Evaluation of moist olive pomace as an antimicrobial agent envisaging leather treatments

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Thesis submitted to
Escola Superior de Tecnologia e Gestão Instituto Politécnico de Bragança

Master Degree in

Chemical Engineering

within the scope of the double diploma with

University of Saida

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Bragança

May 2023

ACKNOWLEDGEMENT

I would like to express my deepest gratitude to the following individuals and organizations who have played a significant role in the completion of this dissertation.

First and foremost, I am immensely grateful to my supervisors, **Dr. Pedro Jorge Louro Crugeira and Prof. Dr. Maria Filomena F. Barreiro**, for their guidance, support, and valuable insights throughout this research journey. Their expertise, encouragement, and unwavering commitment to excellence have been instrumental in shaping the direction of my work.

I would like to extend my heartfelt appreciation to the faculty members of the Polytechnic Institute of Braganca, whose knowledge and expertise have enriched my academic experience. Their guidance, encouragement, and constructive feedback have been invaluable in shaping the development of my research.

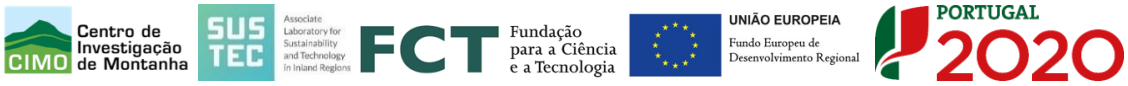
I am grateful to the staff and researchers at CIMO lab, particularly **Heloísa Helena Scorsato de Almeida, Maria João Afonso**, for their assistance, collaboration, and access to resources. Their contribution has greatly facilitated my experiments and data analysis, enabling me to conduct thorough and meaningful research.

I would also like to acknowledge the participants of my study, whose willingness to be involved and share their insights have been vital to the success of this research. Their time and contribution are deeply appreciated.

Finally, I would like to express my gratitude to the Erasmus scholarship program, whose financial support made this research possible. Their investment in my work has allowed me to pursue this study with dedication and passion.

In conclusion, the completion of this dissertation would not have been possible without the support, guidance, and encouragement of the individuals and organizations mentioned above. Their contributions have left an indelible mark on my academic and personal growth, and for that, I am truly grateful.

Foundation for Science and Technology (FCT, Portugal) for financial support through national funds FCT/MCTES (PIDDAC) to CIMO (UIDB/00690/2020 and UIDP/00690/2020), and SusTEC (LA/P/0007/2020). Project RARISS – Raças Autóctones, Rastreabilidade, Inovação e Soluções Sustentáveis (POCI-01-0247-FEDER-069810).



Khadidja Kebaili

DEDICATIONS

To my beloved parents, late grandmother, cherished sisters Maissa, Sara, Yasmine, Marwa and Abir and my loyal friend Assala, and my dear close family and friends,

This dissertation is dedicated to all of you, who have played significant roles in my life and academic journey. Your unwavering support, encouragement, and love have been the foundation of my success. To my parents, your guidance and belief in me have been my guiding light. To the memory of my late grandmother, your wisdom continues to inspire me. To my sisters, your constant presence and unwavering support have kept me going. To my friend Assala, your friendship has been a source of strength and inspiration. To my close family and friends, your encouragement and belief in my abilities have been invaluable.

I am profoundly grateful for the love, guidance, and encouragement I have received from each of you. Your presence in my life has made all the difference, and I am honored to have you by my side on this journey.

With deepest appreciation,

Khadidja.

ABSTRACT

The leather industry faces significant losses due to the impact of antimicrobial resistance on leather products, leading to issues like discoloration, unpleasant odors, and decreased performance. Antimicrobial resistance is a complex problem with implications for public health and various sectors of society, associated with increased morbidity and mortality. The olive oil industry discards valuable compounds found in moist olive pomace, including bioactive polyphenols, presenting an opportunity to utilize them as green, safe, and natural antimicrobial agents. This study aimed to assess the antimicrobial capacity of the moist olive pomace (MOP) and its potential application on leather specimens. The research involved the extraction of three extracts using different concentrations of pasteurized MOP with water as the solvent. The minimum inhibitory concentration against *Escherichia coli* ATCC 8739 and *Staphylococcus aureus* ATCC 6538 were determined through microdilution with a colorimetric assay (INT). The study also quantified the effective microbial reductions achieved with promising MOP concentrations and evaluated the antimicrobial activity of the most effective MOP concentration on leather specimens. By investigating the antimicrobial potential of MOP against *E. coli* and *S. aureus*, this research aimed to introduce this waste product into the sustainable production chain while improving extraction techniques and exploring its applications in the leather industry. This study investigated the effect of different extracts of moist olive pomace (MOP) on the total phenolic compounds (TPC) content. The dried olive pomace was found to have a TPC of approximately 18315 mg.kg-1 GAE (gallic acid equivalent). The main compound identified was oleuropein, which accounted for around 78% of the TPC, followed by hydroxytyrosol and tyrosol, contributing about 19% of TPC. The extraction of MOP was conducted under different concentrations (128, 31.3, and 24 mg/mL) and optimized conditions (45°C and 180 min), using water as the extraction solvent. The extraction yields obtained were 29.5%, 64%, and 83% for the ratios of 1:5, 1:15, and 1:20 w/v respectively. The extracts' antimicrobial effects on Gram-negative bacteria (*Escherichia coli*) and Gram-positive bacteria (*Staphylococcus aureus*) were evaluated using the minimum inhibitory concentration determined by the microdilution technique. The results showed that both bacteria were inhibited at a concentration of 31.3 mg/ml of MOP extract. Furthermore, the MOP extract at 128 mg/ml concentration demonstrated a maximum microbial reduction of 90.22% for *S. aureus* and 85.45% for *E. coli*. Therefore, this concentration was considered the most effective MOP concentration. The antimicrobial activity of the MOP extract at the aforementioned concentration was also evaluated on leather specimens. It was found to exhibit bactericidal activity against both *E. coli* and *S. aureus*. Overall, these findings highlight the potential antimicrobial properties of MOP extracts and their potential application in various fields, including the leather industry.

KEYWORDS:

leather industry, Antimicrobial resistance, olive oil industry, moist olive pomace (MOP), bioactive polyphenols, water, *Staphylococcus aureus*, *Escherichia coli*.

RESUMO

A indústria do couro enfrenta perdas significativas devido ao impacto da resistência antimicrobiana nos seus produtos, levando a problemas como descoloração, odores desagradáveis e diminuição do desempenho. A resistência antimicrobiana é um problema complexo com implicações para a saúde pública e para vários sectores da sociedade, estando associado a um aumento da morbilidade e da mortalidade. A indústria do azeite descarta compostos valiosos existentes no bagaço de azeitona húmido, incluindo polifenóis bioactivos, que possuem potencial de utilização como agentes antimicrobianos ecológicos, seguros e naturais. Este estudo teve como objetivo avaliar a capacidade antimicrobiana do bagaço de azeitona húmido (MOP) e a sua potencial aplicação no tratamento do couro. A investigação envolveu a extração de três extratos a partir de diferentes concentrações de MOP pasteurizada utilizando água como solvente. A concentração inibitória mínima contra a *Escherichia coli* ATCC 8739 e *Staphylococcus aureus* ATCC 6538 foi determinada através do método de microdiluição com um ensaio colorimétrico (INT). Nesse estudo quantificou-se ainda as reduções microbianas efetivas alcançadas com as concentrações mais promissoras de MOP e avaliou-se a atividade antimicrobiana para a concentração mais eficaz de MOP em amostras de couro. Ao investigar o potencial antimicrobiano da MOP contra *E. coli* e *S. aureus*, visou-se introduzir este resíduo na cadeia de produção sustentável, melhorando simultaneamente as técnicas de extração e avaliando a sua aplicação na indústria do couro. Neste estudo, investigou-se o efeito de diferentes extratos de bagaço de azeitona húmido (MOP) no teor de compostos fenólicos totais (TPC). Verificou-se que o bagaço de azeitona seco tinha um TPC de aproximadamente 18315 mg.kg-1 GAE (equivalente de ácido gálico). O principal composto identificado foi a oleuropeína, que representou cerca de 78% do TPC, seguida do hidroxitirosol e do tirosol, correspondendo a 19% do TPC. A extração do MOP foi realizada em diferentes concentrações (128, 31,3 e 24 mg/mL) e em condições otimizadas (45°C e 180 min), utilizando água como solvente de extração. Os rendimentos de extração obtidos foram de 29,5%, 64% e 83% para as proporções de 1:5, 1:15 e 1:20 w/v respetivamente. Os efeitos antimicrobianos dos extratos em bactérias Gram-negativas (*Escherichia coli*) e Gram-positivas (*Staphylococcus aureus*) foram avaliados utilizando a concentração inibitória mínima determinada pela técnica de microdiluição. Os resultados mostraram que para uma concentração de 31,3 mg/ml de extrato de MOP, ambas as bactérias foram inibidas. Adicionalmente, o extracto de MOP a uma concentração de 128 mg/ml demonstrou uma redução microbiana máxima de 90,22% para *S. aureus* e 85,45% para *E. coli*. Assim, esta concentração foi considerada a concentração de MOP mais eficaz. A atividade antimicrobiana do extrato de MOP na concentração acima mencionada foi também avaliada em amostras de couro. Verificou-se que apresenta uma atividade bactericida contra *E. coli* e *S. aureus*. Em geral, estes resultados realçam as propriedades antimicrobianas dos extratos de MOP e a sua potencial aplicação em vários campos, incluindo a indústria do couro.

PALAVRAS-CHAVE:

Indústria do couro, resistência antimicrobiana, indústria do azeite, bagaço de azeitona húmido (MOP), polifenóis bioactivos, água, *Staphylococcus aureus*, *Escherichia coli*.

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List of abbreviations

MOP	Moist olive pomace
PCMC	P-chloro-m-cresol
OPP	O-phenylphenol
OIT	Octylisothiazolinone
TCMTB	2-(Thiocyanomethylthio) benzothiazole
DNA	Deoxyribonucleic acid
EPS	Exopolysaccharide
RNA	Ribonucleic acid
EGCG	Epigallocatechingallate
MDR	Multidrug-resistant
MRSA	Methicillin resistant *staphylococcus aureus*
EU	European union
OMWW	Olive mill wastewater
OP	Olive pomace
IUPAC	International Union of Pure and Applied Chemistry
3,4-DOPET	3,4- Dihydroxyphenylethanol
3,4-DHPEA	3,4-dihydroxyphenolethanol
BHT	Butylatedhydroxytoluene
PVN	Paraventricular nucleus
CD14	Cluster of differentiation 14
HDL	High-density lipoproteins
LDL	Low-density lipoprotein
HPLC	High-Performance Liquid Chromatography
MeOH	methanol
DAD	Diode Array Detector
BHI	Brain heart infusion
MIC	Minimum Inhibitory concentration
CLSI	Clinical and Laboratory Standards Institute
INT	P-iodonitrotetrazolium
CFU	Colony forming units
TPC	total phenolic compounds
GAE	gallic acid equivalent

1. Motivation and Objectives

The quality of the hides obtained after treatment depends on factors ranging from the conditions to which the animal was subjected throughout its life to those used in the industrial process. After slaughter, it is essential to adopt skin conservation procedures to avoid microbial growth, whose presence results in its degradation and consequent loss of properties (Bielak et al., 2020). The high propensity of the skin to support microbial growth is related to the presence of nutrients such as carbohydrates, fats, and proteins, which, together with the high moisture content (greater than 50%), temperature, and pH, guarantee optimal conditions for their development. Microbial growth causes biodeterioration of the leather, resulting in modifications at the level of its surface, diminishing its physical-mechanical properties, and may originate the formation of stains that damage appearance, resulting in a decrease in the quality of the final product and, consequently, a loss of its commercial value and significant losses to the leather industry, which is one of the most active, competitive, and important sectors of the Portuguese economy, accounting for 3% of the national GDP (Costa et al., 2020).

Thus, it is important to develop appropriate conservation procedures with high effectiveness in inhibiting microbial growth to ensure the integrity of the skin both in the initial phase before its processing (from the removal of the carcass) and throughout the shelf life of the final product (Deselnicu & Chirilă, 2018)(Muthusubramanian & Mitra, 2006). Regarding the different microorganisms that can grow on leather, bacterial attack mainly occurs along the stream stages (pre-tanning stage). At the same time, the predominance of fungal contamination is noted from tanning and later stages. The bacterial strains most found in the riffle stage are *Bacillus subtilis, Escherichia coli, Micrococcus spp., Proteus vulgaris, Pseudomonas aeruginosa*, and *Streptomyces sp.,* among others (Orlita, 2004).

Antimicrobial resistance has been declared a global problem, and the World Health Organization updated the Global Report on Antimicrobial Resistance Surveillance in 2015, highlighting that it is a growing threat to public health and a concern in various sectors of society. Antimicrobial resistance has been linked to increased morbidity, mortality, and costs, owing to the risk of loss of efficacy and options in this class of drugs (Davies, 1996; World Health Organization & Anti-Infective Drug Resistance Surveillance and Containment Team., 2001). Microbial colonization capacity and pathogenicity are virulence factors; genomes resist standard antibiotics, implying diverse variables and strategies for evading host defenses

(Sydnor & Perl, 2011). Aside from the ease of multiplication and dissemination, microorganisms produce molecules with high pathogenic power, including enzymes and toxins such as beta-lactamases, coagulases, hyaluronidases, catalases, DNAses, lipases, proteases, and esterases (Luis dos Santos et al., 2007). However, the ability to survive inadequate environmental conditions and to frequently act in quorum sensing forms biofilms with higher antimicrobial resistance (Kokare et al., 2009).

The hunt for antibacterial qualities in plants and their by-products has grown in response to the rise in antimicrobial resistance. The olive oil industry is one of the sectors that produces a lot of waste and by-products. Olive oil extraction units have been reconstructed for environmental and technological reasons, with traditional methods being replaced by centrifugation systems with continuous two-phase extraction. Recalcitrant residual by-products with poor economic value, high moisture content and viscosity, and difficulty transporting, storing, and drying (Medeiros et al., 2016). The most common and important by-product in the olive oil industry today is the moist olive pomace (MOP) (Dermeche et al., 2013); however, MOP is phytotoxic, non-biodegradable, and challenging to remediate due to its high moisture content and rich organic component makeup (Lama-Muñoz et al., 2012). Furthermore, lipids may significantly impact the soil's physical and biochemical characteristics related to fertility, inorganic N immobilization, and plant growth (Riffaldi et al., 1993). The olive pomace is a promising source for the extraction of hydroxytyrosol, tyrosol, and oleuropein, called "Biophenols," a diverse group of secondary metabolites derived from the shikimate pathway and phenylpropanoid metabolism. Polyphenols in olive fruit are highly significant among hydrosoluble compounds due to their broad range of biochemical and pharmaceutical effects, including anticarcinogenic, antiatherogenic, and antimicrobial properties (Bravo, 1998). The amount, diversity, and characteristics of the phenolic compounds present in MOP (catechol, 4-methyl catechol, hydroxytyrosol, tyrosol, oleuropein, p-coumaric and other flavonoids) may interact in the antimicrobial capacity, acting together and synergistically influencing its bioaction efficiency (Gullón et al., 2020; Lesage-Meessen et al., 2001).

1.1 Objectives

1.1.1 General objective

Evaluate the antimicrobial capacity of MOP and test its potential on leather specimens to introduce this waste into the sustainable production chain.

1.1.2 Specific objectives

- Optimize the extraction of three extracts of MOP. The extracts were prepared with the following sample-to-solvent ratios: 1:5 (w/v), 1:15 (w/v), and 1:20 (w/v).
- Determine the minimum Inhibitory concentration of pasteurised MOP against *Escherichia coli* ATCC 8739, *Staphylococcus aureus* ATCC 6538.
- Quantify the effective microbial reductions achieved at the promising MOP concentrations.
- Evaluate the antimicrobial activity of the best MOP concentrations on leather specimens.

Chapter 2 Bibliographic review

2.1 Biocides for the leather industry

2.1.1 Leather industry

The principal aim of the leather industry, which plays a significant role in today's global economy, is to transform animal hides and skins into a physically and chemically stable material by subjecting them to chemical and mechanical sequential processes and, therefore, to obtain products for meeting various needs of people. The leather industry generally uses hides and skins as raw materials, which are by-products of the meat and meat products industry (H. Ozgunay et al., 2009).

The processing of skins or hides into stable materials like leather is known as tanning. Leather is the primary product from the tanning industry, which has applications in making footwear, furniture, bags, etc. The global leather industry generated about 18 billion square feet of leather in 2003, with an estimated price of US$40 billion. Tanning is considered a major process involving chemical reactions and mechanical operations. Chemicals, including chromium, acid solvents, etc., are used (Sai Bhavya et al., 2019).

2.1.2 Leather industry problems

Leather products have been widely used in our daily lives since ancient times. However, they are quickly attacked by microbes, thus resulting in many adverse and undesirable problems, such as discoloration, and unpleasant odor, resulting in lower performance. Employing antimicrobial polymer coatings on a leather surface is a very effective strategy to address biofouling (Xiaobo et al., 2021). However, the leather industry is commonly associated with high pollution due to the foul smell, organic waste, and high-water consumption caused by traditional manufacturing processes. Different forms of waste in quality and quantity that emerge during the transformation of hides and skins into leather in thousands of leather factories, from primitive to modern, all around the world, negatively impact the environment (H. Ozgunay et al., 2009).

These environmental consequences, which must be considered in any tannery, include the load and concentration of traditional pollutants and using specific chemicals like biocides, surfactants, and organic solvents. Additionally, unintentional releases, spills, and leaks of

specific agents and handling effluents and wastes may result in soil and groundwater contamination.

2.1.3 Role of biocides in the leather industry

Microbial activities are responsible for the further degradation of organic substances in nature, leading to undesirable consequences. Specific care must be exercised to avoid such actions in some industrially valued organic products, such as leather. Under microbial attack, leather changes its characteristics, which in turn causes tremendous losses in its economic value (BAYRAMOGLU, 2007).

The perfect technique to prevent microbial degradation and sterilize materials from the textile and leather industries is to use chemicals with antimicrobial properties. These substances are called biocides and are designed to combat, neutralize, and exert control over any harmful organism by chemical or biological means (Coman et al., 2011). However, biocides are essential to maintaining the materials hygienically by destroying molds, microbes, and pests (Roman et al., 2013).

2.2 Biocides types

Biocides are compounds broadly used to protect products and equipment against microbiological damage (Arzani & dos Santos, 2022).

Protect against microbial damage is achieved by applying biocides, substances intended to destroy or control harmful organisms. In the leather industry, biocides usually refer to bactericides and fungicides. Some biocidal substances have both functionalities (W. Zhang, 2020).

2.2.1 Fungicides

Fungicides are used in leather industry production to protect semi-processed and finished leather against mold growth during storage and shipping. These chemicals are essential in leather manufacture to ensure that the leather is not degraded by mold growth and to safeguard the leather’s quality, usefulness, and commercial value (Dalton, 2012).

Due to the procedures' higher pH levels, which are more favorable to mold growth, they are frequently used from pickling until drying. Fungicides must be included in pickled

sheepskins to prevent mold growth while being transported and stored (Sri Bala Kameswari et al., 2016).

2.2.2 Bactericides

In the leather industry, it is important to use bactericides to prevent damage to the materials; commercial bactericides that are widely used in the leather industry contain 7–25% phenol (Bayramoglu, 2006).

2.3 Biocides typically used in the leather industry

Biocides are antimicrobial chemical agents used heavily in domestic, industrial, and healthcare environments for disinfection (Jones & Joshi, 2021). Global leather existed since technologists were concerned about inflicting stability on raw skins and hides. Until the advent of chrome tanning, there were few options for tanners, such as aluminum tanning, smoke tanning, oil tanning, and vegetable tanning. Today, chrome tanning is the most commonly used method, which accounts for the world's leather production. Although chrome tanning has many advantages like high speed, low cost, good hide storage, etc., 40% of the chromium remains in the effluent, ending in the sludge. One of the major problems in the leather industry is chromium disposal, where the treatment results in chromium-contaminated sludge. Due to these disadvantages of chrome tanning, tanners are encouraged to use the eco-friendly process of vegetable tanning (Skwarek et al., 2021). However, tanned wet leather contains several components, such as ammonium salts and fatliquoring agents, that enable fungal growth, causing damage to the leather. Currently, many formulated biocide products are sold commercially, containing various biocides. The following substances: p-chloro-m-cresol (PCMC), o-phenylphenol (OPP), octylisothiazolinone (OIT), and 2-(thiocyanomethylthio) benzothiazole (TCMTB) account for 95% of the active substance in those commercial products. In the leather industry, fungicides represent about 15% of the cost of chemicals in wet blue processing (W. Zhang, 2020).

2.4 Environmental impact of the leather industry

The leather industry plays a major role in the global economy, generating up to 5.4 million tons of solid and liquid waste annually. Moreover, about 75–80% of solid waste arises from processing raw hides into leather (Skwarek et al., 2021). However, the demand for leather and leather products is ever-increasing and, independent of supply, make the leather industry

one of the largest polluters worldwide because of the complex nature of their wastewater (Saxena et al., 2017).

The environmental impact of tannery wastes containing wastewater, hazardous chemicals such as chromium, synthetic tannins, oils, resins, biocides, and detergents, and careless disposal of solid wastes and gaseous emissions creates a negative image of the leather industry. However, it has a significant economic influence (Dixit et al., 2015).

The environmental impact of the tanning industry is generally significant with its waste outputs, i.e., high concentrations of organics, salts, and heavy metals (chromium compounds), both in solid and liquid form (Nazer et al., 2006).

2.4.1 Chrome

Chromium (VI) is one of the heavy metals in water and wastewater with the most toxic characteristics. Consequently, it harms human and environmental health (Ozdemir et al., 2005).

In some tanneries, a considerable quantity of organic pollutants, including 70% chrome (III), is usually discharged, which leads to heavy metal contamination in the water. Chromium release by tanneries is now higher than the mentioned parameters because chromium is a major heavy metal exploited in the leather industry (Sai Bhavya et al., 2019).

Chrome tanning remains the most popular technology in the leather industry worldwide due to its ability to produce leather with attributes desirable for high-quality leather, such as excellent hydrothermal stability, better dyeing characteristics, and softness. Nevertheless, the technology has been censured globally for its environmental detriments and adverse effects on human health and other organisms. Developing alternative eco-friendly tanning technologies capable of producing leather of high quality has remained a challenging scientific inquiry (China et al., 2020).

2.4.2 Tannins

In the leather industry, the art of tanning, i.e., converting animal hide or skin to leather, is considered the first manufacturing process. Aqueous solutions or infusions that contain tannin (known as tan liquors) from plant extract are colloidal in nature with a wide range of particle sizes. They are a mixture of polyphenols and high-molecular-weight compounds.

Tannin reds have been used in the leather processing industries to get better quality leather (Sri Bala Kameswari et al., 2016).

The leather Industry is one of the most polluting industries it is naturally water-intensive. It uses large quantities of chemicals and has a high pollution load in the form of effluents containing chromium and synthetic tannin biocides (Muhammad Qasim et al., 2015).

2.5 Alternative solutions for the leather industry

The leather industry deals with proteinous skin materials for the conversion of leather. It generates vast amounts of solid and liquid waste, giving rise to pollution that needs to be overcome by introducing sustainable, cleaner technologies (Kanagaraj et al., 2015). Getting a proper method of protection for leather against mold during production is very important; it depends on the tanning agents, oils, oil emulsions, fats, and other auxiliary substances (Falkiewicz-Dulik, 2020).

In this way, the leather industry is making significant efforts to apply cleaner processes while substituting chemical products for natural products and searching for alternatives to chrome tanning, such as organic tanning consisting of phenolic synthetic products, aluminum salts, and vegetable tanning. Phenolic acids include vanillic, ferulic, gallic, or caffeic monomeric units, which can confer value added to the leather when polymeric units are generated from these original monomers (Solé et al., 2021).

2.6 Antimicrobial resistance-related issues

Antibiotic resistance has sharply developed over the last few years (Livermore, 2009) and is now acknowledged as a significant medical concern in most healthcare settings. The burden of infectious diseases is increased by the significant mortality attributable to resistance (De Kracker MEA et al., 2011). Resistance is not recent; resistance genes are distributed all over the natural world and interact with a complex ecosystem (Rolain et al., 2012). Antibacterial resistance was barely noticed in the past as novel antibiotics gradually became accessible and were easily changed and enhanced for therapeutic use, even though intensive usage of antimicrobial medications started to exert new survival pressure on important microorganisms. Several significant variables have tipped the scales in favor of the formation and unchecked spread of resistance with the growth of society and technology over the past few decades (Rolain et al., 2012).

The evolution of antibiotic-resistant bacteria can happen in both hospital and community settings. According to Neidell et al. (Neidell et al., 2012), community-associated infections have been a significant cause of patient morbidity and mortality. A potentially fatal public health issue is the rise of nosocomial or healthcare-associated infections. Only in the USA do antibiotic-resistant bacteria like methicillin-resistant *Staphylococcus aureus* cause around 19,000 deaths annually (Pan et al., 2010). These infections affect a large number of individuals worldwide. A few often-found resistant bacteria are included in **Table 1**.

Table 1. Antibiotic-resistance of bacteria commonly associated with infections in hospitals and the community.

Bacteria	Examples of typical diseases	Resistant to	Mechanisms of resistance	Ref
Escherichia coli	Urinary tract infections, blood stream infections	3rd gen. cephalosporins, Fluoroquinolones	Over expression of a TEM or ampC b-lactamase.	(Laura J. V. Piddock et al., 1997)
Staphylococcus aureus	Wound infections, blood stream infections	Oxacillin, vancomycin, linezolid	Alteration of target sites in cell wall of *Staphylococcus aureus*	(Tenover, 2006)
Streptococcus pneumoniae	otitis Diarrhea ("bacillary dysenteria")	non-susceptible or resistant to penicillin.	Alteration of penicillin-binding proteins (PBPs).	(Tarasí et al., 1999)
Neisseria gonorrhoea ***Vancomycin-resistant***	Gonorrhea Urinary tract infections, wound infections and intraabdominal infections	3rd gen. cephalosporins Vancomycin.	Chromosomal mutations. Over expression of VAN A and VAN B.	(Barry & Klausner, 2009; Gold, 2001)

2.7 Mechanisms of antimicrobial resistance

Bacteria can develop resistance to antibacterial drugs via a variety of processes. Some bacteria are naturally resistant to one class of antimicrobial agents. All strains of that bacterial species are then resistant to all members of those antimicrobial classes. Cases of acquired resistance, in which previously susceptible populations of bacteria become resistant to an antibacterial agent and multiply and spread under the selection pressure of that agent's usage, are of growing concern (C Reygaert, 2018).

2.7.1 Enzymes production

Antimicrobial resistance mechanisms are easily transmissible among a broad range of bacterial genera. For instance, an organism may acquire enzymes, such as beta-lactamases, to break down the antibacterial drug, reducing its effectiveness. Additionally, bacteria can develop efflux pumps, which expel antibacterial agents from the cells before they can reach their

intended target. Furthermore, bacteria can acquire multiple genes that empower them to produce modified cell walls, preventing the antimicrobial agent from binding to them. Alternatively, bacteria may gain mutations that diminish the accessibility of the agent to the intracellular target site, caused by the downregulation of porin genes. This has been documented in several studies, including the one by (Vila et al., 1993).

2.7.2 Genetic modification

Ordinarily, susceptible bacterial populations can become resistant to antimicrobial agents through mutation and selection or by acquiring resistance-encoding genetic information from other bacteria. The last event might occur through one of the various genetic pathways, including transformation, conjugation, or transduction. Many bacteria have become resistant to multiple antibacterial agents through genetic exchange mechanisms. These multidrug-resistant bacteria (defined as resistance to three antibacterial drug classes) have become a serious concern, particularly in hospitals and other healthcare institutions where they are most common (Rice & Bonomo, 2011).

As previously stated, susceptible bacteria can develop resistance to an antimicrobial agent through novel mutations. Such spontaneous mutations may result in resistance by (1) changing the target protein to which the antibacterial agent binds by modifying or eliminating the binding site (e.g., change in penicillin-binding protein 2b in pneumococci, resulting in penicillin resistance), or (2) increasing the production of enzymes that inactivate the antimicrobial agent (e.g., erythromycin ribosomal methylase in staphylococci), (3) downregulating or modifying an outer membrane protein channel required for drug entry (e.g., OmpF in *E. coli*), or (4) upregulating pumps that expel the drug from the cell (fluoroquinolone efflux in *S. aureus*). In all these circumstances, antimicrobial usage selects strains of bacteria with resistance-conferring mutations, killing susceptible strains while allowing newly resistant strains to live and develop. Vertical evolution refers to acquired resistance that occurs due to chromosomal mutation and selection (Mcmanus, 1997; Rice & Bonomo, 2011).

Bacteria gain resistance by acquiring additional genetic material from other resistant species. Horizontal evolution can occur across strains of the same species and between distinct bacterial species or genera. Conjugation, transduction, and transformation are examples of genetic exchange mechanisms. Transposons may aid in transferring and integrating acquired resistance genes into the host's genome or plasmids in each stage. During conjugation, gram-negative bacteria transmit plasmid-containing resistance genes to a neighbouring bacterium,

frequently through an extended proteinaceous structure called a pilus that connects the two organisms. Resistance genes are transmitted from one bacterium to another by a bacteriophage (bacterial virus) during transduction. This is currently regarded as a very uncommon occurrence. Finally, transformation, the process by which bacteria acquire and incorporate DNA segments from other bacteria that have released their DNA complement into the environment afterward cell lysis, has the potential to move resistance genes into previously susceptible strains (Mcmanus, 1997)(Matxalen Llosa, 2002).

Many bacterial species can quickly adapt to the introduction of antibacterial agents into their environment through mutation, selection, and genetic exchange mechanisms. Although a single mutation in a critical bacterial gene may only slightly reduce the host bacteria's susceptibility to that antibacterial agent, it may be sufficient to allow its initial survival until it acquires additional mutations or genetic information, resulting in full-fledged resistance to the antibacterial agent. In rare circumstances, however, a single mutation may be enough to bestow high-level, clinically important resistance on an organism (for example, high-level rifampin resistance in *S. aureus* or high-level fluoroquinolone resistance in *Campylobacter jejuni*) (Mcmanus, 1997).

Among all the mechanisms of bacterial resistance to antimicrobial agents, two stand out as the most cunning and dangerous: biofilm formation and sporulation.

2.7.3 Biofilm formation

The following section delves into the introduction of biofilms, shedding light on their formation, structure, and the challenges they pose in the context of bacterial resistance to antimicrobial agents.

A biofilm is a group of cells encased in an exopolysaccharide matrix and forming on a surface. According to Kukhtyn et al. (Kukhtyn et al., 2017), biofilms are a common cause of resistant infections and are notoriously tough to eliminate.

According to Richards and Melander (Richards & Melander, 2009), bacterial biofilms comprise polymeric matrixes created by polysaccharides that can shield the embedded microbe from antimicrobial agents. The antibiotic resistance of biofilms is caused by various mechanisms, including delayed antibiotic penetration, genetic material transfer between biofilms, and altered bacterial cell metabolism (Donlan, 2000).

Organized populations of microorganisms are enclosed in a matrix of EPS that clusters microbial cells together throughout the complex and dynamic process of biofilm formation (Renner & Weibel, 2011). According to Powell et al. (Powell et al., 2018), EPS are primarily made up of polysaccharides, proteins, lipids, and nucleic acids (RNA and extracellular DNA (eDNA)), which combine to form a highly hydrated polar combination that contributes to th' biofilm's overall framework and three-dimensional structure.

The biofilm Lifestyle is an infinite cycle, and according to earlier research (Sharahi et al., 2019), biofilm production may be broken down into the following five primary phases. In Figure 1, (I) Attachment: According to Bos et al. (Bos et al., 1999), microbes are reversibly adsorbed to surfaces by weak contacts (such as the van der Waals forces); (II) Colonization: Via greater hydrophilic/hydrophobic interactions mediated by flagella, pili, lipopolysaccharides, exopolysaccharides, collagen-binding adhesive proteins, etc., microorganisms are permanently adhered to the surface (Limoli et al., 2015); (III) Development: EPS is generated and released, and multi-layered cells accumulate by proliferation (Flemming & Wingender, 2010); (IV) Maturation: the stable development of a three-dimensional community with channels to efficiently distribute nutrition and signalling molecules within the biofilm (Dufour et al., 2012); (V) Active dispersal: microbial cells are detached in clumps or separated as a result of interactions with either intrinsic or extrinsic factors, and the dispersed cells colonize other sites (Srey et al., 2013).

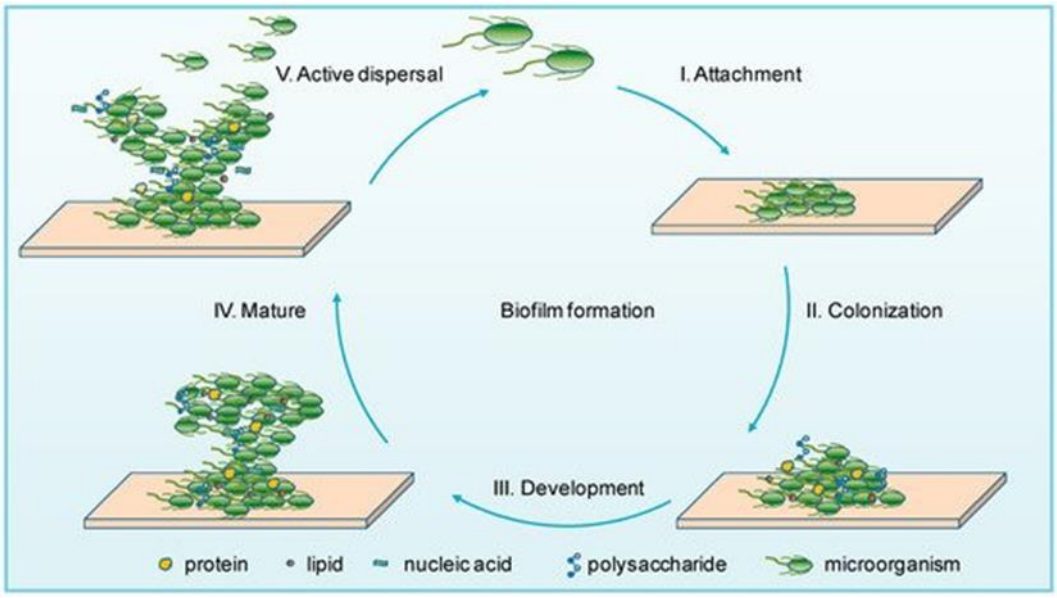


Figure 1.Model of microbial biofilm formation. (Yin et al., 2019).

Biofilm development is a distinct growth mode microorganisms select in response to varied environmental conditions. Previous research has demonstrated that the capacity to create biofilms is critical for bacteria to flourish in various harsh settings (Figure 2), (Yin et al., 2019).

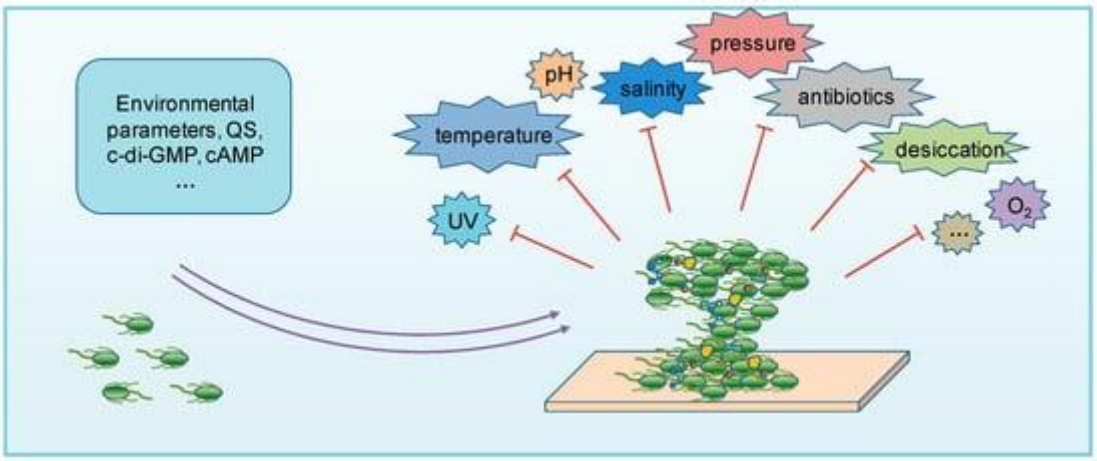


Figure 2. A schematic representation of the biofilm function. Biofilm formation can increase the resistances of microorganisms to various extreme environments (Yin et al., 2019)

As a result, Bacterial Biofilm Resistance is regarded as one of the most cunning and complicated forms of resistance to deal with.

2.7.4 Sporulation

On the other hand, sporulation is a survival strategy employed by certain bacteria under adverse conditions. Through sporulation, bacteria form highly resistant structures called spores, which are dormant and can withstand harsh environments, including antibiotic exposure. Once conditions improve, spores germinate and give rise to fully functional bacteria, perpetuating the cycle of resistance. These two mechanisms, biofilm formation, and sporulation, represent formidable challenges in the fight against antimicrobial resistance, underscoring the urgent need for innovative strategies to combat them effectively. The process of bacterial spore formation can be divided into eight stages: Stage 0 involves the normal vegetative cell; Stage I includes axial filament formation; Stage II encompasses asymmetric septation and the development of the forespore; Stage III involves the engulfment of the forespore; Stage IV focuses on cortex synthesis; Stage V includes coat deposition; Stage VI involves maturation; and Stage VII encompasses the release of the endospore, (Mugadza, 2018), see Figure 3.

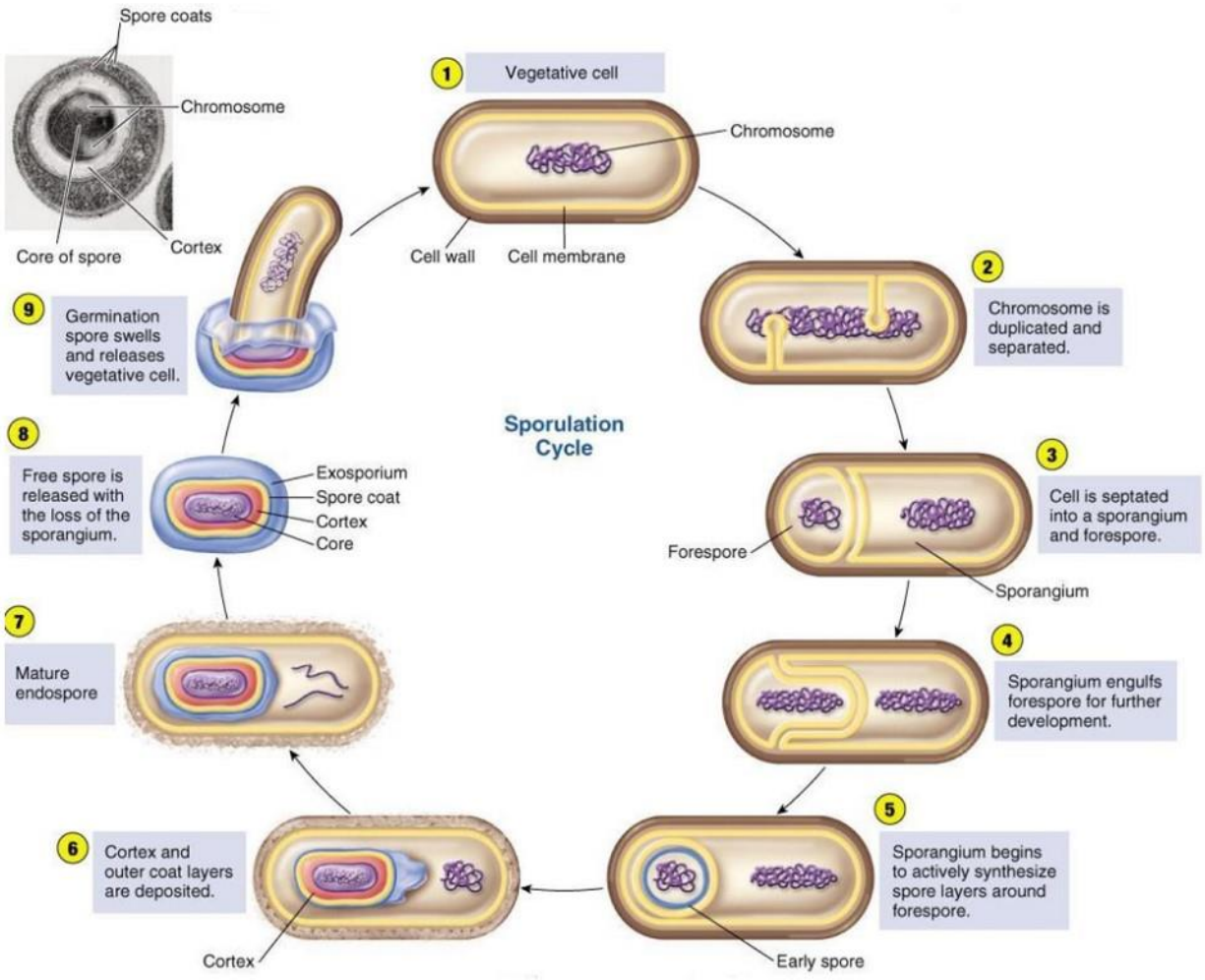


Figure 3. Stages through which a bacterial cell goes through in a sporulation cycle (Mugadza, 2018).

2.8 Antimicrobial resistance prevention techniques

A range of strategies are being developed to combat bacterial resistance. Typical tactics in this field include new antibiotic generations, combination treatments, naturally occurring substances with antibacterial properties, and targeted drug delivery approaches (Khameneh et al., 2016).

2.8.1 Natural compounds

Natural substances, either alone or in combination, are increasingly attracting researchers' attention these days as effective antibacterial agents. **Table 2** provides a summary of several important natural chemicals.

Table 2. Natural compounds with antibacterial properties (Khameneh et al., 2016)

Natural compounds	**Category**	**Antimicrobial spectrum**	**Possible mechanism of action**	**Ref**
Piperine	Alkaloid	Gram-positive bacteria	Bacterial efflux pump inhibition	(Khan et al., 2006)(Poole, 2005)
Tannic acid	Tannins	*Staphylococcus aureus Bacillus cereus, Listeria monocytogenes, Salmonella enterica, Campylobacter jejuni*	Inactivation of microbial adhesins and cell envelope transport proteins	(Scalbert, 1991)
Berberine and harmane	Alkaloid	*Streptococcus agalactiae, Staphylococcus aureus, Listeria monocytogene*	Intercalation with DNA	(Savoia, 2012)
Epigallocatechingallate (EGCG)	Catechins	Both Gram-positive and Gram-negative bacteria	Cell membrane disruption and loss of ATP	(Upadhyay et al., 2014)

2.8.2 Development of novel antibacterial agents

The approach to controlling bacterial infections and battling MDR pathogens includes the development of new antibiotics. The development of new antibacterial agents and scientific methods to introduce new antibacterial compounds have advanced slowly over the past several years, despite the demand for innovative agents (Jabes, 2011). As shown in **Table 3**.

Table 3. creation of new antibiotics based on existing classes

Antibiotic class	Novel antibacterial agents	Mechanism of action	Advantages	Ref
Neoglycoside (aminoglycoside)	Plazomicin (ACHN-490)	Bacterial protein synthesis inhibitor	Resistant to enzymatic inhibition, Able to eradicate hospital acquired resistant infection	(Aggen et al., 2010)(Endimiani et al., 2009)
Oxazolidinones	Tedizolid (TR-701) Radezolid	Protein synthesize inhibitor	Effective against Gram-positive bacteria, including linezolid-resistant strains	(Lemaire et al., 2010) (Moellering, 2014)
Tetracyclines	Eravacycline (TP-434)	Protein synthesize inhibitor	Effective against MRSA infections. Broad-spectrum covering MDR Gram-positive and Gram-negative pathogens	(Sutcliffe et al., 2013)
Glycopeptides	Solithromycin	Bacterial protein synthesis inhibitor	Highly active against Gram-positive organisms,	(Keelan et al., 2014)
Macrolides/ketolides	Cethromycin	Bacterial protein synthesis inhibitor	More reliable coverage against common respiratory pathogens	(Miyashita et al., 2003)

2.9 Olive oil production

The production of olive oil is one of the most important businesses in the Mediterranean area, especially in nations like Spain, Italy, Portugal, Greece, Syria, Morocco, and Tunisia. The leading producer, user, and exporter of olive oil is the European Union (EU), which accounts for roughly 69% of global production. Other nations have recently started producing olive oil, including Argentina, Australia, the United States of America, and South Africa (Figure 4), (Dermeche et al., 2013).

MONTHLY EU OLIVE OIL PRODUCTION (2021-2022)*

(tonnes)

2021/2022	EL	ES	FR	IT	CY	MT	PT	SI	HR	TOTAL
September			110	492						602
October	1 770	51 365	415	42 847			29 079	130	1 585	127 191
November	38 200	288 551	1 249	118 381			85 381	150	1 321	533 233
December	77 500	542 556	1 264	105 216			76 719		138	803 393
January	61 000	470 115	591	49 013			12 993			593 713
February	35 000	107 938	260	8 405			2 062			153 665
March	10 550	19 345	208	2 954						33 057
April	2 100	6 129	255	699						9 183
May	880	1 947	308	201						3 336
June										
Total	227 000	1 487 945	4 660	328 208			206 234	280	3 044	2 257 371
Production Estimation 2021/22	227 000	1 487 945	4 605	329 000	6 100	0	206 235	280	3 044	2 264 209
Consumption Estimation 2021/22	110 000	565 000	110 000	510 000	6 000	200	85 000	2 100	7 000	1 395 300
Final Stock Estimation 2021/22 (on 30.9.)	17 950	501 545	1 200	163 000			0	0	0	683 695
FINAL Production 2021/22										
- of which organic olive oil										
FINAL Consumption 2021/22										
FINAL Stock 2021/22 (on 30.9.)										

Source: MS declarations - Commission Regulation R 2017/1185 Art.12, Annex III.5
CY, MT - NO ISAMM NOTIFICATIONS. Estimated data or IOC.
* Notifications ISAMM 15.08.2022

Figure 4. Monthly EU oil Production (2021-2022)

Recently, the consumption of olive oil has increased significantly all over the world. As the advantages of olive oil for human health become more widely acknowledged, the production and consumption of olive oil are expected to increase steadily in the next few years (Madureira et al., 2022).

2.10 Olive oil extraction

Olive washing, crushing, malaxing of the resultant pastes, and the actual extraction of the olive oil are all steps in making olive oil (Zbakh & el Abbassi, 2012). Both discontinuous (conventional pressing) and continuous (centrifugation) methods can be used to extract olive oil. There are two potential systems for centrifugation processes: three-phase and two-phase systems. A solid cake and two liquids, olive oil and significant volumes of an aqueous fluid known as olive mill wastewater (OMWW), are produced in the three-phase system. When compared to the other approach, the two-phase system produces less OMWW since less water is needed throughout the process. In addition to olive oil, olive husk, and OMWW have a semisolid residue known as “wet pomace" or “olive pomace" (Caporaso et al., 2018). Figure 5 summarizes the steps involved in extracting olive oil. To reduce wastewater volume and energy

needs, two-phase systems are employed in current units in place of three-phase technology (Azbar et al., 2004).

According to research by Azbar et al., (Azbar et al., 2004), two-phase technology can save energy by up to 20% while consuming 80% less process water. However, when they are released into the environment without treatment, their residues have a negative impact on the ecosystem because of their high toxicity and resistance to biological breakdown (Khdair et al., 2019).

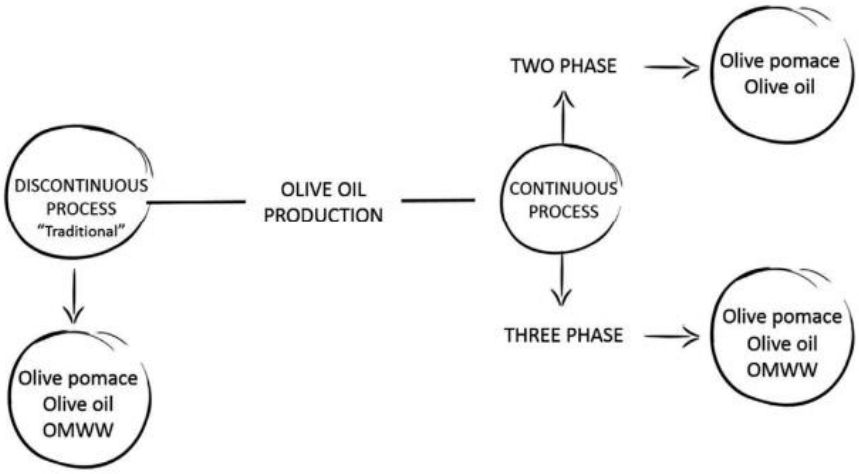


Figure 5. Different processes for olive oil production (Rodrigues et al., 2015).

2.11 Waste and by-product production

The output of olive by-products (Figure 5) starts in olive orchards with the pruning of olive trees, typically done every two years to increase olive oil output by removing old and unproductive branches and creating much biomass. The leaves gathered with the olives are separated after the olives are harvested and taken to the mill. The method of extracting the olive oil involves grinding, mixing, or malaxation and separation in a horizontal centrifuge or decanter (Negro et al., 2017).

The composition of the by-product obtained from the extraction of olive oil from the leftover crushed olive paste can be determined by the method used for extraction (Negro et al., 2017). Traditional discontinuous pressing (used primarily by small producers) was replaced by a continuous process utilizing a three-phase system, which was subsequently replaced increasingly by a two-phase system (Rincón et al., 2012), with the addition of water serving as the primary difference between these last two systems. The result is a decrease in the production

of olive-mill wastewater (OMWW). According to Rodrigues et al. (Rodrigues et al., 2015), the three-phase method produces two primary wastes: a solid cake (olive cake or olive kernel) and significant volumes of liquid phase (OMWW), which include rinse water, olive mill water from the decantation stage, and water from the separator. The two-phase approach, on the other hand, enables the separation of the oil from the olive paste without the use of water and can be thought of as a modified form of the three-phase method. The issue of vegetable wastewater is resolved. Olive pomace (OP), a semisolid residue, and a small quantity of residual water that naturally evaporates in tanks are the sole by-products of the two-phase system (Nunes et al., 2016). The two-phase procedure significantly decreases liquid leftovers and water usage, but it also slightly increases solid waste (Rincón et al., 2012). An olive tree produces roughly 2500 kg of olives per hectare (Rodrigues et al., 2015) and 40–70 kg of OP per 100 kg of olives. The quantity of the principal by-products from the olive oil extraction business is summarized in **Table 4**.

Table 4 . Main characteristics of the different types of olive by-products.

By-product	Location	Olive oil extraction system	Estimated production	Current application	Ref
Olive tree pruning	Olive orchards	-	1.5–3 ton/ha/year	Firewood	(Ruiz et al., 2017)
Olive leaves	Olive mills	Both	4–7% of the olive weight	Animal feed	(Ruiz et al., 2017)
Olive pomace	Olive mills	Two-phase system	40-70% of the olive weight	Production of pomace olive oil and extracted dry olive pomace. Extracted dry olive pomace used as fuel	(Antonio et al., 2015)(Nunes et al., 2016)
Olive stones	Olive mills	Recovered after oil separation in both systems	8–15% of the olive weight	Biofuel	(Romero-García et al., 2014)
Olive cake	Olive mills	Three-phase system	40-45% of the olive weight	Production of pomace olive oil and extracted dry olive cake Extracted dry olive cake used as fue	(Romero-García et al., 2014)(Ruiz et al., 2017)
Olive mill wastewater	Olive mills	Three-phase system	40–50% of the olive weight	None	(Romero-García et al., 2014; Ruiz et al., 2017)

2.12 Olive pomace

2.12.1 Olive pomace composition

Due to its makeup, olive pomace is a desirable by-product since it is an excellent source of beneficial chemicals. Researchers have focused the majority of their attention on polyphenols because of their well-known roles in several processes. Hydroxytyrosol and tyrosol derivatives, iridoids precursors, seicoridoids and derivatives, flavonoids, lignans, and phenolic acids have been identified by other authors by means of liquid chromatography (Alu'datt et al., 2010) (Antónia Nunes et al., 2018). Below is a description of the primary active elements.

2.12.1.1 Hydroxytyrosol

According to the International Union of Pure and Applied Chemistry (IUPAC), hydroxytyrosol (**Figure 6**) also known as 3,4-dihydroxyphenylethanol (DOPET), 3,4-dihydroxyphenolethanol (3,4-DHPEA), or 4-(2-hydroxyethyl)-1,2-benzenediol is one of the most potent naturally occurring antioxidants. It has a higher antioxidant potential than butylatedhydroxytoluene (BHT), Trolox, and vitamins C and E (Pérez-Bonilla et al., 2014)(Zbidi et al., 2009).

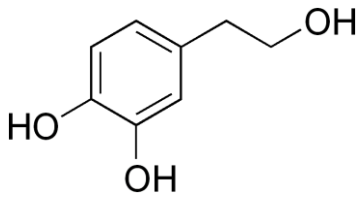


Figure 6. Hydroxytyrosol

The primary outcome of oleuropein's hydrolysis is hydroxytyrosol (Liu et al., 2018). The depth and diversity of the oil and olive flavors are caused by this process, which takes place throughout the ripening of the olives, storage of the oil, and preparation of table olives. Due to its amphipathic nature, hydroxytyrosol can be found in acetate form or as a component of more complex substances like oleoresin, verbascoside, and oleuropein in other olive oil by-products in addition to olive and olive leaf (Madureira et al., 2022). Additionally, it can be created by oleuropein being converted enzymatically (Figure 7).

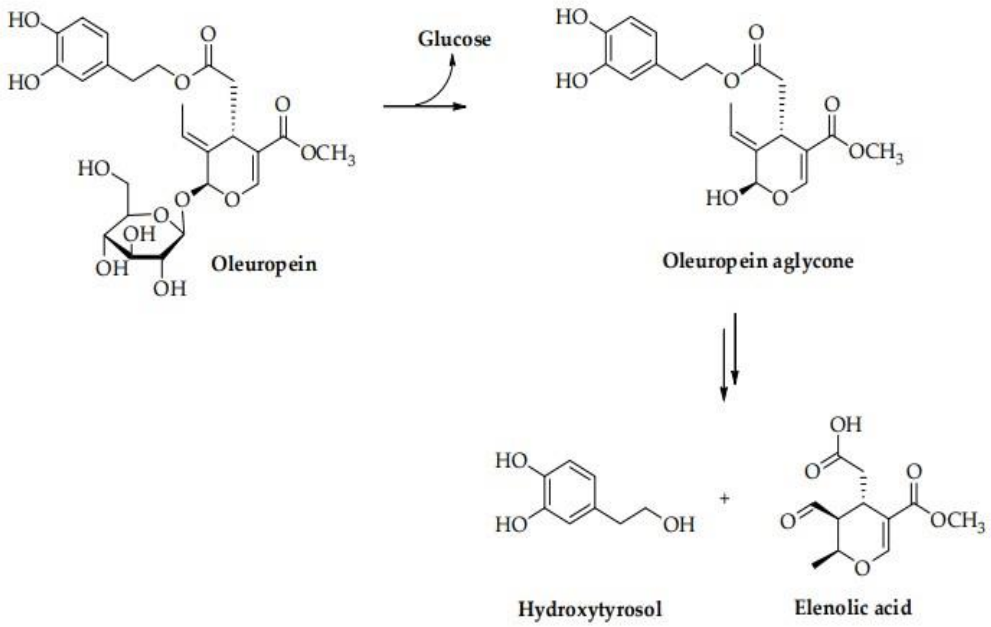


Figure 7. Enzimatic conversion of oleuropein into hydroxytyrosol

2.12.1.2 Oleuropein

Oleuropein (Figure 8) is a member of the secoiridoids class of coumarin-like chemicals and is made up of the molecules hydroxytyrosol, oleanolic acid, and glucose. It is one of the principal polyphenols found in olive waste, which helps explain why olive oil and fruit have a bitter flavor. Oleuropein has several pharmacological advantages, most of which are connected to its potent anti-inflammatory and antioxidant properties (Marković et al., 2019). Oleuropein has hydroxyl groups that can donate hydrogen to inhibit oxidation, which is associated with its antioxidant function (Hassen et al., 2015). According to studies conducted by Zbidi et al. (Zbidi et al., 2009), oleuropein and oleuropein-rich extracts exhibit more antioxidant activity than the synthetic antioxidant BHT. Oleuropein's ability to reduce blood pressure is another appealing quality. Oleuropein has been shown by Sun et al. (Sun et al., 2017) to protect the hypothalamic paraventricular nucleus (PVN) from oxidative stress, making it a promising method for both treating and preventing hypertension. Oleuropein has also been demonstrated to have antibacterial effect (Himour et al., 2017) . Oleuropein may be utilized to treat a variety of human illnesses based on the aforementioned qualities.

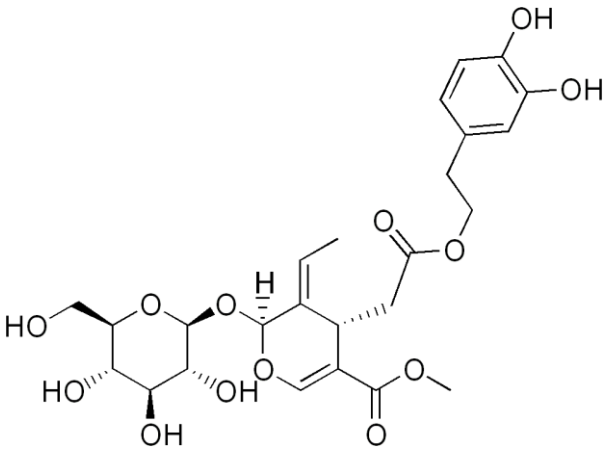


Figure 8. Oleuropein

2.12.1.3 Tyrosol

Tyrosol (Figure 9) is a phenylethanoid compound that is found in naturally occurring quantities in olive fruit, olive oil, and olive waste. It is also known by the IUPAC as 2-(4-hydroxyphenyl)-ethanol, p-hydroxyphenyl alcohol, or 4-(2-hydroxyethyl)-phenol. Tyrosol has the potential to act as an antibacterial, anti-carcinogenic, anti-inflammatory, and antioxidant agent, according to several studies (Madureira et al., 2022). Tyrosol has been suggested to combat insulin resistance, obesity, hypertension, atherosclerosis, coronary heart disease, and chronic heart failure by modifying CD14 upregulation and reducing inflammation (Madureira et al., 2022). In research by Lee et al. (Lee et al., 2018), it was shown that tyrosol was helpful in minimizing muscle damage from oxidative stress brought on by strenuous exercise. Tyrosol may also increase HDL's ability to reduce atherosclerosis (Berrougui et al., 2015) and protect the intestinal mucosa by reducing the oxidative damage caused by LDL, as demonstrated in the human colon adenocarcinoma cell line Caco-2 (Giovannini et al., 1999). Tyrosol may also be useful in the management of diabetes mellitus due to its antioxidant activity, which has been shown to have anti-inflammatory effects on the liver and pancreas in streptozotocin-induced diabetic rats (Chandramohan & Pari, 2016). Tyrosol was also administered intramuscularly to diabetic mice with hind limb ischemia, which dramatically increased the development of blood vessels and improved blood perfusion (J. Zhang et al., 2019). Additionally, according to those authors, tyrosol has a proliferative effect on skeletal muscle cells and acts as a cytoprotectant against oxidative damage brought on by hyperglycemia.

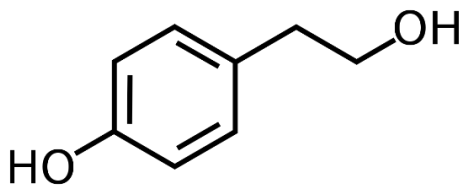


Figure 9. Tyrosol

The phenolic compounds from olives and their by-products possess antimicrobial, anti-inflammatory, and chemo-preventive properties, which enable them to attack microorganisms through the mechanisms discussed in **Table 5**.

Table 5. Olive-pomace bioactive compounds and proposed mechanisms of action

Compounds	Proposed Mechanism	Ref
Phenolics	Disruption of the membrane structure and leakage of the cellular components Hydroxyl groups promote the delocalization of electrons, reducing the gradient across membranes. Reduction of the redox potential of the growth medium, leading to microbial growth constraints.	(Cueva et al., 2010; Stojković et al., 2013)
Flavonoids	Inactivation of microbial adhesion, enzymes, and cell envelope transport proteins. Disruption of microbial membranes (lipophilic flavonoids). Perforation and/or a reduction of the membrane fluidity. Inhibition of nucleic acid synthesis, energy metabolism, and cell membrane synthesis.	(Alghazeer et al., 2017a)
Hydroxytyrosol	The capability of chelating transition metals reducing the reactivity of iron and copper by forming an inert metal-ligand complex, which decreases the bioavailability for bacterial growth. Reduction of intracellular ATP concentrations. Cell membrane depolarization. Reduction of the bacterial protein content.	(Guo et al., 2020a)
Vitamin E (a-tocopherol)	Damage in the cell membrane, affecting the essential components for the integrity of the membrane (reduction in membrane potential and loss of ions, cytochrome C, proteins, and radicals, followed by the collapse of proton pumps and decrease in ATP), increasing the membrane permeability. Interaction with the lipid bilayer of the bacteria cell membrane modifying therespiratory chain and energy production. Capacity of acting in the cell envelope resulting in an imbalance in the fluid mosaic nature of the bacterial membrane	(Andrade et al., 2014)
Fatty acids	Disruption of the electron transport chain by binding to electron carriers Leakage of cell metabolites via cell lysis. Inhibition of nutrient uptake. Formation of peroxidation /auto-oxidation products resulting in cell deactivation.	(Yoon et al., 2018)(Zhou et al., 2020)

Chapter 3 Materials and Methods

3.1 Moist olive pomace and leather samples

The moist olive Pomace was collected at the olive oil extraction unit Olimontes (Macedo de Cavaleiros, Portugal), operating a continuous two-phase centrifugation system. Samples were cooled to 4°C for conditioning at the same temperature (H8 A1E W, Hotpoint-Ariston, Lisbon, Portugal).

The leather used in the study was supplied by the company Couro Azul - Indústria e Comércio de Couros (Portugal, Alcanena).

3.1.1 Extracts preparation

To prepare the extract, 500 g of moist olive pomace were mixed with 300 mL of distilled water, then pasteurized by immersing it in a water bath (Julabo SW22) at 60°C for 30 minutes. The resulting mixture was then strained through a sieve and divided into pots, which were subsequently frozen at -4°C and lyophilized for four consecutive days. The resulting lyophilized olive pomace was stored in a dark desiccator for future use. Three extracts were then prepared from the lyophilized olive pomace using distilled water as the extraction solvent, with the sample-to-solvent ratios consecutively as follows: (1:5), (1:15), and (1:20). The extracts were then subjected to a temperature of 45°C in a water bath for 3 hours. After extraction, the supernatant was separated from the solids by vacuum filtration using filter paper n°4 and analysed for its antimicrobial activity.

3.1.2 Extraction yield

To determine the extraction yield, the extracts were filtered using filter paper n°4, and a volume of 20 mL was subsequently freeze-dried until reaching a constant weight. The extraction yields were then calculated by expressing the grams of extract obtained per 100 g of initial starting material (MOP).

3.1.3 Extraction and HPLC analysis of phenolic compounds from the MOP

The MOP was subjected to freeze-drying (Coolsafe 110-4, Scanvac, Olsztyn, Poland), followed by weighing 1.5 g of the freeze-dried samples, which were then mixed with 50 mL of methanol (MeOH) and left to stir in the dark for an hour. The resulting extract was filtered and transferred to an evaporating flask containing 50 mL of MeOH (Sigma-Aldrich, Germany).

The extraction process was repeated thrice, and upon completion, the MeOH was evaporated using a rotary evaporator set at 35 ºC (RE300DB, Stuart Stone, UK). Subsequently, 5 mL of MeOH were added to achieve a 0.1 mg/mL concentration, filtered through a Whatman Nylon (0.20 µm) filter, and stored in opaque flasks before HPLC analysis. For HPLC analysis, 20 µL of the extract were injected into an analytical HPLC Knauer Smartline separation module equipped with a Knauer Smartline Autosampler 3800, a cooling system set to 4 ºC, and a Knauer Diode Array Detector (DAD). A reversed-phase Shperisorb ODS2 column (250 x 4 mm id, 5 µm particle diameter, end-capped Nucleosil C18 (Macherey-Nagel)) was kept at 30 ºC. The chromatographic separation was carried out using a gradient that consisted of solvent A (water: formic acid (99.8:0.2 (v/v))) and solvent B (MeOH), applied at a flow rate of 0.9 mL/min. The gradient elution program was set as follows: 5% B at 0 min, 15% B at 3 min, 25% B at 13 min, 30% B at 25 min, 35% B at 35 min, 40% B at 39 min, 45% B at 42 min, 45% B at 45 min, 47% B at 50 min, 48% B at 60 min, 50% B at 64 min, 100% B at 66 min, 5% B at 70 min, and 5% B at 75 min. A DAD detector was used for detection, and spectral data from all peaks were accumulated in the 200-600 nm range. Chromatograms were recorded at 254, 280, 320, and 330 nm. The compounds in each extract were identified by comparing their retention times and UV-Vis spectra in the 200-600 nm region with genuine standards examined under the same conditions and with the authors' spectra library. All analyses were carried out in triplicate.

3.2 Microbial cultures

The cryopreserved microbial cultures of *Escherichia coli* ATCC 8739 and *Staphylococcus aureus* ATCC 6538 (Mistracon, Barcelona, Spain) were reactivated in Brain Heart Infusion-BHI broth medium (Liofilchem, Roseto Degli Abruzzi, Italy) and incubated in a bacteriological incubator (Raypa, Incuterm, Barcelona, Spain) at 37 °C for 24 hours. A 10% pre-inoculum was then transferred to the same conditions after standardizing the cell density suspension (1.5×10^8 cells/mL) using a densitometer (DEN-1 McFarland densitometer, Grant-bio, UK) at a wavelength of 550nm.

3.2.1 Antimicrobial Activity

3.2.1.1 Determination of the minimum inhibitory concentration

The minimum inhibitory concentration (MIC) was determined by the microplate dilution technique (96 wells), according to the M7-A6 standard of the Clinical and Laboratory

Standards Institute Manual (CLSI, 2006), using the colorimetric assay with p-iodonitrotetrazolium (INT) described by Kuete et al. (Kuete et al., 2011). The microplates were incubated with the microbial cultures in an incubator at 37°C for 24 hours for both *E. coli* and *S. aureus*.

3.2.1.2 Quantification of microbial reduction

Serial dilutions of the standardized microbial inoculum (*E. coli and S. aureus*) in a 0.85% NaCl solution were performed, and the concentration of MOP giving rise to satisfactory results was added. Subsequently, inoculation was performed in Petri plates with BHI agar culture medium (Liofilchem, Roseto Degli Abruzzi, Italy) for quantifying the colony-forming units (CFU) after 24 hours of incubation at 37°C.

3.2.1.3 Evaluation of antimicrobial activity on leather-specimens

The antimicrobial activity of the most effective MOP concentration, determined to be 1:5 (OP to water), on leather specimens was evaluated. Microbial determination and quantification were conducted using a modified method, PV 3970- Issue 2009-04 of the Volkswagen Group. To assess the effectiveness of the treatment, resistance against both *Escherichia coli* ATCC 8739 (gram-negative) and *Staphylococcus aureus* ATCC 6538 (gram-positive) was examined.

Circular leather pieces and paper filters (Filter-lab 1248, Barcelona, Spain) with a diameter of 1.2 cm were prepared. To ensure decontamination, they were subjected to two cycles in an oven (Scientific, SERIES 9000, SOUTH AFRICA) at 50°C for 30min.

The methodology for the experiment was conducted as follows:

a. Preparation of Microplates and Extracts:

Two 24-well microplates were taken. A single MOP extract was prepared with a ratio of 1:5 (MOP to water) for the assessment. To ensure precise and consistent distribution, 1 mL of the extract was added in triplicate to each well of the microplates.

b. **Immersion of Leather Samples and Initial Incubation:**

One leather sample was immersed in each well of the microplates containing the extract. The plates were then placed in a microbiological incubator set at 37°C and 100 rpm for an initial incubation period of 15 minutes. The incubation time was carefully monitored.

c. **Preparation of Microplates for Bacterial Incubation:**

After the 15-minute incubation, the plates were removed from the incubator. Two additional microplates were prepared for bacterial incubation. In each well of the new plates, 1 mL of standardized inoculum of *S. aureus* and *E. coli* (0.5 McFarland each) was added. The leather samples from the first set of plates (extract immersion) were transferred to the wells of the second set of plates (bacterial inoculation). The plates were then returned to the incubator and incubated at 37°C and 100 rpm for an additional 15 minutes.

d. **Washing Phase:**

Following the bacterial incubation, the plates were taken out of the incubator. Two additional microplates were prepared for the washing phase. In each well of these plates, 1 mL of sterilized distilled water was added. The leather specimens from the plates used for bacterial incubation were placed in the wells of the washing plates. The plates were then returned to the incubator and incubated for 15 minutes at 37°C and 100 rpm.

e. **Preparation of Microplates for Result Analysis:**

After the final 15-minute incubation in the washing phase, the plates were removed from the incubator. Two new microplates were labeled with the name of the bacteria and the concentration of the extract being tested. In each well of these plates, 0.5 mL of sterilized distilled water was added. Filter papers were immersed in each well, and on top of the filter papers, the leather samples taken from the washing plates were placed. These plates were placed in the incubator set at 25°C without agitation.

f. **Result Analysis:**

The plates were monitored after 48 hours to observe the results. Any visible changes, growth inhibitions, or other relevant observations were recorded.

Throughout the entire procedure, a sterile environment was maintained, and adhering to appropriate safety measures was crucial to avoiding contamination and ensuring accurate results.

The experimental design is shown in Figure 10.

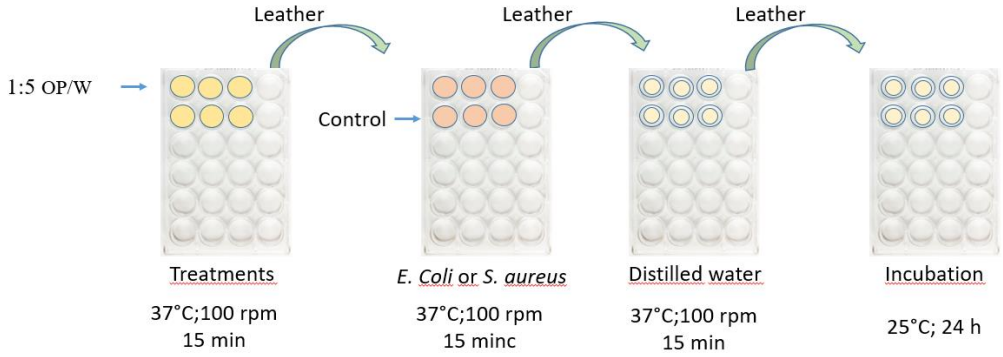


Figure 10. the scheme illustrates the evaluation of antimicrobial activity on leather specimens.

3.3 Statistical Analysis

The analysis of the results obtained in the various tests was performed using the ANOVA statistical test with Tukey's multiple comparison post-test using GraphPad Prism® 8.0 software (San Diego-CA, USA).

Chapter 4 Results and discussion

4.1 HPLC analysis of phenolic compounds from MOP

The reversed phase-HPLC analysis of phenolic compounds extracted from MOP enabled the identification and separation of various phenolic compounds. As illustrated in Figure 11, the total phenolic compounds (TPC) in the dried olive pomace were found to be approximately 18315 mg.kg^{-1} GAE (gallic acid equivalent), which could be classified into three categories: secoiridoids (represented primarily by oleuropein) accounted for around 78% of the TPC. In contrast, phenolic alcohols (hydroxytyrosol and tyrosol) contributed about 19% of TPC. Flavonoids such as luteolin and apigenin were present in trace amounts, comprising approximately 2% of TPC.

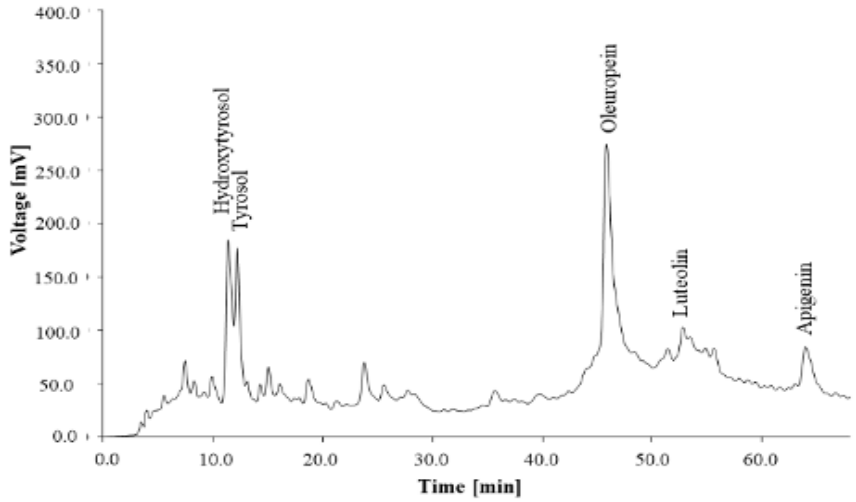


Figure 11. HPLC-DAD chromatogram of MOP acquired at 280 nm. Identified compounds: hydroxytyrosol, tyrosol, oleuropein, luteolin, and apigenin.

In 2020, (Böhmer-Maas et al., 2020) found TPC concentrations ranging from 20886.2 to 23061.2 mg.kg-1 GAE of dried olive pomace in their samples. Similarly, (Cioffi et al., 2010) identified secoiridoids (oleuropein and ligstroside aglycone) as the highest fraction of the TPC, followed by phenolic alcohols (hydroxytyrosol and tyrosol) in all samples. In addition, the authors also detected gallic acid, caffeic acid, ferulic acid, and vanillic acid. However, the phenolic profile of olive pomace varies depending on the chemical composition of the olive oil, which is influenced by factors such as olive cultivar, geographic origin, irrigation technique, and extraction method (Dabbou et al., 2011; Malheiro et al., 2015; Y. Zhang et al., 2022). Therefore, it is challenging to establish a standardized phenolic profile.

4.2 The effect of different extract/water ratios on the extraction yield

The choice of solvents is critical in the extraction of bioactive compounds from biomass in order to design a sustainable method with the least impact on health and the environment (Alexovič et al., 2018). Despite the fact that these organic solvents have traditionally been reported as a safe and advantageous option, there is a growing interest in finding greener alternatives in order to reduce the emission of volatile organic compounds associated with these organic solvents, which contributes to global warming (Chemat et al., 2019). The choice of water as the extraction solvent is noteworthy as it is a safe, cost-effective, and environmentally friendly option. Water is readily available, non-toxic, and can extract a wide range of bioactive compounds from various natural sources. It is also a suitable solvent for extracting hydrophilic compounds, including many phenolic compounds found in olive pomace (Gómez-Cruz et al., 2021).

The effect of the different concentrations on the extraction yield of the olive pomace extracts is shown in **Table 6.**

Table 6. Extraction yield and extract concentrations obtained at different ratios of olive pomace to water.

MOP to Water Ratio	Extract concentration (mg/mL)	Extraction yield (%)
1:5	128.0	29.5
1:15	31.3	64.0
1:20	24.0	83.0

The extraction process of olive pomace using water as the solvent has shown promising results, with higher ratios of olive pomace to water (w/v) leading to increased extraction efficiency. The extraction yields obtained were 29.5%, 64.0%, and 83.0% for the MOP to Water ratios of 1:5, 1:15, and 1:20, respectively. These findings suggest that increasing the amount of olive pomace relative to the used water amount in the extraction process enhances the extraction efficiency.

Moreover, upon comparing these findings with those reported by (Gómez-Cruz et al., 2021), who documented an extraction yield of 37.5%, as well as the results obtained from NREL protocols (Sluiter & Templeton, 2008), which indicated an extraction yield of 41.8%, it can be inferred that the outcomes of this study are superior. The higher extraction yields attained in the present investigation demonstrate enhanced efficacy in extracting valuable constituents from olive pomace using water as the extraction solvent.

The observed trend of increasing extraction yield with higher MOP/water ratios suggests that the extraction process is highly dependent on the availability of the desired compounds in the pomace and their solubility in water. Increasing the amount of olive pomace in relation to the water makes more target compounds likely to be released and dissolved in the solvent, resulting in higher extraction yields.

4.3 . Antimicrobial Activity

4.3.1 Determination of the minimum inhibitory concentration

As mentioned previously, the TPC percentage of individual phenolic compounds was measured in the extracts of the MOP. However, it was decided to conduct antimicrobial activity studies using the entire extract. This decision was based on the understanding that extracts may provide more advantages than isolated components, as other compounds in the extracts can influence the properties of bioactive individual constituents (Borchers et al., 2004). It is well-established that phenolic compounds possess antimicrobial capabilities (Silva et al., 2018).

In the current study, the composition of the extract, particularly the main compounds identified as polyphenols, likely contributed to the observed antimicrobial effect. These polyphenols, primarily represented by secoiridoids such as oleuropein, accounted for approximately 78% of the TPC. Phenolic alcohols, such as hydroxytyrosol and tyrosol, contributed around 19% of the TPC. Trace levels of flavonoids, including luteolin and apigenin, were also detected, comprising roughly 2% of the TPC. Each of these compounds exerts antimicrobial effects using multiple mechanisms of action on the bacteria.

Oleuropein, for example, exhibits potent antimicrobial properties against both Gram-negative and Gram-positive bacteria. Studies have demonstrated that compounds resembling oleuropein's phenolic structures can damage the bacterial membrane and/or disrupt cell peptidoglycans (Omar, 2010). On the other hand, phenolic alcohols (hydroxytyrosol and tyrosol) exert their antimicrobial effects by chelating transition metals, reducing the reactivity of iron and copper, and forming inert metal-ligand complexes that decrease the bioavailability for bacterial growth. They also reduce intracellular ATP concentrations, depolarize cell membranes, decrease bacterial protein content, and cause cytoplasmic leakage (Guo et al., 2020b). Flavonoids, including lipophilic flavonoids, exert antimicrobial effects by inactivating microbial adhesion, enzymes, and cell envelope transport proteins. They can disrupt microbial membranes, leading to perforation and/or a reduction of membrane fluidity. These actions are

followed by inhibiting nucleic acid synthesis, energy metabolism, and cell membrane synthesis (Alghazeer et al., 2017b).

Table 7. Minimum inhibitory concentration (MIC) of different MOP ratio extracts on gram-negative bacteria (*Escherichia coli ATCC 8739) and* gram-positive bacteria (*Staphylococcus aureus ATCC 6538)*

Bacteria	MIC (mg/ml)		
	1:5 (w/v)	1:15 (w/v)	1:20 (w/v)
Staphylococcus aureus ATCC 6538	64	31.3	NI
Escherichia coli ATCC 8739	32	31.3	24

Table 7 presents the minimum inhibitory concentration (MIC) values for different concentrations of olive pomace (MOP) extract against *Staphylococcus aureus* and *Escherichia coli*. The minimum inhibitory concentration (MIC) values for the 1:5 (w/v) olive pomace (MOP) extract were 64 mg/mL for *Staphylococcus aureus* and 32 mg/mL for *Escherichia coli*. In the 1:15 (w/v) MOP extract, the MIC values were 31.3 mg/mL for both *E. coli* and *S. aureus*. However, for the 1:20 (w/v) MOP extract, the MIC value was 24 mg/mL for *E. coli,* while no inhibition was observed for *S. aureus*.

The obtained results demonstrate the antimicrobial activity of the different MOP extracts against *E. coli* and *S. aureus*. In comparison to previous studies, the observed MIC value for *E. coli* (24 mg/mL) was lower than those reported by (Nunes et al., 2021) (62.5 mg/mL) and (Gómez-Cruz et al., 2021) (45 mg/mL). This suggests a stronger inhibitory effect of the tested extracts against *E. coli*. Similarly, for *S. aureus*, the MIC value (31.3 mg/mL) was lower than that reported by (Nunes et al., 2021) (125.0 mg/mL). These findings indicate a potential for enhanced antimicrobial activity against *S. aureus*.

The variation in MIC values among the different extracts can be attributed to differences in the concentration and composition of bioactive compounds present in the extracts. The higher antimicrobial activity observed in the 1:15 (w/v) extract compared to the 1:5 (w/v) extract may be due to the higher concentration of active compounds in the former one. Furthermore, the lack of inhibition for *S. aureus* in the 1:20 (w/v) extract suggests that the concentration of active compounds in this extract may not be sufficient to exert antimicrobial effects against this particular strain.

It is important to consider that the antimicrobial activity of olive pomace extracts can be influenced by various factors, including the extraction method, the used solvent, and the specific bioactive compounds present in it.

4.3.2 Quantification of microbial reduction

The microbial reduction achieved by different MOP extracts at varying concentrations was analysed using the ANOVA statistical test with Tukey's multiple comparison post-test. The results, represented in the histogram Figure 12, were analysed using GraphPad Prism® 8.0 software (San Diego, CA, USA).

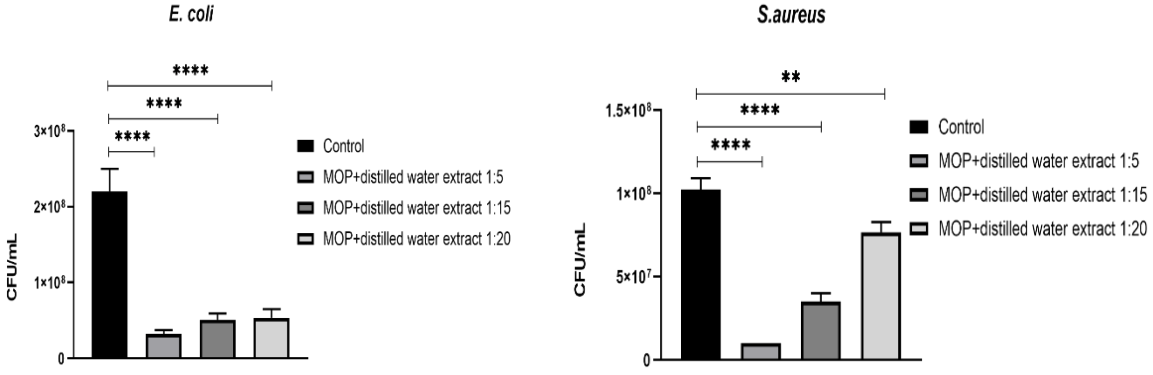


Figure 12. Histogram representing the percentage reduction

According to the statistical analysis, a significant microbial reduction was observed for the 1:5 (w/v) MOP extract. The reduction percentages were calculated as 90.22% for *S. aureus* and 85.45% for *E. coli*, compared to the controls. These findings indicate highly effective antimicrobial activity of the 1:5 (w/v) MOP extract against both bacterial strains.

Furthermore, the 1:15 (w/v) MOP extract also exhibited considerable microbial reduction percentages, with values of 65.70% for *S. aureus* and 76.68% for *E. coli*, compared to the controls. Although slightly lower than the 1:5 extract, these results still demonstrate a significant antimicrobial effect of the 1:15 (w/v) MOP extract.

In contrast, the 1:20 (w/v) MOP extract showed relatively lower microbial reduction percentages, with values of 25.08% for *S. aureus* and 75.75% for *E. coli*, compared to the controls. While the reduction against *E. coli* is noteworthy, the efficacy against *S. aureus* appears to be limited.

Overall, the microbial reduction analysis results indicate that the 1:5 (w/v) MOP extract exhibited the highest antimicrobial activity, followed by the 1:15 (w/v) extract. The 1:20 (w/v) extract demonstrated a lower antimicrobial effect, particularly against *S. aureus*. These findings

suggest that the concentration of the MOP extract plays a crucial role in producing effective antimicrobial extracts.

Based on the statistical analysis outlined above, it is determined that all samples demonstrated a highly significant reduction in microbial activity, with a p-value of <0.0001. The only exception was observed in the case of the 1:20 (w/v) MOP extract against S. aureus, which exhibited a comparatively lower microbial reduction with a p-value of <0.01.

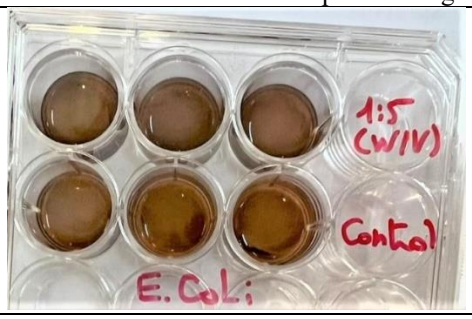
The MIC value obtained for *E. coli* in this study (24 mg/mL) was found to be lower compared to the values reported by Nunes et al. (2021) (62.5 mg/mL) and Gómez-Cruz et al. (2021) (45 mg/mL), indicating a stronger inhibitory effect of the tested extracts against *E. coli*. Similarly, for *Staphylococcus aureus*, the MIC value (31.3 mg/mL) in this study was lower than the value reported by Nunes et al. (2021) (125.0 mg/mL). Moreover, the MIC values observed in this study, which were lower than those reported by previous authors, suggest a higher percentage of antimicrobial reduction.

Overall, the antimicrobial potential of natural extracts is attributed to the synergistic effects of multiple active components, making it difficult to attribute antimicrobial effects to a single compound. Studies on compounds derived from Olea europaea have revealed their efficacy against various microorganisms. For example, tyrosol has shown significant antimicrobial activity against *Streptococcus pyogenes*, *Escherichia coli*, and *Klebsiella pneumoniae*, while oleuropein and verbascoside inhibit the growth of *Listeria monocytogenes*. Hydroxytyrosol has been identified as an important antibacterial compound effective against Propionibacterium acnes, *Staphylococcus aureus*, and *Staphylococcus epidermidis*. These findings support the idea that specific compounds from Olea europaea possess antimicrobial properties, but overall effects are likely a result of their collective action (Gómez-Cruz et al., 2021).

4.4 Evaluation of antimicrobial activity on specimens-leather

A study was carried out on leather specimens to investigate the antimicrobial effects of the MOP extract against *S. aureus* and *E. coli*. The visual inspection of the obtained results is shown in **Table 8**, **Table 9** ,while the percentage microbial reduction is illustrated in Figure 13.

Table 8. Microbial reduction in leather specimens against *E. coli*



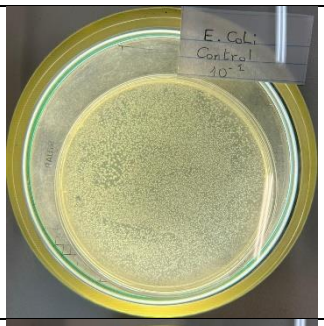
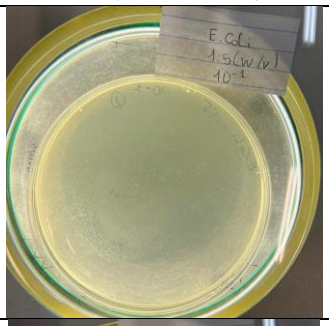
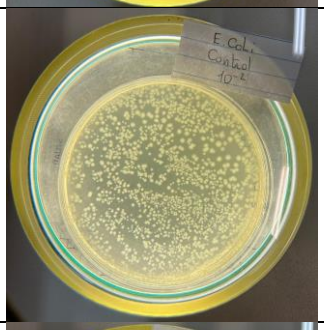
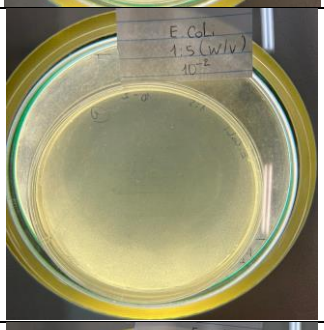
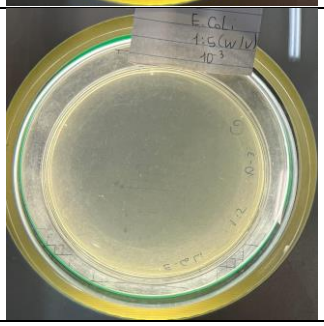
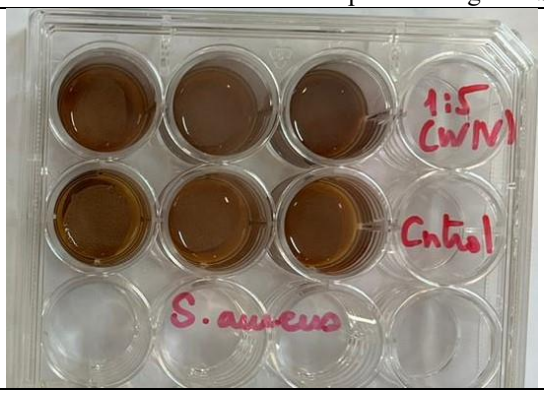
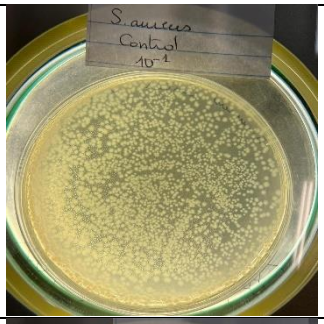
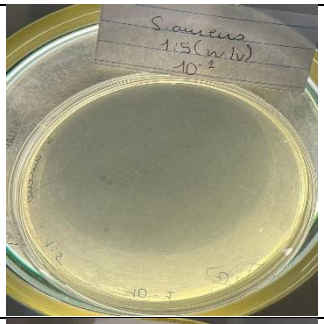
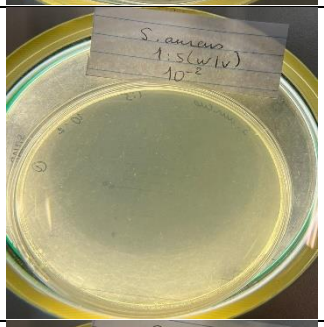
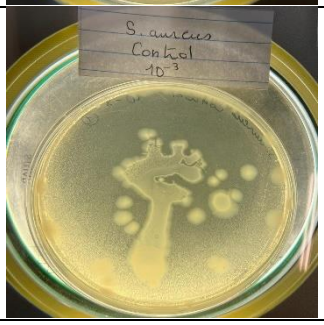
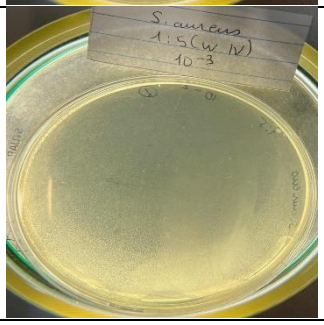
Leather incubation (*E. coli*)		
Control	Dilution	1:5 (MOP/water)
	10^{-1}	
	10^{-2}	
	10^{-3}	

Table 9. Microbial reductions in leather specimens against *S. aureus*



Leather incubation (*S. aureus*)		
Control	Dilution	1:5 (MOP/water)
	10^{-1}	
	10^{-2}	
	10^{-3}	

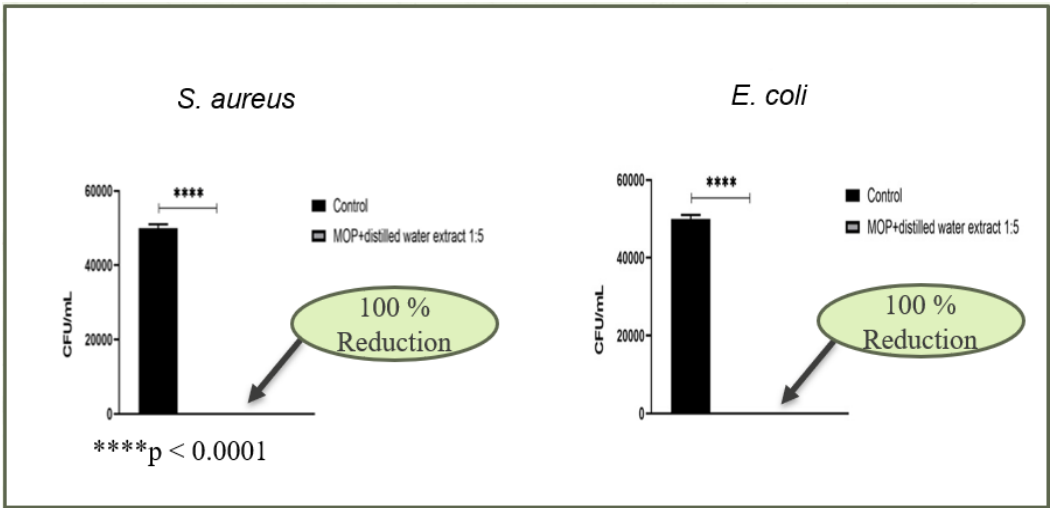


Figure 13.Histogram representing the percentage microbial reduction in leather against *S. aureus* and *E. coli*

After incubating the leather specimens in 24-well plates for two days, a serial dilution technique was used to assess the microbial reduction. Table 8 and Table 9 depict the results of evaluating antimicrobial activity on the leather specimens, utilising the optimised concentration of moist olive extract (MOP) at a ratio of 1:5 (MOP to water), representing a concentration of 128mg/mL. These figures provide valuable insights into the efficacy of the MOP extract in reducing the microbial load on the leather specimens, targeting both *E. coli* and *S. aureus*. Notably, the results demonstrate a remarkable 100% reduction in the microbial count achieved by the MOP extract at the specified concentration. This outcome highlights the potent microbicidal effect of the extract against both *E. coli* and *S. aureus, where* bactericidal activity was determined based on the achievement of a reduction of at least 99.9% (or 3 log10) in the total count of CFU/mL compared to the original inoculum. This criterion for bactericidal activity was defined by the former National Committee for Clinical Laboratory Standards (National Committee for Clinical Laboratory Standards, 1999) (Fernandes et al., 2013). Besides, the histograms in Figure 13 present the percentage reduction in the leather specimens, providing compelling evidence of the extract's significant efficacy ($p < 0.0001$) in reducing the microbial load.

Comparing the results of this study with those of Fernandes et al. (2013) on antimicrobial leather development using chitosan treatment, both studies demonstrated an impressive microbial reduction of 100%. However, taking cost into consideration and comparing the efficacy of chitosan with that of moist olive pomace, it becomes clear that moist olive pomace is a more cost-effective alternative while delivering equivalent results. Derived from olive oil production, moist olive pomace exhibits potent antimicrobial properties and can be obtained at

a lower cost than chitosan. The findings of this study confirm that using moist olive pomace extract at the optimised concentration (128mg/mL) achieved a microbial reduction of 100%, comparable to the results obtained with the chitosan treatment in the previous study. Considering moist olive pomace's cost-effectiveness and comparable efficacy in microbial reduction, it presents an attractive option for industries, including leather manufacturing, seeking to implement antimicrobial treatments. Using moist olive pomace extract not only ensures effective control of microbial contamination but also offers the advantage of reduced production costs.

The observed microbicidal effect in this study highlights the promising antimicrobial properties of the MOP extract, as it effectively eliminates both *E. coli* and *S. aureus*, suggesting its broad-spectrum antimicrobial activity. These findings indicate that the MOP extract has significant potential as a natural and sustainable alternative for controlling microbial contamination, particularly in industries such as leather manufacturing. Utilising natural extracts like MOP in antimicrobial treatments offers numerous advantages, including reduced environmental impact and decreased reliance on synthetic antimicrobial agents. The demonstrated antimicrobial activity against *E. coli* and *S. aureus* further underscores the potential application of MOP extract in various fields, including the leather industry. Overall, the results of this study confirm the successful achievement of a 100% microbial reduction using the optimised concentration of MOP extract (128mg/mL) on leather specimens, emphasising its promising antimicrobial properties and potential for broader applications. Further research and development in this area could pave the way for utilising MOP extract as an effective antimicrobial agent in diverse industries, contributing to improved hygiene and safety standards.

Chapter 5 Conclusion and future work

The analysis of phenolic compounds extracted from MOP using HPLC revealed a diverse composition, with secoiridoids, phenolic alcohols, and flavonoids being the major categories. Secoiridoids, such as oleuropein, were found to be the predominant phenolic compounds. The phenolic profile of olive pomace can vary due to factors like olive cultivar, geographic origin, and extraction method. The use of water as a solvent for extraction showed promising results, particularly when using higher MOP/water ratios, resulting in increased extraction efficiency. This suggests that the extraction process is influenced by the availability and solubility of the desired compounds in the pomace. The olive pomace extracts exhibited significant antimicrobial activity against both Gram-negative (*Escherichia coli*) and Gram-positive (*Staphylococcus aureus*) bacteria. The presence of phenolic compounds, particularly secoiridoids and phenolic alcohols, likely contributed to their antimicrobial effects through various mechanisms of action. The effectiveness of the extracts in reducing microbial growth varied depending on the extract concentration. The 1:5 (w/v) extract showed the highest antimicrobial activity, followed by the 1:15 (w/v) extract. However, the 1:20 (w/v) extract demonstrated lower efficacy, particularly against *S. aureus*. Evaluating the antimicrobial reduction on leather specimens also revealed a remarkable and complete microbial reduction. These findings provide valuable insights into the phenolic composition of olive pomace, the influence of extraction ratios on efficiency, the extracts' antimicrobial potential, and the effectiveness of utilising moist olive pomace in preventing microbial deterioration of leather. Further research can focus on optimizing extraction methods and exploring the potential applications and health benefits of these bioactive compounds. Moreover, confirming the extract’s composition is also important to study to find a relationship between composition and antimicrobial action.

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