d	S1+F(8)	S1+F(8)	S1+F(8)	S2+F(8)	S2+F(8)	S2+F(8)	B F(8)		TSB			
e												
f												
g												
h												

S1 - Sample 1; S2 - Sample 2; F - Flóraszept; B - Blind

Fig. 6b. Description of preparation of samples.

	2xcc TSB (µl)	Disinfectant (in proper conc.) (µl)	Dest water (µl)	Microbe suspension (µl)
Gray marked wells:	135	135	-	30
Orange marked wells	135	135	30	-
Green marked wells	135	-	135	30
Blue marked well	135	-	165	-

The microplate was put into the reader and left for 24 hours, with mixing intervals of 30 minutes, a temperature setting of 30°C and a wavelength of 600 nm. Once this time had passed, the curves obtained were analyzed, determining the MIC value for each microorganism.

3.2.5. Biofilm formation assessment

Biofilm formation ability of the selected six isolates were tested as follows. Suspensions of ~10^8 cell/ml of the isolates were prepared and 0,7 ml was inoculated in square Petri dishes containing 70 ml of TSB; afterwards sterile stainless steel slides were placed into the Petri dishes, and they were left for an hour. Since we worked with three parallels, and three different sampling times were stablished, nine slides were used for each strain.

Once the 60 minutes passed, the slides were washed with 5 ml of sterile distilled water on each side (10 ml per slide), six of the slides were soaked again in sterile TSB, without inoculate; three of them were incubated at 30°C for 2 days, and the other three were incubated at 30°C for 7 days. Meanwhile, the other three slides, already washed, were placed into sterile Petri dishes and each of their surfaces was sampled with a swab, which were put in test tubes with diluents and serial dilutions for each of the parallels were made.

The dilutions were pour-plated with TSA and incubated at 30°C for 48 hours.

The washing, swabbing, and plating procedures were repeated for the remaining six slides, each of them after their corresponding time of incubation.

4. RESULTS AND EVALUATION

4.1. Isolation and identification of isolates

The colony count from the isolation (Table 3), showed a higher number of colonies on PCA and MRS plates, as expected, since *Lactobacillus* spp. (MRS) are abundant in raw milk.

Table 3. Colony count from isolation of raw milk

	PCA	CET	CHR	MRS
CFU/ml	$3.2x10^5$	$9.7x10^2$	$2.3x10^2$	$2.3x10^3$

According to macromorphological observation, 25 different colonies were selected and grouped (Table 4); one isolate from each group (shown in bold) was selected for further investigation, where microscopic observation, KOH, catalase and oxidase tests were carried out (Table 5).

Table 4. Grouping of isolates according to macroscopic characteristics

Group	Isolates	Description
1	**8**,11,5,9,10,14,7	Orange, salmon, rough surface
2	**15**,16	Purple with transparent halo, sticky consistency
3	**4**	Bright yellow, perfectly rounded
4	**13**	Yellowish, translucid
5	23,**19**,24	Opaque yellow/pinkish
6	**6**	Pale yellow
7	**12**	Opaque whitish
8	**21**	Reddish with yellow halo
9	**20**,18,17	Violet with transparent halo
10	**25**,22	Whitish transparent

Table 5. Micromorphological description and results from catalase, oxidase and KOH tests.

Isolate RM	4	6	8	12	13	15	19	20	21	25
Description	Cocci	Cocci in clusters	Rods	Big cocci	Short rods	Rods	Thin rods	Rods	Rods	Rods
Catalase	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
Oxidase	Pos	Neg	Neg	Pos	Neg	Neg	Neg	Neg	Neg	Neg
Gram	Pos	Pos	Neg	Pos	Pos	Neg	Neg	Neg	Neg	Neg

The results from the identification with MALDI-TOF-MS showed a variety of microorganisms; the ones with a high and acceptable score value (see Annex 1.3 and 1.3.1) were selected, obtaining the following results (Table 6).

Table 6. MALDI-TOF-MS identification of isolates.

Code	Source	Microorganism
PM1	Pork meat	*Buttiauxella gaviniae*
PM2	Pork meat	*Aeromonas sp*
PM3	Pork meat	*Kocuria salsicia*
PM5	Pork meat	*Pseudomonas lundensis*
PM6	Pork meat	*Pseudomonas fluorescens*
P2	Pepper	*Pseudomonas extremorientalis*
P6	Pepper	*Brachybacterium conglomeratum*
P7	Pepper	*Pseudomonas oryzihabitans*
P9	Pepper	*Bacillus cereus*
P10	Pepper	*Bacillus cereus*
P12	Pepper	*Staphylococcus hominis*
P14	Pepper	*Escherichia coli*
P16	Pepper	*Micrococcus luteus*
P17	Pepper	*Moraxella osloensis*
P19	Pepper	*Pluralibacter pyrinus*
P21	Pepper	*Pseudomonas flavescens*
P26	Pepper	*Microbacterium arborescens*
P28	Pepper	*Pseudomonas antarctica*
P29	Pepper	*Pseudomonas fulva*
P30	Pepper	*Pseudomonas oryzihabitans*
P32	Pepper	*Bacillus cereus*
P33	Pepper	*Microbacterium arborescens*
RM4	Raw milk	*Kocuria salsicia*
RM6	Raw milk	*Staphylococcus chromogenes*
RM12	Raw milk	*Macrococcus caseolyticus*
RM13	Raw milk	*Acinetobacter johnsonii*
RM15	Raw milk	*Buttiauxella noackiae*
RM19	Raw milk	*Stenotrophomonas maltophilia*
RM20	Raw milk	*Hafnia alvei*
RM21	Raw milk	*Pantoea agglomerans*

Pork meat presented two different species of *Pseudomonas*; this is one of the main genera known for spoilage in pork (Papadopoulou et al., 2020). *Kocuria salsicia* was found both in pork and milk; this bacterium has been isolated from various animals and dairy products (Youn and Seo, 2022) Also in milk, it was found *Staphylococcus chromogenes*, which is often isolated from mastitis of dairy cows (Dos Santos et al., 2016). In pepper it can be observed that *Pseudomonas* spp. Were the most abundant, as they are well known for spoilage of vegetables (Ava and Noor, 2022); while *Bacillus cereus*, a common pathogen of vegetables was also found multiple times (Beuchat, 1996).

4.2 Determination of MIC values of disinfectant on isolates

In the first agar disk diffusion test performed, the concentration of the disinfectant used was the one suggested on the producer's label, which was 3.2%, and no inhibitory effect was observed.

A second test was conducted, increasing 10 times the initial concentration (32%.), the diameter of inhibition was measured obtaining the following data (Table 7).

Table 7. Inhibition diameters of isolates tested with Flóraszept (32%) by agar disk diffusion

Isolate	Microorganism	Inhibition diameter average (mm)	SD
PM1	*Buttiauxella gaviniae*	8.0	0.0
PM2	*Aeromonas sp*	6.0	0.0
PM3	*Kocuria salsicia*	8.5	0.7
PM5	*Pseudomonas lundensis*	8.0	1.0
PM6	*Pseudomonas fluorescens*	8.3	0.6
P2	*Pseudomonas extremorientalis*	6.0	1.0
P6	*Brachybacterium conglomeratum*	9.3	0.6
P7	*Pseudomonas oryzihabitans*	8.0	1.0
P9	*Bacillus cereus*	9.7	0.6
P10	*Bacillus cereus*	17.0	4.2
P12	*Staphylococcus hominis*	9.7	1.2
P14	*Escherichia coli*	8.0	0.0
P16	*Micrococcus luteus*	9.5	0.7
P17	*Moraxella osloensis*	14.0	4.2
P19	*Pluralibacter pyrinus*	8.3	0.6
P21	*Pseudomonas flavescens*	7.7	0.6
P26	*Microbacterium arborescens*	10.0	0.0
P28	*Pseudomonas antarctica*	8.7	0.6
P29	*Pseudomonas fulva*	7.7	0.6
P30	*Pseudomonas oryzihabitans*	6.7	0.6
P32	*Bacillus cereus*	10.0	0.0
P33	*Microbacterium arborescens*	9.0	0.0
RM4	*Kocuria salsicia*	10.0	0.0
RM6	*Staphylococcus chromogenes*	10.0	0.0
RM12	*Macrococcus caseolyticus*	11.0	1.7
RM13	*Acinetobacter johnsonii*	7.0	0.0
RM15	*Buttiauxella noackiae*	10.0	3.6
RM19	*Stenotrophomonas maltophilia*	9.0	1.0
RM20	*Hafnia alvei*	8.3	0.6
RM21	*Pantoea agglomerans*	8.0	0.0

Since the work was made in the frame of a project, the results obtained from the same microorganisms tested against different antimicrobials, were compared in order to select the microorganisms that showed best inhibition in all the tests, which were then tested with different concentrations of disinfectant obtaining the following results (Table 8).

Table 8. Inhibition diameters of microorganisms exposed to different concentrations of Flóraszept

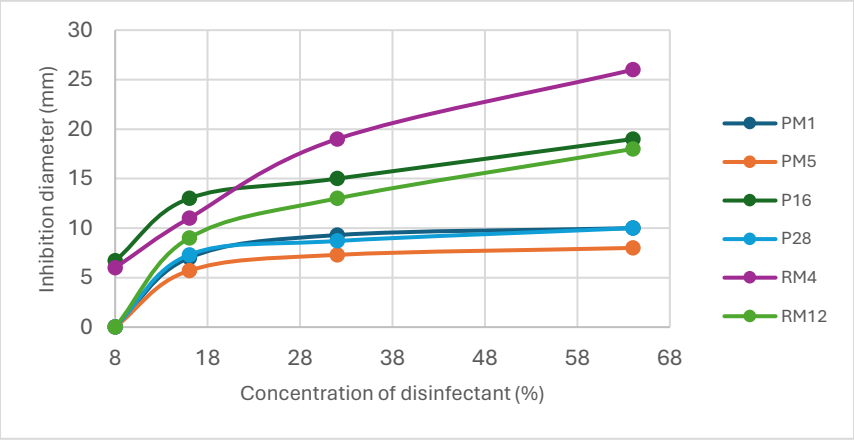
	Inhibition diameter (mm)																	
Strain \ Conc	**64%**			Avg	SD	**32%**			Avg	SD	**16%**			Avg	SD	**8%**			Avg	SD
RM4	30	24	24	**26**	3.5	22	16	18	**19**	3.1	10	8	14	**11**	3.1	6	6	6	**6**	0.0
RM12	15	22	18	**18**	3.5	12	11	15	**13**	2.1	10	8	9	**9**	1.0	0	0	0	**0**	0.0
P16	19	17	22	**19**	2.5	15	12	19	**15**	3.5	12	16	10	**13**	3.1	9	5	6	**6.7**	2.1
P28	9	11	10	**10**	1.0	9	9	8	**8.7**	0.6	9	8	5	**7.3**	2.1	0	0	0	**0**	0.0
PM1	11	10	9	**10**	1.0	9	10	9	**9.3**	0.6	6	8	7	**7**	1.0	0	0	0	**0**	0.0
PM5	9	6	9	**8**	1.7	9	5	8	**7.3**	2.1	6	5	6	**5.7**	0.6	0	0	0	**0**	0.0

From the table above, it can be seen that some microorganisms present a higher sensitivity to the disinfectant at all the concentrations tested, being these RM4, P16, and RM12 (*Kocuria salsicia, Micrococcus luteus,* and *Macrococcus caseolyticus*, respectively), which are Gram positive microorganisms, and as described in the literature review, they show more sensitivity against antimicrobials due to their cell wall structure and composition (DeQueiroz, 2004; Silhavy et al., 2010; Ersoy et al., 2019). In addition, the microorganisms that showed a higher tolerance were PM5, PM1 and P28 (*Pseudomonas lundensis, Buttiauxella gaviniae,* and *Pseudomonas antarctica* respectively) which are Gram negative bacteria.

Another important observation is that two of the microorganisms showed inhibition even at the lowest concentration (8%) making impossible to find the minimum inhibitory concentration by these concentrations in this method.

EUCAST provides important information about the resistance or susceptibility of microorganisms when using agar disk diffusion test, by comparing inhibition diameters with MIC's, allowing to determine the susceptibility of specific strains against specific antimicrobial agents. Unfortunately, these charts are only available for antibiotics, needing other methods to identify with certainty the MIC value for the strains tested, however the inhibition zone diameters give an important insight of their susceptibility. In Fig. 7 the tendencies in inhibition with the different concentrations of 8%, 16%, 32%, and 64% on the selected microorganisms can be observed better.

Fig. 7. Tendencies in inhibition diameter at four different concentrations of disinfectant (8%, 16%, 32%, 64%)



Additionally, the MIC value was investigated by using Multiskan. The graphs created with the absorbance data given by the equipment (Fig. 8a and Fig 8b), showed the growth modelling curve of the microorganisms at each of the different concentrations of disinfectant.

Fig. 8a. Growth curves of microorganisms at different concentrations of disinfectant using Multiskan.

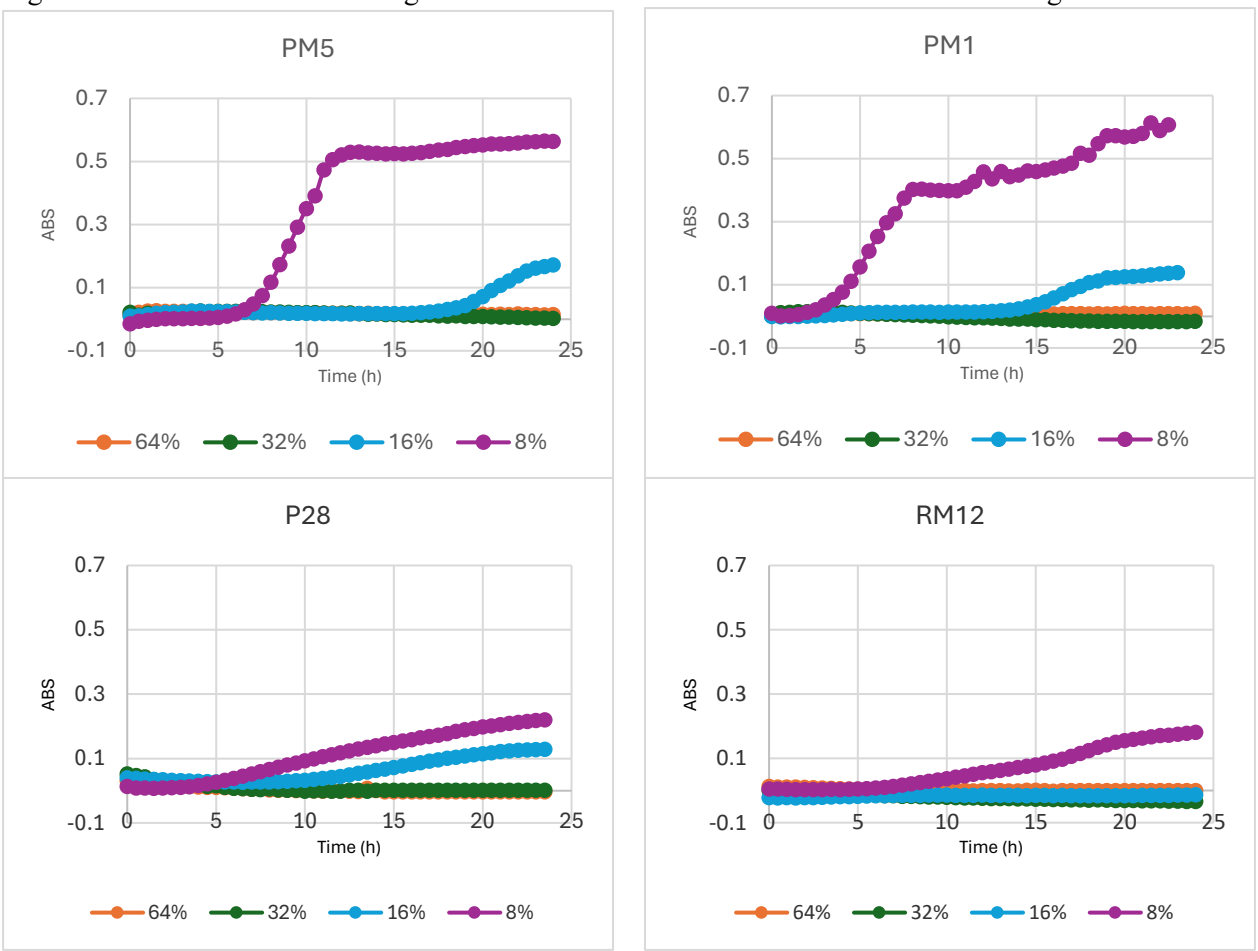
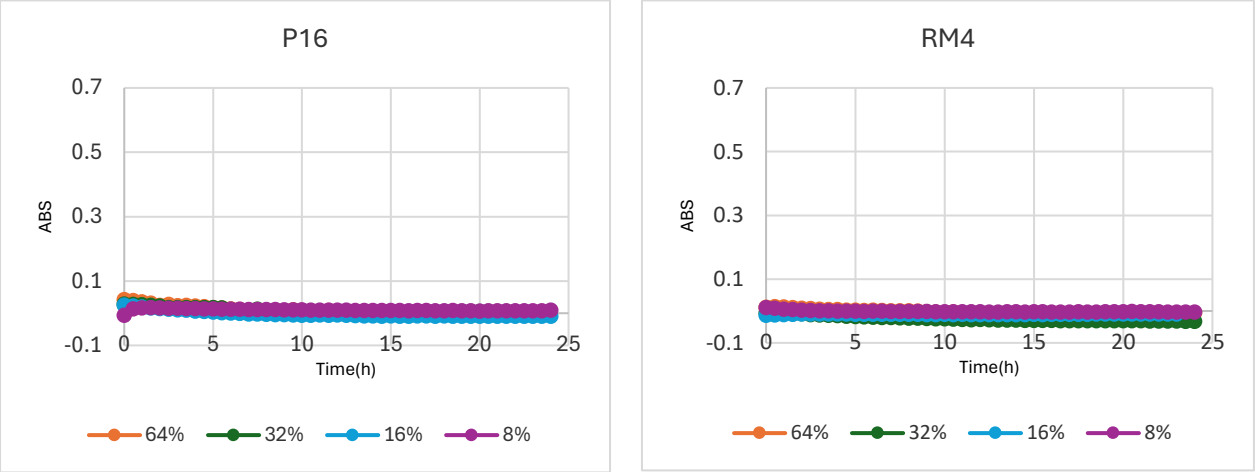


Fig. 8b. Growth curves of microorganisms at different concentrations of disinfectant using Multiskan.



As shown in Fig. 8a and Fig. 8b, the first four graphs (PM5, PM1, P28 and RM12) indicate a notable growth of the microorganism at a concentration of 8% of Flóraszept, which actually match the results from the agar disk diffusion test, where those four isolates did not show inhibitory zone at 8% concentration. In the case of the last two graphs (P16 and RM4), no growth curve is observed at 8% concentration which coincide with the inhibitory zone observed at this same concentration in the agar disk diffusion test.

In case of PM5, PM1 and P28 curves at 16%, an increasing growth rate can be seen, while at a concentration of 32% and 64% no growth was detected.

These initial three graphs correspond to the microorganisms with higher tolerance to the sodium hypochlorite, and their minimum inhibitory concentration is 32% according to the growth curves and the four different concentrations tested.

On the other hand, the last three graphs belonging to the more sensitive microorganisms, show a different behavior at the 16% disinfectant concentration. In RM12's graph, apart from the 8% concentration curve, it showed no signs of growth at any concentration, which indicate that the MIC value is 16%. In the case of P16 and RM4 graphs, there is no growth observed at any concentration of disinfectant, for which it can be inferred that the MIC value is 8%.

The label of Flóraszept states that the recommended use of the product is at a concentration of 3.2%, which would not be enough for inhibit the growth of the microorganisms tested isolated from three major groups of food as are vegetables, meat, and dairy. From the final six microorganisms

selected from testing, only two have a MIC value of disinfectant that could be lower than 8%, but still in the initial test with agar disk diffusion at 3,2% none of them showed growth inhibition, whereas the other four microorganisms would need 3 to 10 times concentration of disinfectant in order to inhibit the growth.

Since the disinfectant was tested at 4 different concentrations which are two-fold diluted between them, even though a growth curve can be observed at certain concentration and in the next one a complete inhibition appears, the exact MIC value can be somewhere between both values, but this method gives a good estimate of where this concentration is.

Resistance to disinfectants as stated previously, can occur due to different reasons such as genetic mutations, horizontal gene transfer, exposure to sublethal concentrations of disinfectant, selective pressure among others (Bragg et al., 2018), and represent an important issue pertaining to food safety and quality. Sodium hypochlorite mode of action targets and degrades peptidoglycan, debilitating the cell wall of bacteria, which leads to damage and death of the cell, being Gram positive bacteria very susceptible; yet in the case of Gram negative, the porins present in their other membrane can act as channels for sodium hypochlorite (DeQueiroz, 2004; Silhavy et al., 2010; Ersoy et al., 2019). This is why sodium hypochlorite is a very effective disinfectant and one of the most used in the industry and at home, and it is important to know how to use it properly so that there are no long-term global health problems.

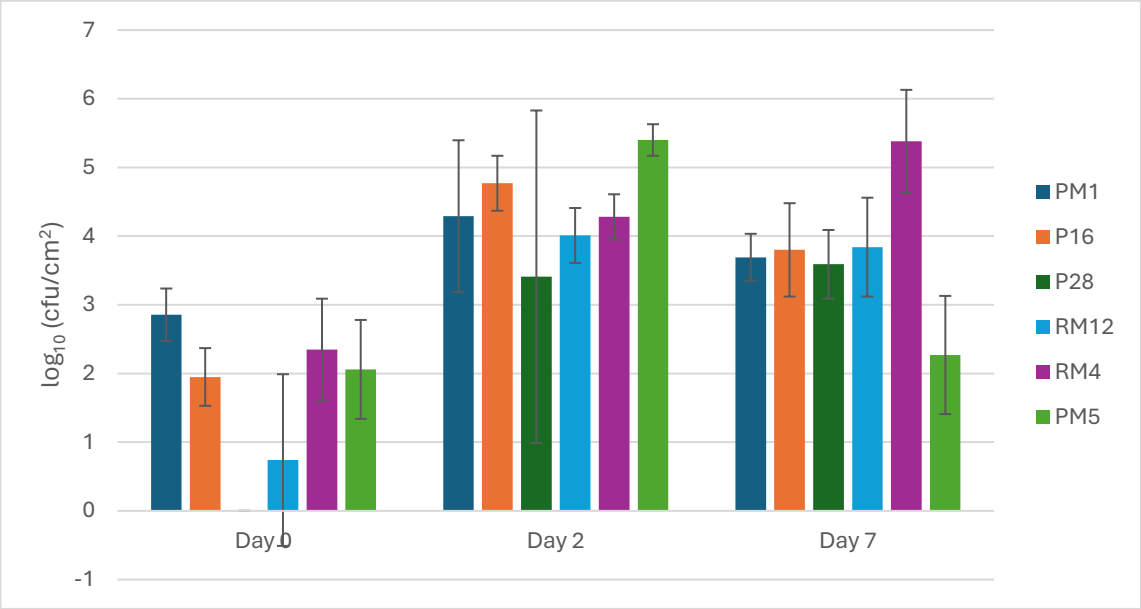
4.3. Biofilm formation assessment

The evaluation of biofilm formation of the microorganisms previously tested against disinfectants was carried out at 0, 2 and 7 days. By day 7, RM4, RM12, and P16 showed intensive biofilm formation activity.

Comparing the results with the literature, was found that *Kocuria salsicia* (RM4) has been studied for its biofilm formation ability and has showed intensive growth at different temperatures (Youn and Seo, 2022); *Macrococcus caseolyticus* (RM12) has also shown high biofilm-forming capacity in the dairy industry (Mnif et al., 2020); and *Micrococcus luteus* has been also subjected to studies showing high biofilm formation activity (Gupta et al., 2022) and the presence of virulent genes responsible for biofilm formation has been found in some strains (Al-Fahham and Motaweq, 2023).

The results of the biofilm formation of the isolates are shown graphically in Fig 9, where it can also be seen in some isolates a notable standard deviation, which can be attributed to the differences in the surfaces of the stainless steel slides in the parallels, since it was observed during the experiment that a few slides showed little scratches on the surface, which can enhance the microorganism capability of forming biofilm.

Fig 9. Biofilm formation of isolates on stainless steel



5. CONCLUSIONS AND SUGGESTIONS

5.1 Conclusions

5.1.1. MIC Values of Disinfectant on Isolates

- Initial testing at the manufacturer-recommended concentration of 3.2% sodium hypochlorite showed no inhibitory effect on the tested microorganisms.
- A tenfold increase in concentration to 32% sodium hypochlorite led to measurable inhibitory effects, with varying degrees of susceptibility observed among the isolates.
- Three Gram-positive microorganisms, RM4 (*Kocuria salsicia*), P16 (*Micrococcus luteus*), and RM12 (*Macrococcus caseolyticus*), exhibited higher sensitivity to sodium hypochlorite across all tested concentrations.
- Gram-negative bacteria PM5 (*Pseudomonas lundensis*), PM1 (*Buttiauxella gaviniae*), and P28 (*Pseudomonas antarctica*) demonstrated higher tolerance to sodium hypochlorite.
- Two isolates showed inhibition even at the lowest tested concentration of 8%, making it impossible to determine their MIC using this method.
- MIC values determined through Multiskan analysis aligned with agar disk diffusion results. The MIC for the most resistant isolates was 32%, while the most sensitive isolates had an MIC of 8% or 16%.
- The concentration recommended by the manufacturer of 3.2% sodium hypochlorite was insufficient to inhibit growth in most tested microorganisms isolated from vegetables, meat, and dairy products.
- The exact MIC value may lie between the tested concentrations due to the twofold dilution method used, providing a range rather than an exact value.

5.1.2. Biofilm Formation Assessment

- *Kocuria salsicia, Macrococcus caseolyticus* and *Micrococcus luteus* exhibited intensive biofilm formation by day 7.

5.2 Suggestions

5.2.1 MIC Testing and Disinfectant Use

- Consider increasing the concentration of sodium hypochlorite used in food industry sanitation to ensure effective disinfection, especially against Gram-negative bacteria that showed higher tolerance.
- Explore alternative disinfectants or combination treatments to enhance efficacy against resistant strains without compromising food safety.

5.2.2. Biofilm Control

- Incorporate biofilm control strategies into food processing to prevent microbial contamination and persistence.
- Investigate the use of biofilm-disrupting agents or technologies to enhance the effectiveness of disinfectants against biofilm-forming microorganisms.

5.2.3. Methodological Improvements

- Develop standardized methods for MIC determination specific to disinfectants, considering the limitations of current methodologies.
- Implement advanced analytical techniques or equipment for more precise MIC determination and resistance profiling.

5.2.4. Further Research

- Investigate the mechanisms of resistance in isolates showing high tolerance to sodium hypochlorite to understand the underlying causes and develop targeted control strategies.
- Assess the long-term effects of sublethal disinfectant exposure on microbial populations to understand the potential for resistance development over time.
- Test and compare efficiency of disinfection methods on biofilms.

5.3 Conclusion

Resistance to disinfectants, particularly sodium hypochlorite, poses challenges to food safety and quality. This study has provided valuable insights into the susceptibility profiles of microorganisms isolated from different food sources and their biofilm-forming capabilities. The findings highlight the need for optimization of disinfectant concentrations and strategies to control biofilm formation

in food industry settings. Further research and methodological improvements are essential to address the emerging issues of disinfectant resistance effectively.

6. CONCLUSIONS

The study aimed to assess the resistance of microorganisms isolated from food against sodium hypochlorite disinfectant and its effect on biofilms produced by these microorganisms. The importance of this study lies in the current problem of microorganisms resistant to antimicrobials, which lead to foodborne illnesses.

The primary objectives were to isolate and characterize microorganisms from raw milk to test them against a disinfectant and determine the minimum inhibitory concentration (MIC) of sodium hypochlorite against the strains isolated as well as microorganisms isolated from red pepper and pork meat. The biofilm formation capacity of the isolates was also studied on stainless steel slides at 30°C.

From the isolation of microorganisms from raw milk, 25 apparently different colonies were obtained, from which 10 of them were subjected to preliminary identification tests including oxidase, catalase and KOH test, followed by identification by MALDI-TOF MS obtaining the typical spoilage and pathogenic microbiota of milk.

Agar disk diffusion tests were conducted using disinfectant concentrations ranging from 3.2% to 64% to determine inhibitory effects. MIC values were obtained using both agar disk diffusion and broth microdilution assays for *Kocuria salsicia* and *Macrococcus caseolyticus* (isolated from raw milk); *Micrococcus luteus* and *Pseudomonas antarctica* (isolated from red pepper); *Buttiauxella gaviniae* and *Pseudomonas lundensis* (isolated from pork meat). Biofilm formation on stainless steel slides was assessed for selected isolates over a 7-day period at 30°C.

Among the main results obtained, it was found that there was no inhibitory effect for any strain at the initial testing at 3.2% concentration which is the manufacturer's recommendation of use; Gram-positive bacteria were generally more sensitive to the disinfectant than Gram-negative bacteria; MIC values ranged from 8% to 32% for the tested microorganisms; intensive biofilm-forming activity was shown in the case of *Kocuria salsicia, Micrococcus luteus,* and *Macrococcus caseolyticus.*

The study revealed varying degrees of resistance to sodium hypochlorite among microorganisms isolated from different food sources. Biofilm-forming capabilities were observed in selected isolates, emphasizing the importance of biofilm control strategies in food safety practices.

This thesis investigated the disinfectant resistance profiles of microorganisms isolated from food and their biofilm-forming capabilities. Through a series of tests and analyses, the study provided valuable insights into the efficacy of the disinfectant and highlighted the need for optimized disinfection strategies in food processing. Further research could be conducted to investigate biofilm susceptibility to disinfectants at different concentrations to have a better insight of the microorganisms in both planktonic and biofilm form. Nevertheless, the findings contribute to the understanding of microbial control in the food industry, aiming to enhance food safety measures and reduce the risk of foodborne illnesses.

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8. LIST OF TABLES AND FIGURES

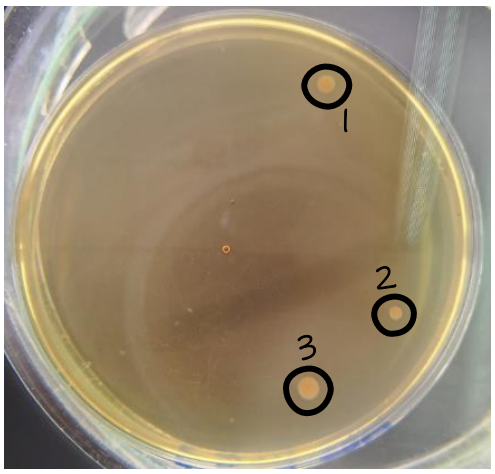
8.1. Tables

8.2. Figures

9. ANNEXES

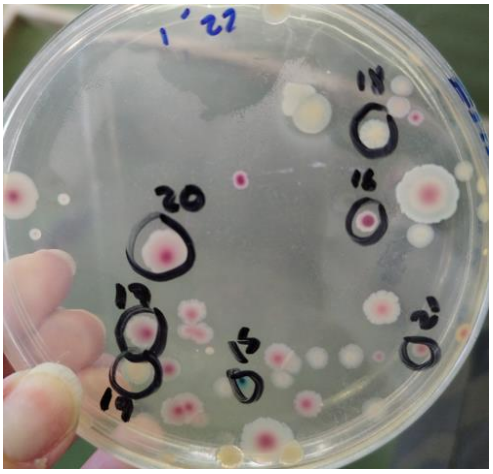
Annex 1: Data obtained during isolation and identification steps.

Annex 1.1: Selection of colonies.

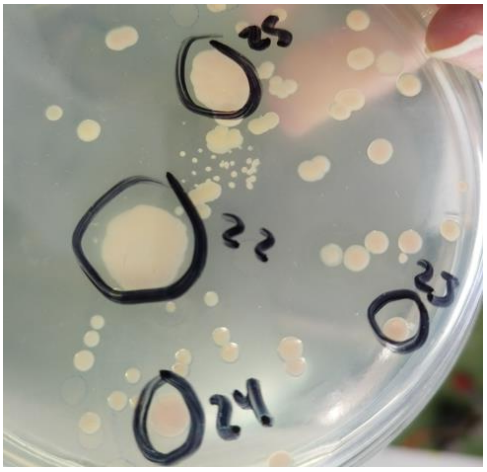


MRS: RM1 - RM3

TGE: RM4 - RM14

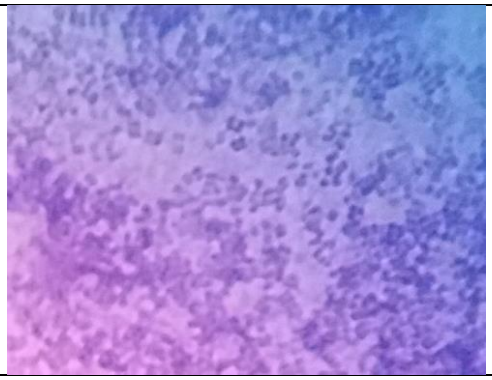
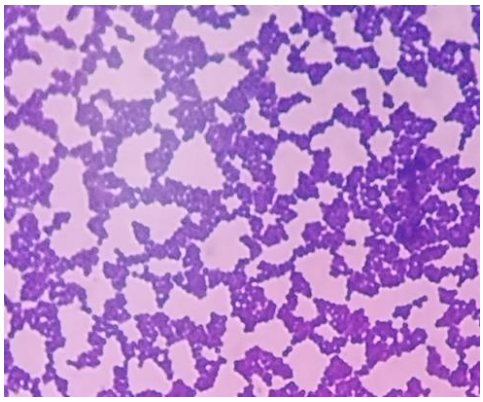
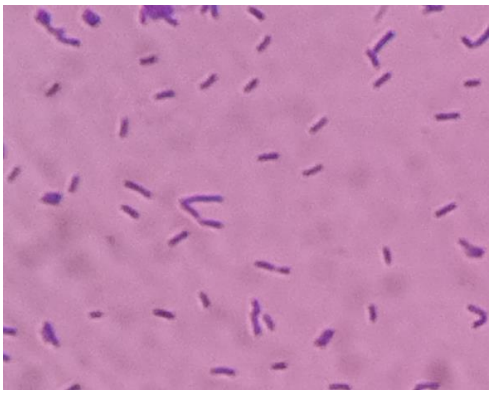


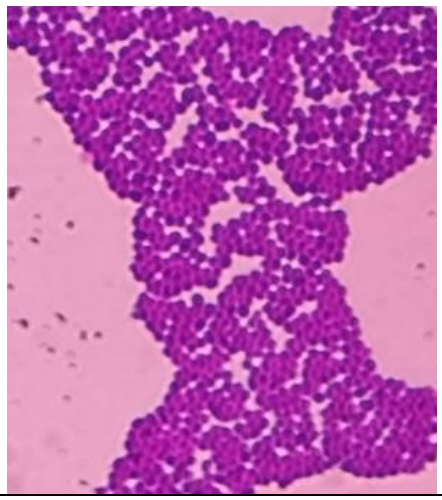
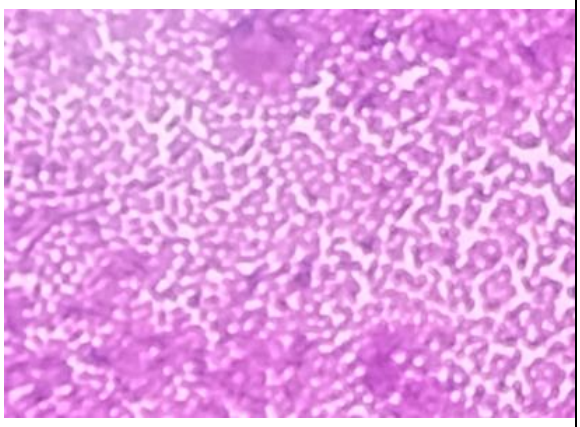
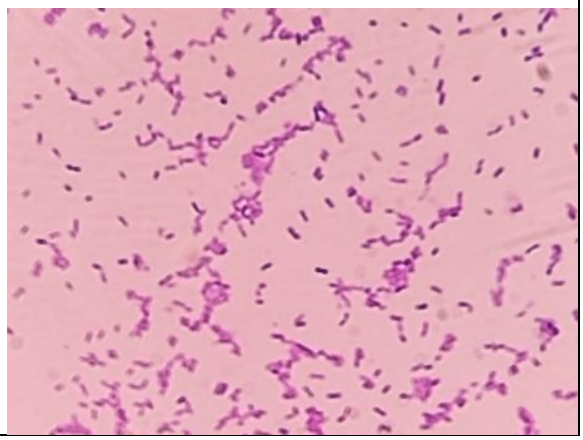
CHR: RM15 - RM21

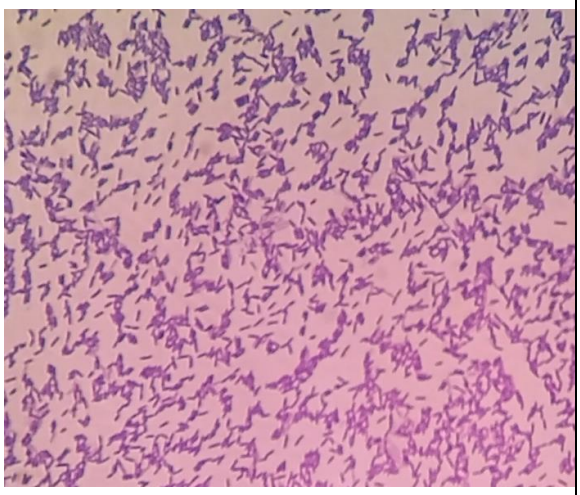
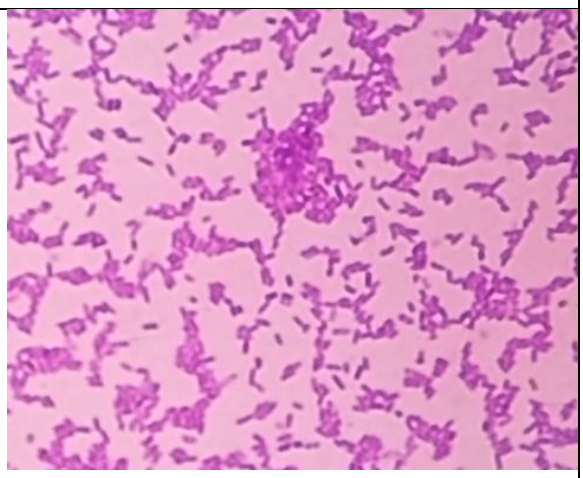
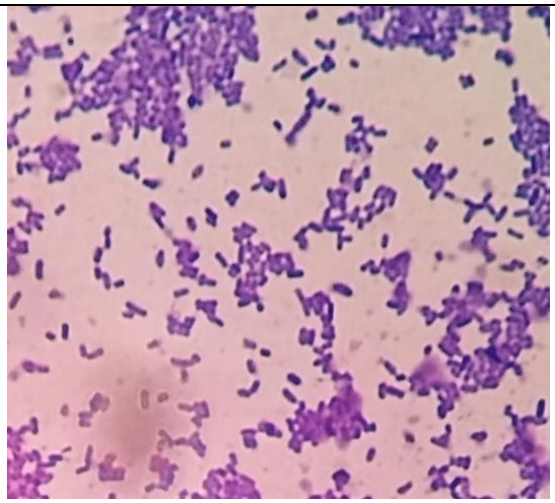


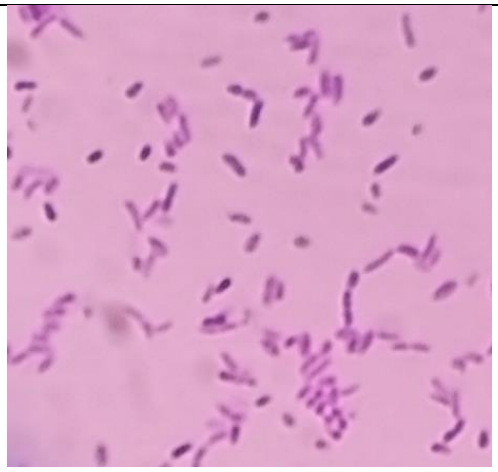
CET: RM22 - RM25

Annex 1.2: Investigation of microorganisms selected.

Code	Isolated from	Microscopic description	Catalase	Oxidase	Gram	
RM4	TGE	Cocci	Pos	Pos	Pos	
RM6	TGE	Cocci in clusters	Pos	Neg	Pos	
RM8	TGE	Rods	Pos	Neg	Neg	

RM12	TGE	Big cocci	Pos	Pos	Pos	
RM13	TGE	Short rods	Pos	Neg	Pos	
RM15	CHR	Rods	Pos	Neg	Neg	

RM19	CHR	Thin rods	Pos	Neg	Neg	
RM20	CHR	Rods	Pos	Neg	Neg	
RM21	CHR	Rods	Pos	Neg	Neg	

RM25	CET	Rods	Pos	Neg	Neg	

Annex 1.4: MALDI-TOF results for isolates from raw milk.

Sample Name	Sample ID	Organism (best match)	Score Value	Organism (second-best match)	Score Value
H6 (+++) (A)	RM20 (Standard)	Hafnia alvei	2.11	Hafnia alvei	1.98
C5 (+++) (A)	RM13 (Standard)	Acinetobacter johnsonii	2.17	Acinetobacter johnsonii	2.11
C6 (+) (B)	RM4 (Standard)	Kocuria salsicia	1.86	Kocuria tytonis	1.70
H9 (+) (B)	RM21 (Standard)	Pantoea agglomerans	1.70	**No Organism Identification Possible**	1.68
H10 (+) (B)	RM12 (Standard)	Macrococcus caseolyticus	1.71	**No Organism Identification Possible**	1.58
H11 (+++) (A)	RM19 (Standard)	Stenotrophomonas maltophilia	2.10	Stenotrophomonas maltophilia	2.00
H12 (+) (B)	RM15 (Standard)	Buttiauxella noackiae	1.71	**No Organism Identification Possible**	1.47
H5 (+++) (A)	RM6 (Standard)	Staphylococcus chromogenes	2.41	Staphylococcus chromogenes	1.92

Annex 1.4.1: MALDI-TOF results for isolates from pepper (P) and pork meat (PM).

Sample Name	Sample ID	Organism (best match)	Score Value	Organism (second-best match)	Score Value
D12 (+++) (B)	P2 (Standard)	Pseudomonas extremorientalis	2.09	Pseudomonas azotoformans	2.07
E4 (+++) (A)	P6 (Standard)	Brachybacterium conglomeratum	2.15	Brachybacterium paraconglomeratum	1.79
E5 (+) (B)	P7 (Standard)	Pseudomonas oryzihabitans	1.92	Pseudomonas oryzihabitans	1.91
E7 (+) (B)	P9 (Standard)	Bacillus cereus	1.87	Bacillus mycoides	1.85
E8 (+++) (A)	P10 (Standard)	Bacillus cereus	2.14	Bacillus cereus	2.00
E10 (+++) (A)	P12 (Standard)	Staphylococcus hominis	2.18	Staphylococcus hominis	2.12
F2 (+++) (A)	P16 (Standard)	Micrococcus luteus	2.19	Micrococcus luteus	2.13
F3 (+++) (A)	P17 (Standard)	Moraxella osloensis	2.12	Moraxella osloensis	1.98
F5 (+) (B)	P19 (Standard)	Pluralibacter pyrinus	1.86	Pluralibacter pyrinus	1.79
C7 (+) (B)	P21 (Standard)	Pseudomonas flavescens	1.89	**No Organism Identification Possible**	1.58
C12 (+) (B)	P26 (Standard)	Microbacterium arborescens	1.99	Microbacterium arborescens	1.94
D2 (+) (B)	P28 (Standard)	Pseudomonas antarctica	1.98	Pseudomonas marginalis	1.90
D3 (+) (B)	P29 (Standard)	Pseudomonas fulva	1.96	Pseudomonas fulva	1.76
D4 (+) (B)	P30 (Standard)	Pseudomonas oryzihabitans	1.72	Pseudomonas oryzihabitans	1.70
D6 (+++) (A)	P32 (Standard)	Bacillus cereus	2.00	Bacillus cereus	1.84
D7 (+) (B)	P33 (Standard)	Microbacterium arborescens	1.70	**No Organism Identification Possible**	1.47
F7 (+++) (A)	PM1 (Standard)	Buttiauxella gaviniae	2.12	Buttiauxella ferragutiae	1.90

F8 (+++) (B)	PM2 (Standard)	Aeromonas sp[2]	2.19	Aeromonas eucrenophila	2.12
F10 (+) (B)	PM3 (Standard)	Kocuria salsicia	1.82	**No Organism Identification Possible**	1.55
F11 (+) (B)	PM5 (Standard)	Pseudomonas lundensis	1.93	Pseudomonas fragi	1.83
F12 (+) (B)	PM6 (Standard)	Pseudomonas fluorescens	1.84	Pseudomonas azotoformans	1.76

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Title of the document:	Resistance of foodborne microorganisms against disinfectants
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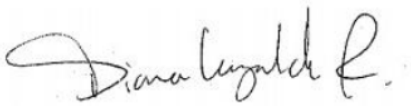

Student's signature

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