UNISS
UNIVERSITÀ
DEGLI STUDI
DI SASSARI

DIPARTIMENTO DI
AGRARIA

UNIVERSITÀ DEGLI STUDI DI SASSARI

CURRICULUM "AGRI-FOOD MICROBIAL BIOTECHNOLOGY"

CYCLE XXXVI

IN VITRO AND IN VIVO BIOLOGICAL ACTIVITIES AND PHYTOCHEMICAL STUDIES OF POMEGRANATE PEEL POLYPHENOLS

AMIRA SALIM ABDELMENAM SALIM

Supervisor: Prof. Severino Zara

Academic Year 2022-2023

Dedication

To all the Palestinian women…

Acknowledgments

It is my privilege to express my thankfulness to my supervisor Prof. Severino Zara, for providing me the opportunity to carry out this work. His constant guidance and constructive criticism immensely shaped my work and the spirit of my scientific thoughts. I would like to thank him for giving me absolute freedom in planning and executing experiments and always considering my views and ideas, which led to healthy discussions throughout the tenure of my study.

I am deeply grateful to Dr. Cassandra L. Quave for generously hosting me during a significant portion of my Ph.D. research in her lab at Emory University, GA, USA. Her guidance, expertise, and support have been invaluable throughout this journey. I also extend my thanks to all the members of Dr. Quave's lab for their assistance, collaboration, and camaraderie during my time there.

I extend my gratitude to Dr. Maria Giovanna Molinu of the National Council of Research, Institute of Food Production Sciences, for her unwavering academic and personal support during my time in her lab, also, Mrs. Gavina Serra for her invaluable technical assistance.

Above all, I am truly grateful for the opportunities and resources provided by my department and would like to thank all and every member of my department for their warm personal support while being away from home. Special thanks to Dr. Francesco Fancello for his past and present guidance, mentorship, and his contributions that have enriched my research experience and shaped my academic growth.

To my parents and sisters, thank you for believing in me and standing by me through every challenge and triumph. Your sacrifices and encouragement have shaped me into the person I am today, and I am forever indebted to you for your unwavering support. This achievement would not have been possible without you, and I am profoundly grateful for your presence in my life.

To my dear friends, I am thankful for the countless moments of laughter, camaraderie, and shared experiences that have lightened the challenges of research and study.

A heartfelt “Thank you” to all,

Amira Salim.

Table of Contents

INTRODUCTION

1. Eating habits of human beings and their feeding behaviour

The importance of food for humanity cannot be overstated. It is a basic necessity that everyone, regardless of age or gender, cannot live without, that's why it is a crucial essential resource for the well-being of people worldwide, where the need for feed has remained constant throughout history. (Broglia and Kapel, 2011; Carocho et al., 2015). Our habits of consuming food have undergone significant transformations (Carocho et al., 2015). Current practices of producing and consuming food are widely viewed as unsustainable. While it is essential to meet the basic nutritional needs of humans, these practices also pose severe environmental threats (Notarnicola et al., 2017). The increase in income and the decrease in food prices have led to a higher availability of food. Consequently, there have been significant shifts in food consumption patterns over the last five decades. At the individual level, various factors, such as food availability, accessibility, and choice, influence food habits and preferences. These factors, in turn, can be influenced by geographical location, demographics, disposable income, urbanization, globalization, marketing strategies, religious beliefs, cultural practices, and consumer attitudes (Kearney, 2010; Reisch et al., 2013).

In primitive times, when humans relied on picking and hunting for food, their diet consisted of raw food items like birds, animals, clams, and fish, as well as plants, leaves, and fruits, all of which were directly handled. The initial shift in eating habits happened when early humans "discovered" fire. This discovery likely enhanced their ability, and that of future generations, to better prepare and utilize food (Zucoloto, 2011). The discovery of fire marked the beginning of civilization and the development of our distinct culinary abilities. Early humans stumbled upon the taste of cooked food by natural fire, realizing it was more flavorful than raw fare. This accidental finding led to the intentional use of fire for cooking. Over time, as people engaged in various tasks, cooking tools emerged, and methods for preserving and storing food were innovated (Zhou et al., 2020). In addition, about 11,000 years ago, a significant shift took place with the rise of agriculture in Southwest Asia. This marked the introduction of grains into the human diet and led to the settling of human communities in specific areas. Agriculture allowed people to cultivate food close to their homes, eliminating the need for nomadic lifestyles (Zucoloto, 2011).

The fast-growing global population has led to a shortage of food, making food security crucial, and resulting in the need for more space for food production (Lindahl and Grace, 2015; Odeyemi et al., 2020; Mokoena et al., 2021). To sustain the projected increase in the global population of 8.4–8.7 billion by 2030, 9.4–10.2 billion by 2050, and 11 billion by 2100, substantial growth in crop and animal production is necessary. This expansion will lead to higher agricultural usage of antibiotics, water, pesticides, and fertilizers (Fitzpatrick, 2013; Godwin et al., 2022). Food security, according to the Food and Agriculture Organization (FAO), refers to the state where every person consistently has the means physically, socially, and economically to obtain enough safe and nutritious food that aligns with their dietary requirements and preferences, enabling an active and healthy life (Schmidhuber and Tubiello, 2007).

Food security and nutrition are deeply intertwined, particularly in areas where food supplies lack safety (Nelluri and Thota, 2018). In order to enhance food supply, and conserve valuable water and nutrient resources, communities are placing a higher importance on sustainable food production. It's crucial, however, to ensure this is done safely to optimize both public health advantages and environmental gains. Nowadays, food safety faces challenges due to the global nature of food supply chains, the necessity to reduce food waste, and the efficient utilization of natural resources like clean water. Food safety involves protecting a nation's own food supply chain from the introduction, growth, or persistence of harmful microbial and chemical hazards (Uyttendaele et al., 2016). As a result, food safety has become a prominent global public health concern where ensuring food safety has gained greater importance, leading to the introduction of many more surveillance systems, preventive and detective measures to identify any risks that represent a health hazard. Crucially, to minimize food safety risks during food processing, it is essential to consider the entire food chain, starting from the farm and ending at the consumer's table (Rivera et al., 2018).

Food systems encompass the various stages of food production, marketing, transformation, and purchase, as well as the consumer practices, resources, and institutions associated with these processes (Waage et al., 2022). Healthy living necessitates the availability of goods of high quality and safe food (Odeyemi et al., 2020). Managing risks related to food involves examining each stage in the process, from raw materials to consumption, because potential hazards can emerge at

any point in the food chain until it reaches the consumer (Tankosić et al., 2022). In the past decade, ensuring food security has become a significant concern for the global community. Every country, to varying degrees, is grappling with the difficult task of providing its population with enough basic agri-food products of appropriate quality. Within the European Union (EU), maintaining food safety stands as a primary goal for European authorities. This involves regulating quality standards while also enhancing, modernizing, and simplifying existing EU regulations in this area. As per European guidelines, each entity involved in the food supply chain is accountable for food quality. This responsibility is affirmed through compliance documents issued by laboratories, overseen by a network of veterinary sanitary and food safety authorities (Ene, 2020).

European legislation encompasses a variety of rules, regulations, and decisions related to ensuring food safety, categorized into areas such as food, animal health, and plant health. In the year 2000, the European Commission established fundamental principles shaping the Food Safety Policy in Europe. These principles advocate for an integrated strategic approach across the entire food production chain. They emphasize clear delineation of roles for everyone involved, from feed manufacturers and farmers to European institutions, Member States, and consumers. Additionally, they promote a policy that is coherent, effective, and adaptable, incorporating the principle of risk analysis, which encompasses assessment, management, and communication of risks. These principles underscore the greater involvement of scientific institutions and the application of precautionary measures in risk management (Tankosić et al., 2022). Despite all the previous efforts, food safety remains a significant concern globally as a considerable number of individuals globally fall ill from consuming contaminated and unsafe food items. Over the past thirty years, the scientific community has encountered at least four significant food safety challenges. These challenges involve the appearance of novel food pathogens, the contamination of food ingredients, uncertainties about the prolonged consumption of genetically modified food, and the existence of substantial levels of chemical pollutants in our food supply. Several factors contribute to the escalation of diverse food safety concerns (Pinu, 2016), and ensuring food safety depends on preventing the presence of any of these food hazards (Martin et al., 2022).

Food losses affect food quality, safety, economic progress, and the environment. The reasons behind these losses differ worldwide, depending on specific conditions and local contexts

within each country. One main reason for food losses is food practices (Rawat, 2015). Current food system practices are making populations susceptible to a range of health problems (Pradyumna et al., 2019), moreover, these practices are believed to be a major factor in food spoilage (Kadariya et al., 2014). Food spoilage occurs when the original sensory qualities of food degrade from their state during processing. It is defined as a deterioration in food quality that makes it unappealing and unsuitable for consumption. Factors like storage temperature, pH, water content, presence of spoilage microorganisms, processing methods, transportation, and food handling all impact the rate of spoilage. According to the FAO report, one-third of the food produced for human consumption is either spoiled or wasted, making food spoilage a significant global issue requiring attention from various stakeholders (Gram et al., 2002; Odeyemi et al., 2020).

Food hygiene encompasses the conditions and steps required to ensure food safety from production to consumption. Contamination can occur at any stage, including harvesting, processing, storage, distribution, transportation, and preparation. The risk of contamination and cross-contamination is higher in lower socio-economic groups due to inadequate environmental conditions, lack of personal hygiene, insufficient and poor-quality water supplies, unhygienic food preparation and storage, and improper feeding practices. (Abebe et al., 2020; Kamboj et al., 2020). Every community has a vested interest in both food safety and disease prevention. Over the past ten years, the focus on food safety concerns has shifted from chemical problems back to microbial threats. This change is attributed to the identification of new, potentially fatal microorganisms, ongoing widely reported outbreaks transmitted through food and water, and the seeming inability of public health authorities to effectively curb them (Todd, 1997). Microbial food spoilage incurs significant financial losses for the food industry, indirectly affecting consumers by wasting valuable resources, costing millions of Euros annually. While it's technically feasible to produce completely microbe-free food using established methods like gamma irradiation, this drastic approach contradicts the current consumer preference for minimally processed, 'fresh' foods. Consequently, the challenge lies not only in guaranteeing food safety but also in creating high-quality foods that are convenient to prepare, reduce the need for frequent grocery trips, and rely less on chemically produced preservatives (Roller, 1999). The link between food consumption and

human infectious diseases was identified in the early stages of human civilization (Bintsis, 2017). Across history, dietary habits have played a significant role in triggering outbreaks of infectious diseases. If unhealthy eating practices, such as consuming raw or undercooked food and meat from wild animals, persist, infectious diseases will continue to pose a significant risk for outbreaks and epidemics globally. Consequently, it is crucial to alter people's perceptions and understanding of healthy eating to completely eradicate these risky habits and prevent the resurgence of infectious diseases (Butt et al., 2004; Zhou et al., 2020).

2. Infectious diseases associated with agriculture

At the beginning of the twentieth century, food safety faced numerous challenges such as diseased animals, improper food handling, the consumption of raw foods, contaminated water, and lack of sanitation (Cohen, 2000). The bigger market size and broad geographical dispersion of products indicate that if issues arise, a substantial number of people are vulnerable. This scenario has led to major outbreaks, with thousands of cases documented (Todd, 2013). Diseases, particularly infectious ones, pose a significant hurdle to biologically efficient livestock production. Both common and unfamiliar diseases lead to animal deaths and illnesses, resulting in less food than what should ideally be produced in existing farming systems. Moreover, a substantial number of these diseases impact the safety of food supplies, sometimes even more so than their influence on the quantity and quality of food products (Fitzpatrick, 2013).

The COVID-19 situation has refocused health researchers' attention on the significant role food systems can have in contributing to the burden of human diseases. Nevertheless, the potential for new pandemic threats is just a single aspect of the intricate connection between agriculture and infectious diseases (Waage et al., 2022). Even after a century of frequently effective prevention and control attempts, infectious diseases continue to be a significant global public health challenge (Cohen, 2000; Assob et al., 2011; Abdullah et al., 2012). As per the World Health Organization's (WHO) estimates, there were 600 million reported cases of illnesses caused by tainted food in 2010, with nearly 350 million attributed to pathogenic bacteria (Chlebicz and Śliżewska, 2018). Societal shifts, technological advancements, and alterations in microorganisms are all factors leading to the emergence of new diseases, the resurgence of previously controlled ones, food-borne

diseases, and the rise of antimicrobial resistance. Successfully managing infectious diseases in the coming years demands robust public health systems that can swiftly identify, respond to, and prevent these emerging issues (Cohen, 2000). Four major sets of infectious diseases associated with agriculture are endemic and epidemic zoonoses, emerging and re-emerging zoonoses, and foodborne diseases.

2.1 Endemic and epidemic zoonoses

Animal-derived protein serves as a vital nutritional source for numerous individuals globally. However, ensuring its sustainable production poses a significant challenge in the upcoming decades (Nespolo, 2021). One of the significant health concerns associated with livestock involves zoonotic diseases. These diseases can be transmitted between animals and humans. Zoonotic diseases, whether they are common or occur as outbreaks are caused by various pathogens like viruses, bacteria, fungi, chlamydia, rickettsia, and parasites. While some diseases are directly transmitted between animals and humans, many others are spread through consumption of contaminated food products (such as milk and meat), water, and animal waste (Grace, 2019).

Livestock diseases lead to economic losses due to decreased animal populations, reduced productivity, and higher veterinary expenses. Additionally, these diseases pose a risk to human health. The growing demand for food due to the increasing global population has exposed us to pathogens found in the production environment, leading to food-related illnesses (Chlebicz and Śliżewska, 2018). The rise and spread of disease-causing agents that can be transmitted from animals to humans are rapidly increasing. This trend is anticipated to persist due to global connections and human-induced alterations in land use. The threat posed by these zoonotic agents to public health cannot be emphasized enough, as highlighted by the COVID-19 pandemic. COVID-19, which originated in animals and crossed over to humans, serves as a clear example. In fact, some of the most severe disease outbreaks in humans are caused by these zoonotic agents (Owen et al., 2021). Recent zoonotic epidemics have been fueled by a rise in interactions between animals and humans, globalization, agricultural intensification, climate change, and inadequately funded healthcare systems (Judson and Rabinowitz, 2021). A study revealed that more than half of the emerging infectious diseases in humans, around 60%, stem from animals, with the majority,

72%, originating in wildlife. Another estimate suggested an even higher rate, indicating that 75% of emerging pathogens are zoonotic. These diseases, capable of causing global pandemics, have historically led to extensive disruptions, causing massive loss of life, economic instability, and social and political turmoil (Naguib et al., 2021).

2.2 Emerging Infectious Diseases (EIDs)

From the start of the 20th century, infectious diseases have been identified as the leading global cause of mortality (Felgueiras, 2021), this includes emerging pathogens that can be classified as infectious agents that can be newly emerging pathogens, pathogens that have extended to new regions or populations, pathogens that have existed throughout human history but are now recognized as distinct due to infectious agents, and pathogens that were once major health concerns, declined, but are resurging as significant health issues for a considerable portion of the population (Koluman, 2013; Grace, 2019; Sileshi and Gebeyehu 2021). New infectious diseases, caused by the presence and proliferation of pathogens like viruses, bacteria, parasites, and fungi within the host organism, are emerging or re-emerging nearly every year (Felgueiras, 2021), where the rise in the world's population and its aging, along with climate shifts, globalization, and urbanization, has facilitated the emergence, development, and dissemination of these novel pathogens (Lindahl and Grace, 2015; Felgueiras, 2021) causing losses in both human and animal lives, as well as large costs to society (Lindahl and Grace, 2015; Rohr at al., 2019).

From the 1950s onwards, healthcare communities have dealt with the challenge of emerging and reemerging infectious diseases. Emerging pathogens are now widely acknowledged as a significant public health concern in the field of microbiology. Over the past four decades, around 50 emerging infectious agents have been recorded, with approximately 10% of them being bacterial agents (Assob et al., 2011; Vouga and Greub, 2016). EIDs are becoming a greater threat to worldwide food security and public health. Despite advancements in technology, our struggle against these reemerging diseases is proving challenging, leading to escalating treatment expenses and production losses (Brooks et al., 2022). Food safety standards were established to prevent known and re-emerging pathogens, but they have not adequately anticipated new EIDs that affect consumers' worldwide supply chain. In contrast, food security has a broader scope, ensuring the

availability of the following elements global supply chains for domestic animals, crop and plant-based resources, and managed systems in freshwater and marine environments. EIDs restrict food production and distribution, directly and indirectly contributing to global food insecurity. The recent global report on food crises indicates that approximately 103.2 million people in ten countries are currently experiencing food crises due to EIDs, which jeopardize the availability and access to nutritious food, impacting global security and human health (Trivellone et al., 2022).

2.3 Foodborne Diseases (FBD)

The issue of pathogens contaminating the food supply and their ability to persist, grow, multiply, or produce toxins has become a significant worry for public health. The majority of these challenges can be managed through the efforts of food handlers, whether they work in processing plants, restaurants, or other food-related settings. Addressing this contamination issue is crucial for safeguarding public health (Sousa, 2008). Foodborne illnesses and organisms causing food spoilage remain significant threats to both public health and the food industry. Outbreaks of these diseases have led to a considerable loss of life, as well as imposing substantial financial burdens due to healthcare expenses Table 1(Elmi, 2004; Abebe et al., 2020; Ibrahim et al., 2021; Pires et al., 2021). The WHO characterizes foodborne disease as an illness caused by infectious or toxic agents, suspected to be linked to the consumption of contaminated food or water (Kadariya et al., 2014). Over 250 foodborne hazards have been identified, including infectious microorganisms like bacteria, fungi, viruses, protozoa, and parasites, to noninfectious substances such as chemicals and toxins (Martinović et al., 2016; Hoffmann and Scallan, 2017; Castro et al., 2018). These agents can originate from naturally occurring components within the food or can be added accidentally during its production, processing, or preparation. Additionally, many of these agents can be transmitted through means other than food, such as water or contact with infected farm animals, pets, and humans Table 2 (Hoffmann and Scallan, 2017)

As stated in the WHO report, approximately 70% of foodborne illnesses result from contamination by foodborne pathogens (Ge et al., 2022). As these pathogens grow, they release various substances, including toxins, into their surrounding environment. Additionally, when food pathogens break down, other harmful substances can be released, contaminating the food. Many

of these molecules play roles in cellular processes and can reveal diverse mechanisms behind the pathogenicity of foodborne organisms (Martinović et al., 2016). Bacteria are widely considered the primary cause of food- and waterborne outbreaks where, bacterial toxins ranked second, accounting for 19.5% of cases, while in 2015, viruses, which were the most commonly reported agents in 2014, caused 9.2% of total outbreaks. Parasites and other agents, including histamine, were responsible for less than 3% of the outbreaks. Notably, the cause of the outbreak remained unknown in 34% of reported cases (Bintsis, 2017; Elbehiry et al., 2023). Several bacteria like *Escherichia coli*, *Salmonella*, *Staphylococcus aureus, Listeria monocytogenes, Campylobacter, and Clostridium*, as well as viruses like norovirus, are responsible for acute foodborne illnesses that pose a growing concern for both the global food industry and healthcare systems Figure 1 (Castro et al., 2018; Ge et al., 2022; Khan et al., 2023).

The WHO Report states that roughly ten percent of the global population falls ill annually due to food contamination, leading to an average of 1.8 million deaths each year caused by foodborne illnesses (Wei and Zhao, 2021). Zoonotic bacteria, primarily transmitted through meat, dairy products, and eggs, pose significant risks. *S. aureus, Salmonella* species, *Campylobacter* species, *L. monocytogenes*, and *E. coli* are the main bacterial pathogens causing foodborne illnesses and deaths worldwide due to contaminated animal products, where the production of toxins and virulent factors contributes to the pathogenicity of these bacteria (Abebe et al., 2020; Ene, 2020; Lee and Yoon, 2021). According to the WHO, without significant intervention, the worldwide death toll from foodborne diseases is projected to rise to approximately 10 million people each year by 2050 (Ibrahim et al., 2021). Various factors have played a role in altering patterns of foodborne diseases. These factors include the rapid growth of the population and a shift toward an aging demographic. Additionally, globalization in the food supply chain has led to the widespread distribution of foods globally. Changes in dietary habits, such as the preference for raw or lightly cooked food, and the demand for exotic cuisine, have also contributed. Economic development has led to a shift from low- to high-protein foods. Farming practices have changed, including intensification for cheaper food production and a shift to free-range/organic animal farming in response to consumer welfare concerns. Furthermore, climate change has influenced these trends (Newell et al., 2010; Camino et al., 2017).

Table 1: Estimations of the global and certain national levels' annual burden of foodborne illnesses spanning from 2006 to 2016. (Kassem, 2018).

Country-Author	Years of study	Leading Foodborne Pathogens	All Foodborne illnesses/year	Hospitalizations	deaths	(DALYs)*	Unknown Pathogen included	Cost
World, WHO (2015)	2015	Campylobacter spp., nontyphoidal Salmonella enterica, Salmonella Typhi, Taenia solium, hepatitis A virus and Fungi producing aflatoxins.	600 Million of 6.9 Billion (2010) ((8.7%)	NA	420000	18 Millions DLAYs	No	NA
Kirk et al., (2010)	Circa 2010	Norovirus, pathogenic Escherichia coli, Campylobacter spp. and nontyphoidal Salmonella spp.	4.1 Million Of 22.03 Million (2010), 8.65%)	31,920	86	NA**	Yes	NA
Canada, Thomas et al. (2013)	Circa 2006 (2000-2010)	Norovirus, Clostridium perfringens, Campylobacter spp., and nontyphoidal Salmonella spp.	4.0 Million Of 32.5 Million (2006), (12.3%)	NA	NA	NA	Yes	NA
UK, Food Standard Agency (2011)	Circa 2010	Campylobacter, Listeria Monocytogenes, E. coli O157, Salmonella, Norovirus, Clostridium perfringens	1 Million Of 62.77 Million (2010), 1.59%)	20.000	500	NA	NA	£1.5 billion (2008)
Bouwknegt et al., (2017)	2016	Norovirus, Campylobacter spp. And Staphylococcus aureus	672.785 Thousands of 17.02 Million (2016) (4%)	NA	90	4,708 DALYs	No	€171 Million Euro

* DALYs (Disability Adjust Life Years), **NA, Not Applicable

The most effective approach to prevent and manage human foodborne illnesses is through constant monitoring and precise identification of the responsible pathogens. Accurate identification of these pathogens enables food producers and distributors to take necessary measures, ensuring the safety of their products for consumption (Elbehiry et al., 2023). The European surveillance system for multistate foodborne outbreaks combines aspects from public health, animal health, and the food chain to promptly detect, assess, and control these incidents (Sarno et al., 2021). Ensuring food safety involves safe production and handling practices. A successful food safety policy needs to allocate resources where they can most efficiently minimize the risk of foodborne illnesses (Hoffmann and Scallan, 2017).

Table 2: Incidence of foodborne pathogens associated with fresh produce outbreaks in Europe and North America. (Aiyedun et al., 2021)

Foodborne Pathogens		Europe	North America
Bacteria	Campylobacter	-	1 (0.43%)
	Escherichia coli	8 (18.18%)	43 (18.53%)
	Listeria	-	12 (5.17%)
	Salmonella	8 (18.18%)	121 (52.16%)
	Shigella	4 (9.09%)	-
	Yersinia	5 (11.36%)	-
Viruses	Hepatitis A	5 (11.36 %)	4 (1.72%)
	Norovirus	2 (4.55%)	13 (5.60%)
Parasites	Cryptosporidium	9 (20.45%)	1 (0.43%)
	Cyclospora	3 (6.82 %)	37 (15.95%)
	TOTAL	44	232

Maintaining proper hygiene, following effective operational sanitation procedures, and adopting standardized Hazard Analysis and Critical Control Points (HACCP) protocols and pasteurization methods (Abebe et al., 2020), in addition to consumer education about basic principles of food safety, all are effective procedures and methods for foodborne pathogens control and prevention (Tauxe, 1997).

Certainly, it is crucial to identify foodborne pathogens to prevent significant damage to both human and environmental health, (Elbehiry et al., 2023) and it is necessary to quickly track the identification of pathogens responsible for food-borne disease outbreaks (Wei and Zhao, 2021). Hence, food testing is crucial not only for the final food products but also for pre-harvest and food processing settings. This emphasizes the significance of the scientific approaches used in food detection methods and their application. Both the food industry and the government face a significant challenge in obtaining swift information about pathogens in the food chain.

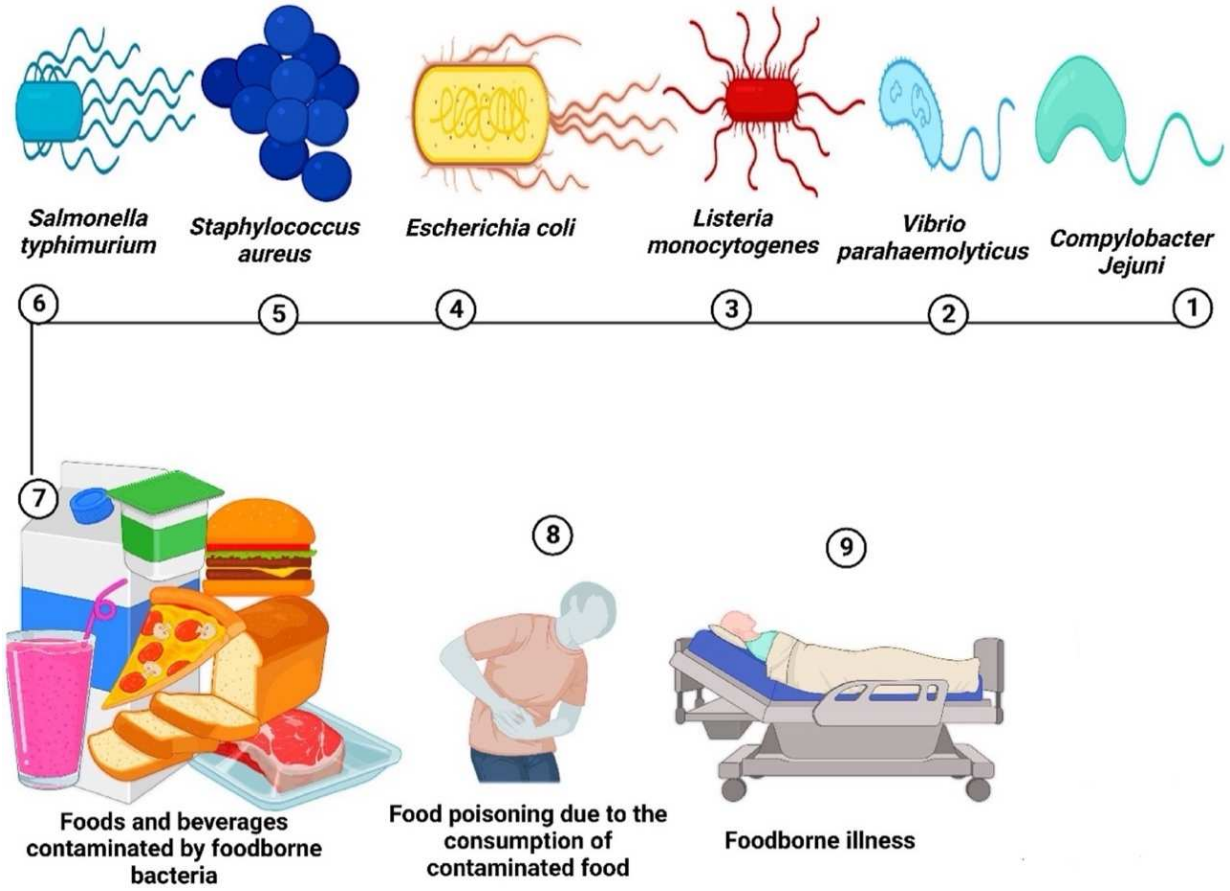

Figure 1: Food contamination by food-borne pathogenic bacteria, resulting in food poisoning and potentially serious illnesses related to food consumption (Khan et al., 2023).

Fast testing techniques enable food producers to respond promptly, preventing the distribution of contaminated food batches that could be linked to an outbreak (Rivera et al., 2018). There is a growing consensus that traditional microbiological diagnostic methods are excessively laborious and time-consuming, especially when it comes to swiftly identifying foodborne pathogens during the urgent requirement for outbreak detection (Wei and Zhao, 2021; Elbehiry et al., 2023). Therefore, there is a demand for new analytical rapid methods to identify contamination from foodborne pathogens. Recent progress in quickly diagnosing and characterizing these pathogens has been facilitated through the application of nucleic acid-based techniques, immunological methods, and biosensor-based technologies, each with distinct targets, speeds, accuracy, and other characteristics (Elbehiry et al., 2023) Figure 2. In recent years, these analytical techniques have experienced rapid advancement, contributing to the food testing industry's projected market

growth of $15 billion by 2019. This accelerated progress is directly related to the rising significance of food safety, particularly in developed nations, as consumers prioritize it. Food safety and quality assurance programs necessitate food microbiology testing as an integral part of their safety systems. In the food industry's monitoring efforts, delayed test outcomes can lead to the distribution of contaminated food products or a surge in human cases (Newell et al., 2010).

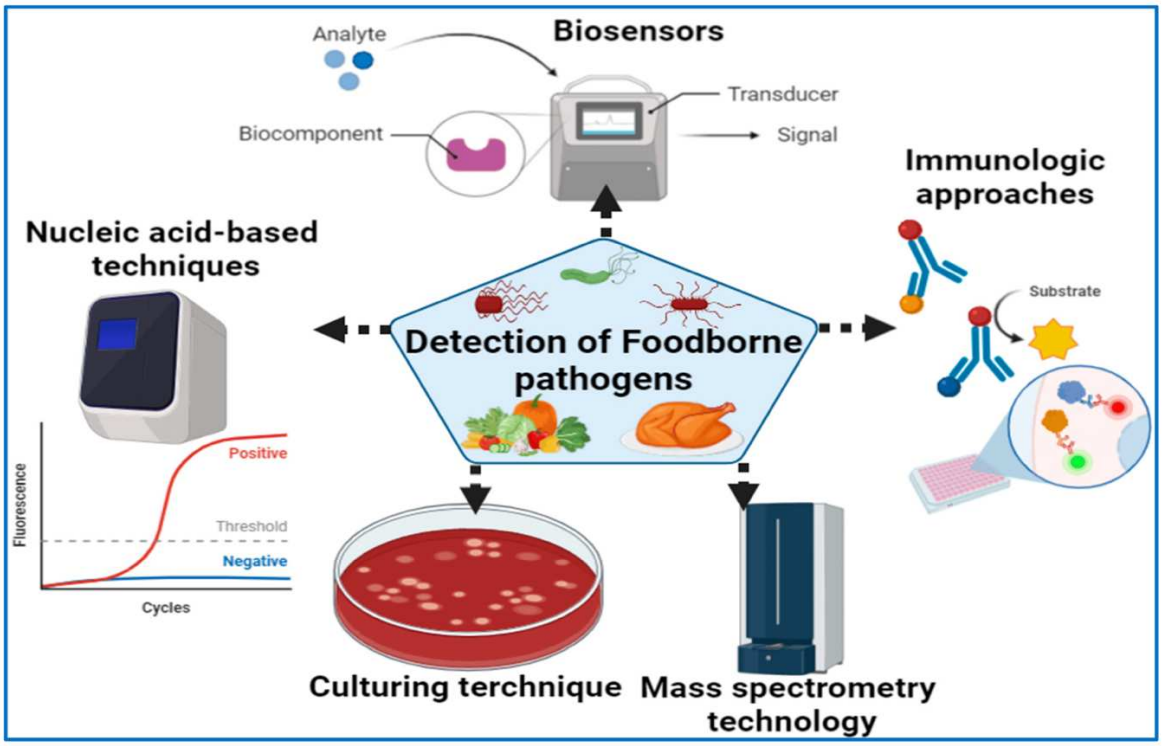

Figure 2: Identification of foodborne pathogens through diverse diagnostic methods (Elbehiry et al., 2023).

3. Food spoilage bacteria

Spoilage microbes invade food in diverse ways, contingent on the food type. High-water-content products like meat, dairy, and seafood are susceptible to bacterial spoilage, in contrast to low-water-content foods, where foods are typically spoiled by molds or yeasts as well (Odeyemi et al., 2020). Food spoilage refers to any change in texture, appearance, smell, or taste those consumers consider to be unacceptable. Spoilage can happen at any point in the food supply chain and may result from factors like insect or physical damage, natural enzyme activity within plants or animals, or microbial infections (Gram et al., 2002; André et al., 2017). Most natural foods have

a finite shelf life. Perishable items like fish, meat, and bread have a brief duration before spoiling. While some foods can be stored for a longer period, they eventually decay. Enzymes break down polymers in certain foods, while chemical processes like oxidation and rancidity degrade others. However, the primary cause of food spoilage is the infiltration of microorganisms such as molds, yeast, and bacteria. Bacterial contamination poses a greater risk because contaminated food often appears normal even if severely infected. Dangerous toxins and bacterial spores often go unnoticed until a food poisoning outbreak occurs, and laboratory analysis reveals the infecting agent. The variety of spoilage microorganisms in food products varies significantly due to different practices followed in their production, formulation, processing, packaging, storage, distribution, and handling (Rawat, 2015). There are three main types of bacterial foodborne illnesses: intoxications, infections, and toxic infections. Bacterial intoxication occurs when preformed bacterial toxins, like those produced by *S. aureus* and *C. botulinum*, are ingested with food. Foodborne infections happen when viable bacteria such as *Salmonella* and *Listeria* are ingested, multiply in the host, and cause illness. Foodborne toxic infections occur when bacteria like *Clostridium* perfringens, present in food, are ingested and later produce toxins in the host's body (Mokoena et al., 2021).

3.1 Staphylococcus aureus

Staphylococcal foodborne poisoning continues to be a widespread global health issue, caused by consuming food contaminated with *S. aureus* enterotoxin. Due to the frequent presence of staphylococci in raw animal-derived foods, it is crucial to process these foods effectively to ensure safety. The interconnections between *Staphylococcus*' taxonomy, ecosystem, and virulence factors play a significant role in understanding its impact on both food safety and public health (Sousa, 2008; Mokoena et al., 2021). Many cases of staphylococcal food poisoning can be linked back to food contamination during preparation due to insufficient refrigeration, improper cooking or heating, and inadequate personal hygiene. The presence of *S. aureus* in food poses a significant public health risk due to its capacity to produce enterotoxins, which can result in food poisoning. In 2014, outbreaks caused by staphylococcal toxins in different foods accounted for 7.5% of all reported outbreaks in the European Union (Bintsis, 2017; Castro et al., 2018). The existence of harmful microorganisms in food items poses potential risks to consumers, leading to serious economic losses and decreased human productivity due to foodborne illnesses. *S. aureus* stands

out as a major contributor to foodborne diseases, causing approximately 241,000 illnesses annually in the United States (Kadariya et al., 2014). Numerous types of staphylococcal bacteria generate enterotoxin, the reason behind staphylococcal food poisoning. Furthermore, staphylococcal intoxication continues to be a prevalent form of food poisoning (Sousa, 2008). According to European Union food regulations, safety standards specifically target staphylococcal enterotoxins (SEs) in dairy products, as even a minute amount of SEs can lead to staphylococcal food poisoning. For instance, a significant outbreak occurred due to chocolate milk contaminated with SEs at a concentration as low as 0.5 ng/mL. SEs comprise a group of nine major types of heat-resistant toxins (SEA, SEB, SEC, SED, SEE, SEG, SEH, SEI, and SEJ) belonging to the extensive pyrogenic toxin superantigen family. The heat stability of *S. aureus* poses a major concern for the food industry. Although the precise mechanisms behind SEs causing food poisoning remain unclear, it is believed that these toxins directly impact the intestinal lining and the vagus nerve, leading to stimulation of the emetic center (Kadariya et al., 2014; Castro et al., 2018). Cooking food thoroughly is crucial, yet the most effective means of preventing staphylococcal food poisoning involves avoiding contamination and cross-contamination and maintaining specific control points. Preserving the cold chain is vital to curb the growth of *S. aureus* in food items. Additional preventive measures encompass regulating raw ingredients, proper handling and processing, thorough cleaning, and disinfection of equipment used in food preparation. Rigorous adherence to microbiological guidelines such as HACCP, Good Manufacturing Practices (GMPs), and Good Hygienic Practices (GHPs) set forth by the WHO and the United States Food and Drug Administration plays a pivotal role in preventing *S. aureus* contamination (Kadariya et al., 2014).

3.2 Listeria monocytogenes

L. monocytogenes is the only species within the *Listeria* genus posing a threat to human health. This facultative intracellular gram-positive bacterium has the potential to induce severe invasive diseases, known as listeriosis, in both humans and animals (Kathariou, 2002). Listeriosis is a severe infection typically contracted by consuming food contaminated with *L. monocytogenes*. This pathogen is widely present in nature and has become a significant concern in foodborne pathogens over the past decade, causing severe illnesses. Although rare, it has a high mortality rate (20–30%), making it one of the most lethal foodborne risks. It particularly endangers pregnant

women, newborns, the elderly, and individuals with weakened immune systems. Contamination occurs when food is mishandled and is often carried asymptomatically by various animal species (Teuber, 1999; Bintsis, 2017; Yeni et al., 2017). The presence and survival of *L. monocytogenes* in the body after consuming contaminated food depend on a complicated interaction involving the food's composition, the susceptibility of the host, and strain phylogeny (Kallipolitis et al., 2020). *L. monocytogenes* causes approximately 1600 cases of illness annually in the United States and around 2500 confirmed invasive human infections in European Union countries (Ceruso et al., 2020). This bacterium can be present in ready-to-eat foods like meats, salads, dairy, and vegetables that people consume, leading to serious infections (Quinlan, 2013; Khan et al., 2023). *L. monocytogenes* continues to pose a significant challenge to the food industry. This bacterium can endure harsh environmental conditions and withstand different stresses such as heat, high salt levels, low pH, and cold temperatures. It can persist on food-contact surfaces for extended periods, enhancing the risk of contamination and food product growth. These traits present challenges for control measures. Therefore, the presence of *L. monocytogenes* in food products remains a major global food safety concern (Shamloo et al., 2019; Abebe et al., 2020; Ceruso et al., 2020). Therefore, implementing GMP in food industries is essential. This includes enhancing food products, refining storage and handling methods, and providing food safety training programs, especially for employees in restaurants, distribution centers, and other food industry staff. Additionally, it's crucial to conduct thorough environmental monitoring in food processing facilities to effectively manage *L. monocytogenes* contamination (Shamloo et al., 2019; Kallipolitis et al., 2020).

3.3 Escherichia coli

E. coli is a Gram-negative, non-spore-forming rod that comprises a large and diverse group of bacteria with the majority being harmless. However, certain strains have developed characteristics, such as toxin production, making them harmful to humans. These pathogenic forms of *E. coli* (referred to as pathovars or pathotypes) are responsible for a significant number of illness and death globally. Many of these pathotypes pose a significant public health risk due to their low infectious doses and their ability to spread through common mediums like food and water. *E. coli* spreads when contaminated food or water, containing fecal matter from infected humans or

animals, is consumed (Notarnicola et al., 2017). A distinct strain of *E. coli* was first identified in 1982 and emerged as a significant zoonotic pathogen related to a US outbreak causing severe illnesses linked to the consumption of undercooked hamburgers. *E. coli*, being zoonotic, poses a public health threat, leading to severe diseases and substantial economic losses. Shiga toxin-producing *E. coli* is one strain that has been linked to life-threatening food-borne outbreaks worldwide. *E. coli* O157:H7 is one of the most important food-borne pathogens that has been reported increasingly from all parts of the world. This strain is an important emerging food-borne pathogen of humans causing outbreaks worldwide, causing outbreaks in countries such as the United States, Canada, Asia, Australia, Europe, and various African nations. Reports suggest that approximately 74,000 cases and 61 deaths annually in the USA are attributed to *E. coli* O157:H7 (Abebe et al., 2020; Khan et al., 2023).

Enterohemorrhagic Escherichia coli (EHEC) induces illness by generating a toxin known as Shiga toxin, which results in severe diseases. Other strains of *E. coli* possessing various virulence traits can also cause foodborne illnesses. *Enterotoxigenic E. coli* (ETEC), for instance, can cause infections in the small intestine when ingested through food. This strain produces enterotoxins and impacts the intestinal epithelial cells in the region it inhabits. ETEC has the capability to produce both heat-stable and heat-labile endotoxins (Ma et al., 2019). In the last decade, most foodborne outbreaks linked to *E. coli* were caused by consuming raw foods contaminated by harmful *E. coli* both at the source and during preparation. These incidents led to significant economic losses and had severe consequences on human health, including fatalities. Numerous studies have aimed to create new antimicrobial drugs and vaccines to combat pathogenic *E. coli* and its associated diseases. However, these therapies and environmental disinfectants are only temporary fixes. Enhancing environmental cleanliness and personal hygiene might be the most effective approach to prevent infections from harmful *E. coli* and subsequent foodborne outbreaks (Yang et al., 2017).

3.4 Salmonella

Salmonella spp. are Gram-negative, rod-shaped, facultative anaerobic organisms (Keba et al., 2020). *Salmonella* is widely acknowledged as one of the most significant pathogens globally. There are more than 2,500 serotypes of Salmonella, some of which can lead to human enteric

infections, commonly referred to as salmonellosis (Ehuwa et al., 2021; Khan et al., 2023). Despite the discovery of new microbiological hazards in food, *Salmonella* spp. remains a crucial indicator of food product safety and is widely spread. These bacteria are common in the environment and can be found in domestic and wild animals, either as pathogens or as normal inhabitants. They can infect humans, primarily through contaminated food. In the EU, salmonellosis, caused by consuming *Salmonella*-contaminated foods, is the second most frequently reported gastrointestinal disorder and a significant cause of foodborne outbreaks. According to the European Centre for Disease Prevention and Control (ECDC), *Salmonella* led to the highest number of human infections, causing illnesses in 91,857 people in the EU in 2018 (Maka and Popowska, 2016; Ehuwa et al., 2021). In the United States *Salmonella* spp. is the primary bacterial source of foodborne illnesses. According to the CDC, over 1 million individuals in the US are affected by *Salmonella* annually. Initially transmitted through contaminated food, *Salmonella* enters the body, traverses the digestive system, and endures the acidic conditions of gastric juice (Bintsis, 2017; Ma et al., 2019).

Salmonella enterica, a species within the *Salmonella* genus, can be categorized into six subspecies, with *S. enterica* subspecies enterica causing 99% of infections in both humans and animals. Meat and meat products, although rich in nutrition and high-quality protein, are major sources of human dietary intake. Paradoxically, they are significant culprits in foodborne illnesses, particularly salmonellosis infections. Outbreaks of *Salmonella* have been linked to various foods such as poultry, beef, fruits, vegetables, and prepared meals. Previous investigations have also identified dairy products like raw milk and soft unpasteurized cheese as sources of Salmonella. The primary mode of transmission for human Salmonella infections is the consumption of foods contaminated through contact with animal feces or natural environmental matter (Sousa, 2008; Keba et al., 2020; Ghaly et al., 2021). Addressing salmonellosis requires collaboration between the public and private sectors. Government regulations and enhanced measures can establish guidelines for both domestic production and international imports. Adhering to proper food hygiene practices, including maintaining cleanliness, segregating raw meat from other foods, cooking at appropriate temperatures, refrigerating foods before and after cooking, and

implementing HACCP protocols at all production stages, significantly can reduce the risk of *Salmonella* spp. contamination (Ehuwa et al., 2021).

4. Antimicrobial resistance

Antibiotics refer to a diverse array of chemical compounds that are naturally occurring, semi-synthetic, or entirely synthetic in origin (Manyi-Loh et al., 2018) that has been employed since the 1950s to treat and prevent diseases, and enhance food production efficiency, meeting the growing demand for food (Allen et al., 2013). Antibiotics are classified according to their impact, either inhibiting bacterial growth or killing bacteria outright. They are also categorized based on their range of effectiveness, falling into either narrow or broad-spectrum categories (Manyi-Loh et al., 2018). Being among the most significant medical breakthroughs of the 20th century, antibiotics played a vital role in the treatment of microbial pathogens (Ge et al., 2022). However, as a result of the inappropriate use of antibiotics in agriculture, as well as in the treatment of humans and animals, instances of infections caused by drug-resistant bacteria have become widespread globally, leading to an ecological imbalance. A report from the WHO indicates that antibiotic-resistant bacteria have reached a critical level in the last decade, posing a severe threat to human health and safety (Teuber, 1999; Zalewska et al., 2021; Ge et al., 2022). The rapid advancement in antibiotic development, known as the "Golden Age," which began in the 1940s, has significantly slowed down. Between the 1980s and the early 2000s, there was a drastic 90% decrease in the approval of new antibiotics and the discovery of new classes. The approval of systemic antibiotics by the U.S. Food and Drug Administration (FDA) also dropped by 90% over the past 30 years. Antimicrobial resistance (AMR) is estimated to cause 25,000 deaths annually in the European Union and 700,000 worldwide. Predictions suggest that by 2050, AMR will surpass cancer as a leading cause of death. The increasing threat of antimicrobial resistance, coupled with the decline in antibiotic development, has raised concerns about entering a post-antibiotic era (Stanton, 2013; Luepke et al., 2017; Zalewska et al., 2021).

European nations have enforced prohibitions on the utilization of antibiotics for promoting growth, and in the USA, this practice is facing growing regulatory and political scrutiny (Allen et al., 2013) AMR, primarily caused by the imprudent use of antibiotics in agriculture, environment,

and both animal and human medicine, is widely acknowledged as a major global health issue. It poses threats to food security and the health of humans and animals, leading to significant economic losses. According to the WHO, the level of antibiotic resistance observed in the past decade is a grave concern for global health. The extensive use of antibiotics in agriculture, for purposes such as growth promotion and disease prevention, has serious consequences for producing farm animals, food safety, and human health. In the EU and the USA, agriculture accounts for more than 75% of the annual antibiotic consumption (Caniça et al., 2019). As a result, resistance developed in agriculture can add to the overall public health risk where food has the potential to carry both antimicrobial-resistant bacteria and resistance genes that can provide a direct infection hazard following ingestion or food handling (Newell et al., 2010). One famous example of AMR is Methicillin-resistant *Staphylococcus aureus* (MRSA) which emerged in 1961 and has caused significant illness and death globally. Apart from causing staphylococcal infections, *S. aureus* is also responsible for food poisoning due to ingesting toxins in contaminated foods. Despite the availability of effective antibiotics against staphylococcal infections, MRSA remains a significant threat, leading to high rates of illness and death. Compared to methicillin-susceptible *S. aureus* (MSSA), MRSA strains generally result in more severe illness, longer hospital stays, and higher healthcare costs (Castro et al., 2018).

The WHO has published the first-ever list of antibiotic-resistant priority pathogens that pose a significant threat to human health. This list includes *Pseudomonas aeruginosa*, *E. coli*, *S. aureus*, *Campylobacter* spp., *Salmonellae*, and *Streptococcus pneumoniae*. Hence, there is an urgent requirement for new antibacterial agents to combat the rise of microbial antibiotic resistance. Plants could offer a promising solution, providing a wide array of chemotherapeutic compounds in the form of secondary metabolites. These metabolites, also known as phytochemicals, encompass alkaloids, flavonoids, quinones, phenols, coumarins, and more. Many of these phytochemicals have demonstrated effectiveness against the molecular factors responsible for drug resistance in pathogens, such as membrane proteins, biofilms, efflux pumps, and bacterial cell communications (Khare et al., 2021).

5. *Plant-based phytochemicals as a possible alternative to antibiotics*

Traditional medicine has a history of utilizing natural sources to address infectious diseases. Various screening methods, including analyzing plant components like roots, leaves, stems, and fruits, have revealed powerful compounds believed to enhance their therapeutic properties (Abdullah et al., 2012; El-Saadony et al., 2023). Preventing, reducing, or reversing antimicrobial resistance can be achieved through various methods, and using medicinal plant extracts with inherent antimicrobial properties has proven to be relatively effective. According to a survey conducted by the United Nations Conference on Trade and Development, over 33% of drugs produced by industrialized nations are derived from plants. The WHO has identified more than 20,000 species of medicinal plants with diverse potential uses. Medicinal plants are often more cost-effective, safer in terms of side effects, and readily accessible compared to their synthetic counterparts. These bioactive compounds, also known as phytochemicals, include substances like tannins, alkaloids, carbohydrates, glycosides, terpenoids, steroids, flavonoids, and coumarins (Abdallah, 2011; Cheesman et al., 2017).

The phytochemicals' activity varies significantly based on the species, geographical location, and climate of their country of origin, potentially containing diverse categories of active components. (Assob et al., 2011; Savoia, 2012). Many of these phytochemicals consist of chemical compounds that demonstrate antioxidant, antimicrobial, anti-inflammatory, anticoccidial, and anthelmintic properties (Chandra et al., 2017; Denli and Demirel, 2018; Bittner et al., 2021; Łojewska and Sakowicz, 2021).

From 2015 to 2019, approximately 11,000 research papers were published globally, exploring the antibacterial characteristics of plants. These studies, conducted both in laboratory settings and living organisms, showcased the antibacterial effects of numerous plants against different types of bacteria (Savoia, 2012; Arsene et al., 2021). For example, allicin, derived from garlic *Allium sativum*, serves as a potent inhibitor against a wide array of bacteria, both gram-negative and gram-positive. Its impact extends to bacteria such as *Escherichia, Salmonella, Streptococcus, Staphylococcus, Klebsiella, and Proteus*. Notably, all serogroups of *E. coli*, particularly

Enterotoxigenic E. coli (specifically serogroup O8), have been found to be susceptible to the effects of garlic extract (Łojewska and Sakowicz, 2021).

Clove water extract demonstrates effectiveness against a range of bacteria, including *E. coli, S. aureus, and P. aeruginosa* (Gupta and Sharma, 2020). Scientific literature research findings indicate that methanolic extracts from the guava plant *Psidium guajava* have antimicrobial properties against various bacteria, both gram-positive like *B. subtilis* and *S. aureus* and gram-negative such as *E. coli* and *S. enteritidis*. The phenolic compounds in *P. guajava* were found to disrupt the structure and function of bacterial cell membranes, explaining their antibacterial effect (El-Saadony et al., 2023). In another study, the inhibitory activity of concentrates from 14 Brazilian plants against MRSA strains was reported (Betoni et al., 2006).

The mechanisms by which plant-derived compounds function could offer alternative resistance-modifying agents due to the diverse secondary metabolites they contain. It is theorized that these compounds disrupt the organization of cellular membranes, reducing membrane potential and ATP synthesis. Additionally, they might alter membrane permeability, chelate metal ions, and interfere with the activity of membrane-bound ATPase. These actions change the physiological state of bacteria, leading to bacterial death (AlSheikh et al., 2020; Ghaly et al., 2021). Moreover, plant extracts can bind to protein domains, altering or inhibiting protein-protein interactions. This capability allows these herbal extracts to effectively regulate essential host-related cellular processes like immune response, mitosis, apoptosis, and signal transduction. Consequently, their impact extends beyond simply killing microorganisms; they influence crucial stages in the pathogenic process. As a result, bacteria, fungi, and viruses might find it harder to develop resistance against botanical substances (Gupta and Birdi, 2017). The active components found in natural remedies such as garlic, ginger, and turmeric share a common method of affecting bacteria. They work by decreasing or preventing the growth of biofilms, making bacteria as responsive as antibacterial herbal medications. Similarly, the active elements in clove and tulip function in a comparable manner, hindering the production of genetic materials like DNA and proteins and impeding bacterial growth Figure 3 (AlSheikh et al., 2020; Gupta and Sharma, 2020).

6. *Application of by-products in the food industry*

Nowadays, the agro-food industry produces substantial by-products containing valuable compounds with significant functionality and/or bioactivity. Moreover, there has been a growing consumer demand for healthier food products in recent years, prompting the food industry to respond to this demand. By-products, typically originating from primary agro-food production processes, serve as valuable and cost-effective sources of potential functional ingredients, including peptides, carotenoids, and phenolic compounds. This approach promotes the concept of a circular economy (Ayala-Zavala et al., 2011; Gowe, 2015; Faustino et al., 2019).

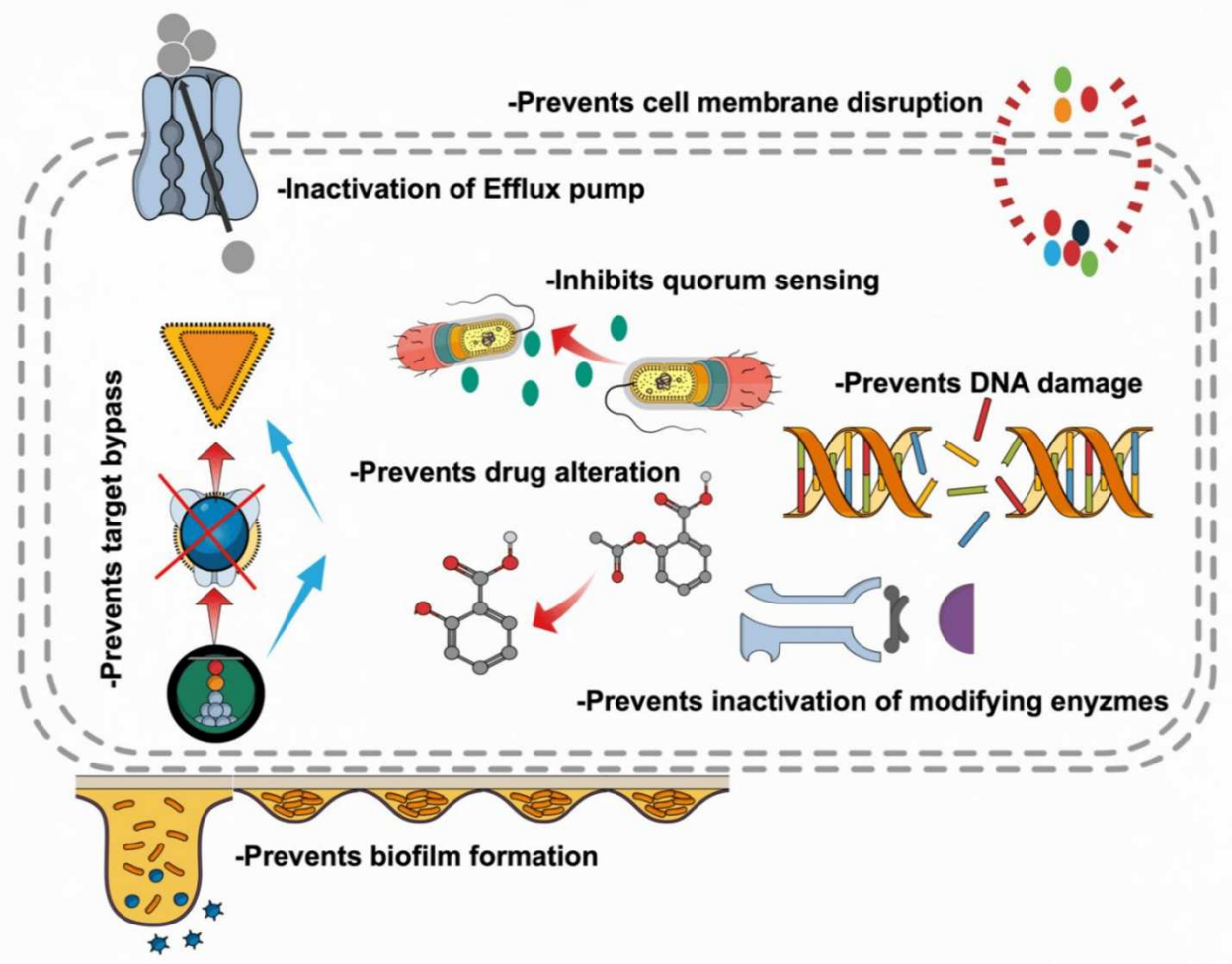

Figure 3: Phytochemicals' role as antimicrobials: their mode of action and efficacy against microorganisms (AlSheikh et al., 2020).

Recent research from FAO reveals that approximately 1.3 billion tons of food are wasted globally each year, constituting one-third of the total food industry output. Fruits and vegetables account for the majority of this loss, totalling 0.5 billion tons (Gowe, 2015). The EU has devised an action plan within the circular economy framework to curb food waste. This plan focuses on strategic measures involving the reduction, reuse, recovery, and recycling of materials and energy. Its objective is to enhance the value and extend the useful lifespan of products, materials, and resources within the economy. Repurposing by-products from the agro-industry can serve as a renewable reservoir for existing food additives or even give rise to new high-value ingredients containing functional compounds. This approach benefits the entire food system. These by-products contain polysaccharides, organic acids, proteins, and various other compounds. Utilizing them incurs no additional production costs and reduces industrial expenses, making them a plentiful and cost-effective source of natural compounds potentially applicable in the food industry as additives (Faustino et al., 2019).

Previous research has demonstrated the successful integration of by-products and their extracts into food products. For example, phenolic compounds extracted from eggplant could serve as a versatile food additive possessing antimicrobial, antioxidant, and food coloring properties. Avocado peel and seed extracts exhibited activity against yeast, as well as both gram-negative and gram-positive bacteria. Various antimicrobial packaging systems, including those utilizing lemon by-product extracts, have been employed to preserve Mozzarella cheese. The outcomes revealed an extended shelf life for all Mozzarella cheeses packaged with these active methods, indicating that lemon extract could inhibit the microorganisms responsible for spoilage without affecting the product's essential microorganisms (Ayala-Zavala et al., 2011; Faustino et al., 2019).

Pomegranate (*Punica granatum* L.) constitutes a naturally rich source of phenolic compounds. It is usually consumed fresh or commercialized in the form of juice. Global pomegranate production reached approximately 3.8 million tons in 2017. During the processing of pomegranate juice, a significant amount of waste is generated, primarily consisting of peel (about 78%), which holds potential for further utilization (Kaderides et al., 2021). The health benefits of pomegranates vary significantly based on factors like varieties, growing conditions, cultivation methods,

developmental stages, and extraction techniques. PPE has been tested alone and in combination with edible films and coatings for food preservation (Chen et al., 2020).

Pomegranate by-products, particularly the peels resulting from pomegranate food processing, have gained significant interest because of their scientifically verified medicinal properties (Chen et al., 2020). Pomegranate peels contain substantial quantities of secondary metabolites, primarily phenolic acids (such as gallic acid, ellagic acid, and caffeic acid), flavonoids (including flavonols like catechin, gallocatechin, and epicatechin, as well as anthocyanins), and hydrolyzable tannins (like punicalagin and gallotannins). Numerous studies have linked these phenolic compounds to various biological activities and health advantages, including antioxidant, antimicrobial, anti-inflammatory, wound healing, antimutagenic, anticarcinogenic, and antihypertensive effects Figure 4 (Ayala-Zavala et al., 2011; Kaderides et al., 2021; Singh et al., 2023).

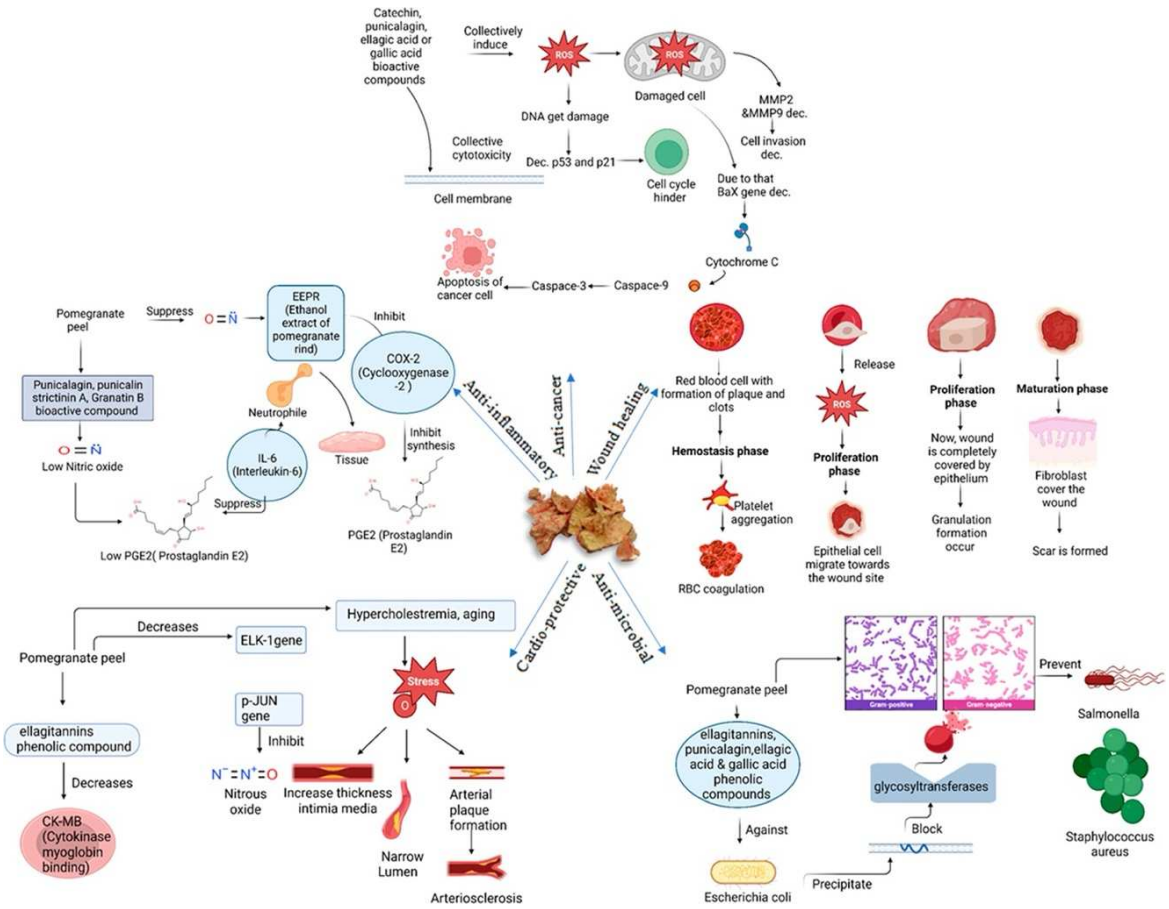

Figure 4: Pharmacological characteristics of the peel of the pomegranate fruit peels (Singh et al., 2023).

The broad variation in content can be attributed to factors such as the specific pomegranate variety, sample preparation methods, extraction solvent, and techniques utilized, among other variables (Kaderides et al., 2021). Several scientific studies have indicated that pomegranate peel extract (PPE) displays strong antimicrobial properties against various foodborne pathogens and enhances the shelf life of food products after harvest. The antioxidant properties of PPEs are attributed to their content of phenolic acids, flavonoids, and tannins, with ellagitannins being particularly responsible for the peels' antioxidant effects. It is noted that both the levels of these phytochemicals and the antioxidant activity are significantly influenced by the solvents utilized during peel extraction. The concentrations of phenolic acids, flavonoids, and tannins in PPEs are primarily determined by the extraction method employed, which significantly impacts the biochemical composition. Traditional methods typically require substantial solvent quantities and yield low extraction rates. Furthermore, the use of elevated temperatures during extraction has been reported to lead to degradation in the resulting extracts (Chen et al., 2020).

These polyphenolic compounds not only contribute to the antioxidant properties but also enhance the antimicrobial activity of the extracts against various microorganisms. These include *S. aureus, E. coli, L. monocytogenes, S. Enteritidis, Aspergillus niger, Saccharomyces cerevisiae, Fusarium sambucinum, Penicillium italicum, B. subtilis, K. pneumoniae, S. Typhi, Yersinia enterocolitica*, and *Candida albicans* (de Almeida and de Aquino, 2023; Salim et al., 2023). An aqueous extract from pomegranate peels was tested as an additive to prolong the shelf life of meat products. The extract was observed to inhibit protein and lipid oxidation and demonstrate antimicrobial effects against various pathogenic strains. Another study found that water-based pomegranate peel extracts displayed significant in vitro antimicrobial activity against microorganisms like *B. subtilis, S. aureus, E. coli, Enterobacter aerogenes, Serratia marcescens, Brucella spp., Saccharomyces cerevisiae, Rhodotorula glutinis,* and *S. Typhimurium*, comparable to the effects of acetone and ethanol extracts (Betoni et al., 2006; Chen et al., 2020; Salim et al., 2023).

In this context, employing fruit by-products as a valuable reservoir of natural food additives presents a promising avenue for discovering new phenolic antimicrobial compounds. This not only provides fresh business prospects for the food industry but also emerges as a viable solution for addressing environmental challenges. Additionally, it offers an opportunity to create food additives or supplements with substantial nutritional value, proving economically attractive. The transformation of these by-products into high value "products" enables companies to cut treatment costs and potentially generate extra revenue, thereby enhancing their competitiveness in the market.

7. References

Abdallah, E. M. (2011). Plants: An alternative source for antimicrobials. *Journal of Applied Pharmaceutical Science*, (Issue), 16-20.

Abdullah, E., Raus, R. A., & Jamal, P. (2012). Extraction and evaluation of antibacterial activity from selected flowering plants. *American Medical Journal*, *3*(1), 27-32.

Abebe, E., Gugsa, G., & Ahmed, M. (2020). Review on major food-borne zoonotic bacterial pathogens. *Journal of tropical medicine*, *2020*.

Aiyedun, S. O., Onarinde, B. A., Swainson, M., & Dixon, R. A. (2021). Foodborne outbreaks of microbial infection from fresh produce in Europe and North America: a systematic review of data from this millennium. International Journal of Food Science & Technology, 56(5), 2215-2223.

Allen, H. K., Levine, U. Y., Looft, T., Bandrick, M., & Casey, T. A. (2013). Treatment, promotion, commotion: antibiotic alternatives in food-producing animals. *Trends in microbiology*, *21*(3), 114-119.

André, S., Vallaeys, T., & Planchon, S. (2017). Spore-forming bacteria responsible for food spoilage. *Research in Microbiology*, *168*(4), 379-387.

AlSheikh, H. M. A., Sultan, I., Kumar, V., Rather, I. A., Al-Sheikh, H., Tasleem Jan, A., & Haq, Q. M. R. (2020). Plant-based phytochemicals as possible alternative to antibiotics in combating bacterial drug resistance. *Antibiotics*, *9*(8), 480.

Arsene, M. M. J., Jorelle, A. B., Sarra, S., Viktorovna, P. I., Davares, A. K., Ingrid, N. K., ... & Carime, B. Z. (2021). Short review on the potential alternatives to antibiotics in the era of antibiotic resistance. *Journal of Applied Pharmaceutical Science*, *12*(1), 029-040.

Assob, J. C., Kamga, H. L., Nsagha, D. S., Njunda, A. L., Nde, P. F., Asongalem, E. A., ... & Penlap, V. B. (2011). Antimicrobial and toxicological activities of five medicinal plant species from Cameroon Traditional Medicine. *BMC complementary and alternative medicine*, *11*(1), 1-11.

Ayala-Zavala, J. F. N., Vega-Vega, V., Rosas-Domínguez, C., Palafox-Carlos, H., Villa-Rodriguez, J. A., Siddiqui, M. W., ... & González-Aguilar, G. A. (2011). Agro-industrial potential of exotic fruit byproducts as a source of food additives. *Food Research International*, *44*(7), 1866-1874.

Betoni, J. E. C., Mantovani, R. P., Barbosa, L. N., Di Stasi, L. C., & Fernandes Junior, A. (2006). Synergism between plant extract and antimicrobial drugs used on Staphylococcus aureus diseases. *Memórias do Instituto Oswaldo Cruz*, *101*, 387-390.

Bintsis, T. (2017). Foodborne pathogens. *AIMS microbiology*, *3*(3), 529.

Bittner Fialová, S., Rendeková, K., Mučaji, P., Nagy, M., & Slobodníková, L. (2021). Antibacterial activity of medicinal plants and their constituents in the context of skin and wound infections, considering European legislation and folk medicine—A review. *International Journal of Molecular Sciences*, *22*(19), 10746.

Broglia, A., & Kapel, C. (2011). Changing dietary habits in a changing world: emerging drivers for the transmission of foodborne parasitic zoonoses. *Veterinary parasitology*, *182*(1), 2-13.

Brooks, D. R., Hoberg, E. P., Boeger, W. A., & Trivellone, V. (2022). Emerging infectious disease: an underappreciated area of strategic concern for food security. *Transboundary and Emerging Diseases*, *69*(2), 254-267.

Butt, A. A., Aldridge, K. E., & Sander, C. V. (2004). Infections related to the ingestion of seafood. Part II: parasitic infections and food safety. *The Lancet infectious diseases*, *4*(5), 294-300.

Camino Feltes, M. M., Arisseto-Bragotto, A. P., & Block, J. M. (2017). Food quality, food-borne diseases, and food safety in the Brazilian food industry. *Food Quality and Safety*, *1*(1), 13-27.

Caniça, M., Manageiro, V., Abriouel, H., Moran-Gilad, J., & Franz, C. M. (2019). Antibiotic resistance in foodborne bacteria. *Trends in Food Science & Technology*, *84*, 41-44.

Carocho, M., Morales, P., & Ferreira, I. C. (2015). Natural food additives: Quo vadis?. *Trends in food science & technology*, *45*(2), 284-295.

Castro, A., Silva, J., & Teixeira, P. (2018). Staphylococcus aureus, a food pathogen: Virulence factors and antibiotic resistance. In *Foodborne Diseases* (pp. 213-238). Academic Press.

Ceruso, M., Clement, J. A., Todd, M. J., Zhang, F., Huang, Z., Anastasio, A., ... & Liu, Y. (2020). The inhibitory effect of plant extracts on growth of the foodborne pathogen, Listeria monocytogenes. *Antibiotics*, *9*(6), 319.

Chandra, H., Bishnoi, P., Yadav, A., Patni, B., Mishra, A. P., & Nautiyal, A. R. (2017). Antimicrobial resistance and the alternative resources with special emphasis on plant-based antimicrobials—a review. *Plants*, *6*(2), 16.

Cheesman, M. J., Ilanko, A., Blonk, B., & Cock, I. E. (2017). Developing new antimicrobial therapies: are synergistic combinations of plant extracts/compounds with conventional antibiotics the solution?. *Pharmacognosy reviews*, *11*(22), 57.

Chen, J., Liao, C., Ouyang, X., Kahramanoğlu, I., Gan, Y., & Li, M. (2020). Antimicrobial activity of pomegranate peel and its applications on food preservation. *Journal of Food Quality*, *2020*, 1-8.

Chlebicz, A., & Śliżewska, K. (2018). Campylobacteriosis, salmonellosis, yersiniosis, and listeriosis as zoonotic foodborne diseases: a review. *International journal of environmental research and public health*, *15*(5), 863.

Cohen, M. L. (2000). Changing patterns of infectious disease. *Nature*, *406*(6797), 762-767.

de Almeida Soares, L., & de Aquino Santana, L. C. L. (2023). Physicochemical Characterization, Antioxidant and Antimicrobial Potential of Biodegradable Chitosan-Based Films Containing Pomegranate (Punica granatum L.) Peel Extract. *Journal of Polymers and the Environment*, 1-12.

Denli, M., & Demirel, R. (2018). Replacement of antibiotics in poultry diets. *CABI Reviews*, (2018), 1-9.

Ehuwa, O., Jaiswal, A. K., & Jaiswal, S. (2021). Salmonella, food safety and food handling practices. *Foods*, *10*(5), 907.

Elbehiry, A., Abalkhail, A., Marzouk, E., Elmanssury, A. E., Almuzaini, A. M., Alfheeaid, H., ... & Abu-Okail, A. (2023). An Overview of the Public Health Challenges in Diagnosing and Controlling Human Foodborne Pathogens. *Vaccines*, *11*(4), 725.

Elmi, M. (2004). Food safety: current situation, unaddressed issues and the emerging priorities. *EMHJ-Eastern Mediterranean Health Journal, 10 (6), 794-800, 2004*.

El-Saadony, M. T., Zabermawi, N. M., Zabermawi, N. M., Burollus, M. A., Shafi, M. E., Alagawany, M., ... & Abd El-Hack, M. E. (2023). Nutritional aspects and health benefits of bioactive plant compounds against infectious diseases: a review. *Food Reviews International*, *39*(4), 2138-2160.

Ene, C. (2020). Food security and food safety: Meanings and connections. *Econ Insights Trends Challs IX (1)*, 59-68.

Faustino, M., Veiga, M., Sousa, P., Costa, E. M., Silva, S., & Pintado, M. (2019). Agro-food byproducts as a new source of natural food additives. *Molecules*, *24*(6), 1056.

Felgueiras, H. P. (2021). An insight into biomolecules for the treatment of skin infectious diseases. *Pharmaceutics*, *13*(7), 1012.

Fitzpatrick, J. L. (2013). Global food security: the impact of veterinary parasites and parasitologists. *Veterinary parasitology*, *195*(3-4), 233-248.

Ge, H., Wang, Y., & Zhao, X. (2022). Research on the drug resistance mechanism of foodborne pathogens. *Microbial Pathogenesis*, *162*, 105306.

Ghaly, M. F., Nasr, Z. M., Abousaty, A. I., Seadawy, H. G., Shaheen, M. A., Albogami, S., ... & Bendary, M. M. (2021). Alternative and complementary therapies against foodborne Salmonella infections. *Antibiotics*, *10*(12), 1453.

Godwin, E. J. O., Chandrasekaran, V., Smah, A. C., & Faith, E. O. (2022). Emerging Infectious Food System Related Zoonotic Foodborne Disease-A Threat to Global Food Safety and Nutrition Security.

Gowe, C. (2015). Review on potential use of fruit and vegetables by-products as a valuable source of natural food additives. *Food Science and Quality Management*, *45*(5), 47-61.

Grace, D. (2019). Infectious diseases and agriculture. *Encyclopedia of food security and sustainability*, 439.

Gram, L., Ravn, L., Rasch, M., Bruhn, J. B., Christensen, A. B., & Givskov, M. (2002). Food spoilage—interactions between food spoilage bacteria. *International journal of food microbiology*, *78*(1-2), 79-97.

Gupta, P. D., & Birdi, T. J. (2017). Development of botanicals to combat antibiotic resistance. *Journal of Ayurveda and integrative medicine*, *8*(4), 266-275.

Gupta, R., & Sharma, S. (2020). Herbal antibiotics: A Review. *Bull. Env. Pharmacol. Life Sci*, *9*, 136-142.

Hoffmann, S., & Scallan, E. (2017). Epidemiology, cost, and risk analysis of foodborne disease. In *Foodborne Diseases* (pp. 31-63). Academic Press.

Ibrahim, S. A., Ayivi, R. D., Zimmerman, T., Siddiqui, S. A., Altemimi, A. B., Fidan, H., ... & Bakhshayesh, R. V. (2021). Lactic acid bacteria as antimicrobial agents: Food safety and microbial food spoilage prevention. *Foods*, *10*(12), 3131.

Judson, S. D., & Rabinowitz, P. M. (2021). Zoonoses and global epidemics. *Current Opinion in Infectious Diseases*, *34*(5), 385-392.

Kadariya, J., Smith, T. C., & Thapaliya, D. (2014). Staphylococcus aureus and staphylococcal food-borne disease: an ongoing challenge in public health. *BioMed research international*, *2014*.

Kaderides, K., Kyriakoudi, A., Mourtzinos, I., & Goula, A. M. (2021). Potential of pomegranate peel extract as a natural additive in foods. *Trends in Food Science & Technology*, *115*, 380-390.

Kallipolitis, B., Gahan, C. G., & Piveteau, P. (2020). Factors contributing to Listeria monocytogenes transmission and impact on food safety. *Current Opinion in Food Science*, *36*, 9-17.

Kamboj, S., Gupta, N., Bandral, J. D., Gandotra, G., & Anjum, N. (2020). Food safety and hygiene: A review. International Journal of Chemical Studies, 8(2), 358-368.

Kassem, A. Z. E. (2018). Cost of Foodborne Illnesses: A literature Review. Food Safety Science, Health and Behaviour, 1-10.

Kathariou, S. (2002). Listeria monocytogenes virulence and pathogenicity, a food safety perspective. *Journal of food protection*, *65*(11), 1811-1829.

Kearney, J. (2010). Food consumption trends and drivers. *Philosophical transactions of the royal society B: biological sciences*, *365*(1554), 2793-2807.

Keba, A., Rolon, M. L., Tamene, A., Dessie, K., Vipham, J., Kovac, J., & Zewdu, A. (2020). Review of the prevalence of foodborne pathogens in milk and dairy products in Ethiopia. *International dairy journal*, *109*, 104762.

Khan, F. M., Chen, J. H., Zhang, R., & Liu, B. (2023). A comprehensive review of the applications of bacteriophage-derived endolysins for foodborne bacterial pathogens and food safety: recent advances, challenges, and future perspective. *Frontiers in Microbiology*, *14*.

Khare, T., Anand, U., Dey, A., Assaraf, Y. G., Chen, Z. S., Liu, Z., & Kumar, V. (2021). Exploring phytochemicals for combating antibiotic resistance in microbial pathogens. *Frontiers in pharmacology*, *12*, 720726.

Koluman, A., & Dikici, A. (2013). Antimicrobial resistance of emerging foodborne pathogens: status quo and global trends. *Critical reviews in microbiology*, *39*(1), 57-69.

Lee, H., & Yoon, Y. (2021). Etiological agents implicated in foodborne illness world wide. *Food science of animal resources*, *41*(1), 1.

Lindahl, J. F., & Grace, D. (2015). The consequences of human actions on risks for infectious diseases: a review. *Infection ecology & epidemiology*, *5*(1), 30048.

Łojewska, E., & Sakowicz, T. (2021). An alternative to antibiotics: selected methods to combat zoonotic foodborne bacterial infections. *Current microbiology*, 1-13.

Luepke, K. H., Suda, K. J., Boucher, H., Russo, R. L., Bonney, M. W., Hunt, T. D., & Mohr III, J. F. (2017). Past, present, and future of antibacterial economics: increasing bacterial resistance, limited antibiotic pipeline, and societal implications. *Pharmacotherapy: The Journal of Human Pharmacology and Drug Therapy*, *37*(1), 71-84.

Ma, Y., Ding, S., Fei, Y., Liu, G., Jang, H., & Fang, J. (2019). Antimicrobial activity of anthocyanins and catechins against foodborne pathogens Escherichia coli and Salmonella. *Food Control*, *106*, 106712.

Maka, L., & Popowska, M. (2016). Antimicrobial resistance of Salmonella spp. isolated from food. *Roczniki Państwowego Zakładu Higieny*, *67*(4).

Manyi-Loh, C., Mamphweli, S., Meyer, E., & Okoh, A. (2018). Antibiotic use in agriculture and its consequential resistance in environmental sources: potential public health implications. *Molecules*, *23*(4), 795.

Martin, C. S., Jubelin, G., Darsonval, M., Leroy, S., Leneveu-Jenvrin, C., Hmidene, G., ... & Dubois-Brissonnet, F. (2022). Genetic, physiological, and cellular heterogeneities of bacterial pathogens in food matrices: Consequences for food safety. *Comprehensive Reviews in Food Science and Food Safety*, *21*(5), 4294-4326.

Martinović, T., Andjelković, U., Gajdošik, M. Š., Rešetar, D., & Josić, D. (2016). Foodborne pathogens and their toxins. *Journal of proteomics*, *147*, 226-235.

Mokoena, M. P., Omatola, C. A., & Olaniran, A. O. (2021). Applications of lactic acid bacteria and their bacteriocins against food spoilage microorganisms and foodborne pathogens. *Molecules*, *26*(22), 7055.

Naguib, M. M., Li, R., Ling, J., Grace, D., Nguyen-Viet, H., & Lindahl, J. F. (2021). Live and wet markets: food access versus the risk of disease emergence. *Trends in microbiology*, *29*(7), 573-581.

Nelluri, K. D., & Thota, N. S. (2018). Challenges in emerging food-borne diseases. In *Food safety and preservation* (pp. 231-268). Academic Press.

Nespolo, N. M. (2021). The behavior of consumers and producers of food of animal origin and their impacts in one health. *Frontiers in Veterinary Science*, *8*, 641634.

Newell, D. G., Koopmans, M., Verhoef, L., Duizer, E., Aidara-Kane, A., Sprong, H., ... & Kruse, H. (2010). Food-borne diseases—the challenges of 20 years ago still persist while new ones continue to emerge. *International journal of food microbiology*, *139*, S3-S15.

Notarnicola, B., Tassielli, G., Renzulli, P. A., Castellani, V., & Sala, S. (2017). Environmental impacts of food consumption in Europe. *Journal of cleaner production*, *140*, 753-765.

Odeyemi, O. A., Alegbeleye, O. O., Strateva, M., & Stratev, D. (2020). Understanding spoilage microbial community and spoilage mechanisms in foods of animal origin. *Comprehensive reviews in food science and food safety*, *19*(2), 311-331.

Owen, J. C., Landwerlen, H. R., Dupuis, A. P., Belsare, A. V., Sharma, D. B., Wang, S., ... & Kramer, L. D. (2021). Reservoir hosts experiencing food stress alter transmission dynamics for a zoonotic pathogen. *Proceedings of the Royal Society B*, *288*(1956), 20210881.

Pinu, F. R. (2016). Early detection of food pathogens and food spoilage microorganisms: Application of metabolomics. *Trends in Food Science & Technology*, *54*, 213-215.

Pires, S. M., Desta, B. N., Mughini-Gras, L., Mmbaga, B. T., Fayemi, O. E., Salvador, E. M., ... & Devleesschauwer, B. (2021). Burden of foodborne diseases: Think global, act local. *Current Opinion in Food Science*, *39*, 152-159.

Pradyumna, A., Egal, F., & Utzinger, J. (2019). Sustainable food systems, health and infectious diseases: concerns and opportunities. *Acta tropica*, *191*, 172-177.

Quinlan, J. J. (2013). Foodborne illness incidence rates and food safety risks for populations of low socioeconomic status and minority race/ethnicity: a review of the literature. *International journal of environmental research and public health*, *10*(8), 3634-3652.

Rawat, S. (2015). Food Spoilage: Microorganisms and their prevention. *Asian journal of plant science and Research*, *5*(4), 47-56.

Reisch, L., Eberle, U., & Lorek, S. (2013). Sustainable food consumption: an overview of contemporary issues and policies. *Sustainability: Science, Practice and Policy*, *9*(2), 7-25.

Rivera, D., Toledo, V., Reyes-Jara, A., Navarrete, P., Tamplin, M., Kimura, B., ... & Switt, A. I. M. (2018). Approaches to empower the implementation of new tools to detect and prevent foodborne pathogens in food processing. *Food microbiology*, *75*, 126-132.

Rohr, J. R., Barrett, C. B., Civitello, D. J., Craft, M. E., Delius, B., DeLeo, G. A., ... & Tilman, D. (2019). Emerging human infectious diseases and the links to global food production. *Nature sustainability*, *2*(6), 445-456.

Roller, S. (1999). Physiology of food spoilage organisms. *International journal of food microbiology*, *50*(1-2), 151-153.

Salim, A., Deiana, P., Fancello, F., Molinu, M. G., Santona, M., & Zara, S. (2023). Antimicrobial and antibiofilm activities of pomegranate peel phenolic compounds: Varietal screening through a multivariate approach. *Journal of Bioresources and Bioproducts*, *8*(2), 146-161.

Sarno, E., Pezzutto, D., Rossi, M., Liebana, E., & Rizzi, V. (2021). A review of significant European foodborne outbreaks in the last decade. *Journal of food protection*, *84*(12), 2059-2070.

Savoia, D. (2012). Plant-derived antimicrobial compounds: alternatives to antibiotics. *Future microbiology*, *7*(8), 979-990.

Schmidhuber, J., & Tubiello, F. N. (2007). Global food security under climate change. *Proceedings of the National Academy of Sciences*, *104*(50), 19703-19708.

Sileshi, G. W., & Gebeyehu, S. (2021). Emerging infectious diseases threatening food security and economies in Africa. *Global Food Security*, *28*, 100479.

Singh, J., Kaur, H. P., Verma, A., Chahal, A. S., Jajoria, K., Rasane, P., ... & Durul, M. S. (2023). Pomegranate Peel Phytochemistry, Pharmacological Properties, Methods of Extraction, and Its Application: A Comprehensive Review. ACS omega, 8(39), 35452-35469.

Shamloo, E., Hosseini, H., Moghadam, Z. A., Larsen, M. H., Haslberger, A., & Alebouyeh, M. (2019). Importance of Listeria monocytogenes in food safety: A review of its prevalence, detection, and antibiotic resistance. *Iranian journal of veterinary research*, *20*(4), 241.

Sousa, C. P. D. (2008). The impact of food manufacturing practices on food borne diseases. *Brazilian Archives of biology and Technology*, *51*, 615-623.

Stanton, T. B. (2013). A call for antibiotic alternatives research. *Trends in microbiology*, *21*(3), 111-113.

Tankosić, J. V., Puvača, N., Giannenas, I., Tufarelli, V., & Ignjatijević, S. (2022). Food safety policy in the European Union. *Journal of Agronomy, Technology and Engineering Management*, *5*(2), 712-717.

Tauxe, R. V. (1997). Emerging foodborne diseases: an evolving public health challenge. *Emerging infectious diseases*, *3*(4), 425.

Teuber, M. (1999). Spread of antibiotic resistance with food-borne pathogens. *Cellular and Molecular Life Sciences CMLS*, *56*, 755-763.

Todd, E. C. (1997). Epidemiology of foodborne diseases: a worldwide review. *World health statistics quarterly 1997; 50 (1/2): 30-50*.

Todd, E. C. (2013). Globalization and epidemiology of foodborne disease. *Guide to Foodborne Pathogens*, 1-25.

Trivellone, V., Hoberg, E. P., Boeger, W. A., & Brooks, D. R. (2022). Food security and emerging infectious disease: risk assessment and risk management. Royal Society Open Science, 9(2), 211687.

Uyttendaele, M., Franz, E., & Schlüter, O. (2016). Food safety, a global challenge. *International Journal of Environmental Research and Public Health*, *13*(1), 67.

Vouga, M., & Greub, G. (2016). Emerging bacterial pathogens: the past and beyond. *Clinical Microbiology and Infection*, *22*(1), 12-21.

Waage, J., Grace, D., Fèvre, E. M., McDermott, J., Lines, J., Wieland, B., ... & Chan, K. (2022). Changing food systems and infectious disease risks in low-income and middle-income countries. *The Lancet Planetary Health*, *6*(9), e760-e768.

Wei, X., & Zhao, X. (2021). Advances in typing and identification of foodborne pathogens. *Current Opinion in Food Science*, *37*, 52-57.

Yang, S. C., Lin, C. H., Aljuffali, I. A., & Fang, J. Y. (2017). Current pathogenic Escherichia coli foodborne outbreak cases and therapy development. *Archives of microbiology*, *199*, 811-825.

Yeni, F., Acar, S., Soyer, Y., & Alpas, H. (2017). How can we improve foodborne disease surveillance systems: A comparison through EU and US systems. *Food Reviews International*, *33*(4), 406-423.

Zalewska, M., Błażejewska, A., Czapko, A., & Popowska, M. (2021). Antibiotics and antibiotic resistance genes in animal manure–consequences of its application in agriculture. *Frontiers in Microbiology*, *12*, 640.

Zhou, M., Zhang, N., Zhang, M., & Ma, G. (2020). Culture, eating behavior, and infectious disease control and prevention. *Journal of Ethnic Foods*, *7*, 1-7.

Zucoloto, F. S. (2011). Evolution of the human feeding behavior. *Psychology & Neuroscience*, *4*, 131-141.

AIM OF THIS STUDY

The Ph.D. research project overarching aim is to comprehensively explore the biological potentials of the pomegranate peel extracts and derive valuable insights for various applications in food industry. By systematically investigating and screening phenolic compounds from different pomegranate varieties. The thesis has been divided into three studies with the following aims:

- **Aim 1**: The first study is to systematically investigate and screen the antimicrobial and antibiofilm activities of phenolic compounds derived from pomegranate peels across different varieties using a multivariate approach to identify the most potent varieties and phenolic profiles with significant activity against a range of microbial strains, shedding light on the potential use of pomegranate peel extracts as natural antimicrobial agents.

- **Aim 2**: The second study focuses on employing bioassay-guided fractionation techniques to isolate and identify specific antimicrobial bioactive compounds from Punica Granatum peels, with a particular emphasis on their effectiveness against *Staphylococcus aureus* to uncover the molecular basis of antimicrobial activity, paving the way for the development of targeted antimicrobial agents derived from pomegranate peels.

- **Aim 3**: The third study seeks to investigate the effects of different maturity stages on the physicochemical and phytochemical composition of pomegranate peels and juice from various cultivars to delineate the variations in chemical profiles at different developmental stages, offering a comprehensive understanding of how maturity influences the quality and bioactive content of pomegranate peels and juice. This knowledge can guide optimal harvesting practices, ensuring the production of pomegranate-derived goods with enhanced quality and health-promoting properties.

CHAPTER I

Journal of Bioresources and Bioproducts 8 (2023) 146–161

Contents lists available at ScienceDirect

Journal of Bioresources and Bioproducts

journal homepage: https://www.keaipublishing.com/en/journals/journal-of-bioresources-and-bioproducts/

Research Article

Antimicrobial and antibiofilm activities of pomegranate peel phenolic compounds: Varietal screening through a multivariate approach

Amira Salim[a,1], Pierfrancesco Deiana[a,1], Francesco Fancello[a,1], Maria Giovanna Molinu[b,*], Mario Santona[a,*], Severino Zara[a,*]

[a] Department of Agricultural Sciences, University of Sassari, Sassari 07100, Italy
[b] National Council of Research, Institute of Food Production Sciences, Sassari 07100, Italy

ARTICLE INFO

Keywords:
Punica granatum
Agrobiodiversity
OPLS-DA
Punicalagin
Staphyloccocus aureus
Listeria monocytogenes

ABSTRACT

Pomegranates are rich in phenolic compounds and known for their antioxidant, anti-inflammatory, and anticancer properties. The highest concentration of these compounds is found in the peel (exocarp and mesocarp), which constitutes about 50% of the whole fresh fruit. These bioactive phytochemicals exhibit a broad spectrum of antimicrobial effects against both Gram-negative and Gram-positive bacteria, as well as fungi. In the present paper, the chemical composition and antimicrobial activity of the peel (exocarp and mesocarp) from seven *Punica granatum* varieties (Wonderful, Mollar de Elche, Primosole, Sassari 1, Sassari 2, Sassari 3, and Arbara Druci) grown in Sardinia (Italy) were evaluated. Polar phenols, flavonoids, condensed tannins, and anthocyanin contents were evaluated by extraction with water at 20 and 40 °C. Orthogonal projections to latent structures discriminant analysis (OPLS-DA) was used to characterize each variety according to the chemical composition of the pomegranate peel extracts (PPEs). The antimicrobial and antibiofilm activities of each PPE were further tested in vitro against *Staphyloccocus aureus*, *Listeria monocytogenes*, *Salmonella bongori*, *Escherichia coli*, *Lacticaseibacillus casei* and *Limosilactobacillus reuteri*. Gram-positive species were more sensitive than Gram-negative to the extracts tested. Antimicrobial activity was shown against *S. aureus* and *L. monocytogenes* strains, whereas less, even no activity was found against *S. bongori* and *E. coli* strains. The PPEs from Mollar de Elche, Primosole, and Sassari 3 showed the highest antimicrobial activities at concentrations that varied from 0.19 to 1.50 mg/mL, with biofilm activity being reduced by more than 70%. These activities were positively related to the punicalagin, flavonoid, and chlorogenic acid content of the extracts. Finally, regarding the pro-technological bacterial strains, *La. casei* and *Li. reuteri* showed very low, even no sensitivity to the used of the specific PPEs with high concentrations. This study proposes a formulation of pomegranate peel extract that valorizes agro-industrial waste in the context of sustainability and circular economy. Pomegranate extracts should be considered potential sources of natural, plant-derived antimicrobials, providing an alternative to artificial antimicrobial products.

* Corresponding authors.
E-mail addresses: mariagiovanna.molinu@cnr.it (M.G. Molinu), msantona@uniss.it (M. Santona), szara@uniss.it (S. Zara).
[1] Amira Salima, Pierfrancesco Deiana, and Francesco Fancello contributed equally to this work and should be considered co-first authors.

https://doi.org/10.1016/j.jobab.2023.01.006

Available online 14 January 2023

1. *Introduction*

The pomegranate (*Punica granatum* L.) is one of the oldest species of domesticated fruit. Originating in Iran and surrounding areas, this ancient fruit has spread across the world and been part of the human diet for around 5,000 years (Chandra et al., 2010). Its cultivation has been adapted to a wide range of environmental conditions, from the Mediterranean to desert climates, and it is presently produced at commercial levels in many areas of the world (Schwartz et al., 2009). The market demand for pomegranate fruit and its derived products, primarily in the form of juice, minimally processed fruits, jam, and dietary supplements, has increased greatly over the last decade. An important factor contributing to the expansion of its popularity, commercial production, and consumption habits has been the emergence of scientific evidence demonstrating the health-promoting benefits of this fruit (Kandylis and Kokkinomagoulos, 2020).

The pomegranate is rich in bioactive phytochemicals (present in different parts of the fruit) which are known for their antioxidant, anti-inflammatory, antimicrobial, and anticancer properties. The pomegranate peel (exocarp and mesocarp), which constitutes about 50% of the whole fresh fruit, contains the highest concentration of phenolic compounds, mainly hydrolyzable ellagitannins, anthocyanins, and flavonoids (Akhtar et al., 2015). As a consequence, this part of the pomegranate, considered a by-product in the agri-food sector, should instead be designated a co-product from which a host of chemical compounds can be extracted for numerous applications as food additives, nutraceuticals, and supplements in the pharmaceutical, food, and cosmetics industries (Singh et al., 2018; Puneeth and Chandra, 2020; Kaderides et al., 2021; Gigliobianco et al., 2022).

Hydrolyzable ellagitannins (ellagic acid, punicalagin, punicalin, and gallic acid) are the predominant phenolic compounds in pomegranate peel, and they are also the constituent phytochemicals exhibiting the highest antioxidant capacities (Gigliobianco et al., 2022). For example, the chemical structure of punicalagin, which contains sixteen phenolic hydroxyl groups, underlies its ability to scavenge free radicals, as well as its anti-proliferative, anti-inflammatory, hepatoprotective, and antigenotoxic activities (Oudane et al., 2018). Ellagic acid is the main molecule present in pomegranate peel exhibiting anticancer activity (Puneeth and Sharath, 2020),

whereas the anthocyanins are known for their antioxidant, anti-inflammatory, and chemopreventative properties (Li et al., 2017). Moreover, the magnitude of the antioxidant and antitumor activities of pomegranate peel extract is stronger than the sum of the individual activities of its constitutive bioactive molecules, indicating a possible synergistic effect resulting from the mixtures of phenolic compounds present in the pomegranate (Orgil et al., 2014; Kandylis and Kokkinomagoulos, 2020).

Infectious diseases and food decomposition caused by pathogenic microorganisms are two of the principal causes of morbidity and death worldwide (Endo et al., 2018; Celiksoy and Heard, 2021). Notably, food poisoning is predominantly linked to bacterial contamination by Gram-negative bacteria, such as *Escherichia coli, Pseudomonas aeruginosa,* and *Salmonella typhi*. However, Gram-positive bacteria, including *Staphylococcus aureus* and *Bacillus cereus*, have also been reported as the causal agents of food spoilage (Mostafa et al., 2018).

The widespread use of antibiotics to control life-threatening infectious diseases in humans and animals has resulted in the rise and spread of antibiotic-resistance mechanisms among bacterial pathogens. Moreover, the phenomenon of recalcitrant infections, involving the growth of bacteria or fungi in biofilms, has given rise to a second major challenge in dealing with microbial resistance (Slobodníková et al., 2016). Biofilms play a central role in the phenomenon of multi-drug resistance as they shield the microbes behind a protective coat made of extracellular polymeric substances (Bakkiyaraj et al., 2013). Furthermore, the antibiotics available at present are ineffective at treating biofilm-related infections due to their high minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC), meaning that biofilm-related infections have the potential to result in vivo toxicity (Roy et al., 2018).

The search for natural antimicrobials, especially plant-derived compounds, as an alternative to artificial antimicrobial products for the treatment of certain enteric infections is presently enjoying a surge in research attention (Pai et al., 2011; Xu et al., 2017). The peels of P. granatum present promising antimicrobial activity against antibiotic-resistant microbial strains such as methicillin-resistant *Staphylococcus aureus* (Pagliarulo et al., 2016; Xu et al., 2017; Singh et al., 2019). Several studies have reported pomegranate extracts to exhibit a broad spectrum of antimicrobial

effects against both Gram-negative and Gram-positive bacteria, as well as fungi (Singh et al., 2019; Chen et al., 2020; El-Beltagi et al., 2022). Different parts of pomegranate fruit (peel, seeds, juice, and whole fruits) were tested for their antimicrobial activity against a range of bacterial species (*S. aureus, B. cereus, B. subtilis, B. coagulans*, *E. coli, Klebsiella pneumoniae,* and *P. aeruginosa*), and the highest inhibitory values were consistently obtained using the peel extracts (Singh et al., 2019; Chen et al., 2020).

In order to recover as many antioxidants from a plant source as possible, the most appropriate extraction solvent must be used. Methanol or hydroalcoholic solutions are effective solvents for phenolic compounds, and they are generally the solvent of choice in the laboratory; however, for large-scale industrial applications, the possibility of using deionized water as a more economically sustainable and eco-friendly alternative should be considered (Russo et al., 2018; Wu et al., 2021; El-Beltagi, 2022). Wang et al. (2011) reported that water extraction at 40 °C for 4 h was an efficient medium for extracting the antioxidants from pomegranate peel, due to the polar nature of these compounds, and produced comparable results to methanol. They concluded that water should be the solvent of choice, especially when considering the significant impact of methanol in terms of cost and toxicity. Another advantage of using water as extract solvent is that the antimicrobial properties of the resulting pomegranate peel extract can be tested directly without the need for further steps to remove alcoholic solvent (e.g., by vacuum distillation or freeze-drying).

Aqueous pomegranate peel extract was tested as an additive to extend the shelf-life of meat products. It was found to retard protein and lipid oxidation and induce antimicrobial activity against a range of pathogenic strains (Pirzadeh et al., 2021). Another study used extracts obtained using boiling water and demonstrated them to exhibit antimicrobial activity in vitro against microorganisms such as *B. subtilis, S. aureus, E. coli,* and *S. typhimurium* at levels comparable to those of acetone and ethanol extracts (Nuamsetti et al., 2012).

In a recent paper, Wu et al. (2022) demonstrated that aqueous extracts of pomegranate peels had a significant inhibitory effect on the growth of two *S. enterica* strains. Moreover, El-Beltagi et al. (2022) confirmed that water extracted pomegranate peel extract (PPE) had a high antimicrobial activity compared with ethanol extracted PPE against *S. aureus* and *E. coli*. As reported by

Melgarejo-Sánchez et al. (2021), few studies have compared the phytochemical composition of different varieties of *P. granatum* or evaluated the nutraceutical effects as a function of genotype. Since the number of bioactive compounds is known to distinguish the different pomegranate varieties, the authors recommended carrying out specific studies to elucidate the biological activities of individual compounds and their synergistic actions related to genetic variability.

On these bases, the aim of the present work was to characterize the chemical composition of aqueous extracts of pomegranate peel from Italian and local Sardinian varieties and to study their antimicrobial and antibiofilm properties against pathogenic and protechnological microorganisms. Using a multivariate statistical approach, the final objective was to identify the chemical components responsible for the biological action. As the bioactive compound content of the fruit is also related to its growing region, growth stages, pedo-climatic, and ecological conditions (Russo et al., 2018), the environmental, bioclimatic, and agronomic conditions were kept the same for all varieties.

2. Materials and methods

2.1 Chemicals

The analytical reagents Folin-Ciocalteu reagent, aluminium chloride, 2,2′-azino-bis-(3-ethylbenzothia zoline-6-sulphonic acid) (ABTS), and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma-Aldrich Co. (Milan, Italy). The high-performance liquid chromatography (HPLC) grade standards for punicalin α and β, punicalagin α (Punα) and punicalagin β (Punβ), hydroxybenzoic acid, catechin, epicatechin, chlorogenic acid, caffeic acid, rutin, ellagic acid, cyanidin 3,5-diglucoside chloride, and delphinidin 3,5- diglucoside chloride were purchased from Sigma-Aldrich (Milan, Italy). The standard for cyanidin 3-O-glucoside was purchased from Extrasynthese (Genay, France). Gallic acid was from Carlo Erba Reagenti SpA (Rodano, Milan, Italy). Ultrapure water was prepared using a Milli-Q system (Millipore Corporation, Billerica, USA).

2.2 Preparation of pomegranate peel extracts

The chemical composition and antimicrobial activities of the peel from seven pomegranate varieties were evaluated. Fruits were harvested on October 20th, 2020, from the following P. granatum varieties: Wonderful (WF), Primosole (PS), Mollar de Elche (ME), Sassari 1 (SS1), Sassari 2 (SS2), Sassari 3 (SS3), and Arbara Druci (AD). All trees are located in the pomegranate varietal collection field of the University of Sassari's Experimental Station "A. Milella" in Oristano, Sardinia (San Quirico-Fenosu, 39°54′12″N, 8°37′19″E), situated 13 m above sea level. The pomegranate orchard was planted in 2016 according to a 6.0 m × 4.5 m planting distribution. Trees are pruned annually, and spontaneous vegetation is controlled by mowing. Drip irrigation is activated during the summer (approx. 2 500 m3 per hectare per year). The bioclimate of the study area is classified as thermo-Mediterranean, with annual mean, maximum and minimum average temperatures of 17.1 °C, 25.4 °C (July), and 9.6 °C (February), respectively. Precipitation is concentrated in the autumn and winter seasons, with mean annual precipitation of 581 mm (data from the Environmental Protection Agency of Sardinia, Fig. S1).

Five pomegranate fruits per genotype were washed with distilled water. The peel and the arils were manually separated, and the peel was chopped into small pieces using a sharp knife, then dried using a vacuum freeze dryer for 72 h at –55 °C. The dried peel was ground into a fine powder using a laboratory blender (Waring Commercial Blender 7011S). The 1.5 g pomegranate powder was extracted with 25 mL ultrapure water with the solvent: sample mass ratio of 15:1) at 20 or 40 °C for 4 h in a thermostatic bath. Then, samples were centrifuged at 5000 r/min for 10 min, and the filtrate passed through a 0.45 mm hydrophilic nylon membrane. Samples of PPE were stored at –20 °C until the analysis.

2.3 Determination of PPE total phenolic content

The PPE total phenolic content was assessed by Folin-Ciocalteu assay (Deiana et al., 2019). Briefly, aliquots of the diluted samples were mixed in a 25 mL volumetric flask with Folin-Ciocalteu reagent (1:1) and 10 mL sodium carbonate solution 7.5% and incubated for 2 h at room temperature. Total phenolic content was determined by spectrophotometric analysis (8453 UV-Vis Spectrophotometer, Agilent Technologies, USA) as absorbance at 750 nm, and expressed as mg of

gallic acid equivalents per gram of freeze-dried matter (mg GAE per g DW) using gallic acid calibration curve (10−100 mg/L, R2 =0.989). Samples were analyzed in triplicate.

2.4 Determination of PPE total flavonoid content

Total flavonoids were quantified by colorimetric assay according to the AlCl3 method and following previously reported procedures (Re et al., 2019). Quantification was carried out using a catechin calibration curve (2.5−20.0 µg/mL, R2 =0.996). Results are expressed as mg of catechin equivalent per g of freeze-dried matter (mg CE per g DW).

2.5 Determination of PPE total tannin content

Analysis of condensed tannins was carried out by vanillin assay, as reported by Melito et. al (2016). The absorbance of vanillintannin adducts was detected spectrophotometrically at 500 nm, and concentrations were calculated using a catechin calibration curve (1–6 μg/mL, R2 =0.998). Results are expressed as mg CE per g DW.

2.6 The PPE antioxidant capacity

The antioxidant capacity of pomegranate extract was evaluated using both DPPH and ABTS methodologies according to the procedures reported in Piluzza et al (2020). Briefly, for each assay, 0.1 mL of appropriately diluted PPE was mixed with 3.9 mL of 60 μmol/L DPPH or 7 mmol/L ABTS, and then stored in the dark for 120 or 6 min, respectively. For both assays, a calibration curve was generated for Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) (2–12 μmol/L; R2 =0.997 for DPPH assay and R2 =0.998 for ABTS assay). Spectrophotometric readings (8453 UV-Vis Spectrophotometer, Agilent Technologies, USA) were carried out at 515 nm for DPPH and at 734 nm for ABTS. Results are expressed as mmol of Trolox equivalents per 100 g of dry weight, (mmol TEAC per 100 g DW).

2.7 The HPLC analysis of phenolic compounds

Reverse-phase HPLC analysis of phenolic compounds was performed using a liquid chromatography system (Agilent 1100, Agilent Technologies, Palo Alto, USA) equipped with a quaternary pump (G1311A), degasser, column thermostat, auto-sampler (G1313A), and a diode

array detector (G1315 B, DAD). Chromatographic separation was achieved with a Luna C18 column (250 mm × 4.6 mm, 5 μm) from Phenomenex (Torrance, USA) with a security guard cartridge (4 mm × 2 mm). The flow rate was set at 1 mL/min, and the column temperature was set to 30 °C. Elution was carried out with a binary mobile phase of solvent A (water and 0.1% trifluoracetic acid) and solvent B (acetonitrile). The gradient elution program was as follows: 0 min, 99% A; 5 min, 95% A; 6 min, 93% A; 10 min, 85% A; 15 min, 75% A; 20 min, 10% A; 25 min, 99% A, with a post-time of 2 min. Detection was performed at 280, 360, and 520 nm. Phenolics were identified according to the retention time of a mixture of standards and quantified using the respective calibration curves. Samples were appropriately diluted before injection. The results are presented as milligrams per gram of dry weight (mg/g DW). To detect anthocyanins, 2 mL pomegranate extract was loaded into C18-Sep-Pak cartridges (Strata C-18-E, 500 mg per 6 mL, Phenomenex) previously conditioned with 2 mL methanol, followed by 5 mL of 5 mmol/L H2SO4. After washing with 5 mL of 5 mmol/L H2SO4, the anthocyanins were eluted with 5 mL MeOH followed by 5 mL ultrapure water into a 10 mL calibrated flask.

2.8 Antimicrobial activity of PPE

2.8.1 Bacterial strains

The following bacterial strains were used in this study: *Staphylococcus aureus* DSM 20231, *Staphylococcus aureus* DSM 2569, *Staphylococcus aureus* DSM 6148, *Listeria monocytogenes* DSM 20600, *Listeria monocytogenes* DSM 15675, *Salmonella bongori* DSM 13772, E. coli DSM 30083 and DSM 4415, *Lacticaseibacillus casei* Shirota, and *Limosilactobacillus reuteri* DSM 17938. All strains, with the exception of the lactic acid bacteria (LAB), were cultivated overnight at 37 °C in brain heart infusion agar (BHI) for the determination of PPE antimicrobial activities and to detect biofilm formation. The LAB were cultivated overnight at 30 °C in De Man, Rogosa, and Sharpe (MRS) agar.

2.8.2 Determination of minimum inhibitory concentration

The antimicrobial activities of PPE were quantitatively evaluated in vitro by measuring the MIC (Petretto et al., 2018). Concentrations ranging from 0.09 to 3.00 mg/mL of each extract (from both room temperature (20 °C) and warm (40 °C) water extractions) were prepared. Subsequently, 100 µL of each concentration were added to wells of a 96-well microtiter plate containing a total volume of 100 µL Müller-Hinton broth concentration 2X (MHB 2X) plus bacterial inoculant of approximately 5 × 105 CFU/mL final concentration of the tested bacteria. Negative control wells contained non-inoculated medium plus PPEs. Positive control wells contained inoculated MHB with no PPE. Plates were then incubated at 37 °C for 24 h. Inhibition of bacterial growth was determined visually, and the MIC was defined as the lowest concentration of the extract that inhibited microorganism growth at the end of the incubation period. All the bacterial strains were tested in triplicate, and the assay was repeated twice for each strain.

2.9 Antibiofilm activity of PPE

The quantitative analysis of the ability of pathogenic bacteria strains to form biofilms was evaluated by crystal violet (CV) assay, according to the methods reported in Xu et al. (2016) with some modifications. Three PPEs (ME, PS, and SS3) were processed further to test the extracts' activities against planktonic bacteria and their ability to block biofilm formation. Concisely, 100 μL of the bacteria cell suspensions containing 5 × 106 cells per mL Tryptic Soy Broth (TSB) were transferred to microtiter plates. Then, 100 μL of different concentrations of PPEs, ranging from 0.09 to 3.00 mg/mL, according to the MIC of each strain (the MIC and two sub-MIC concentrations), were added to each well. The bacterial strains tested were selected according to their biofilm formation ability. Negative controls were wells containing non-inoculated medium. Positive controls were wells containing inoculated culture medium but no PPE. The microplates were incubated at 30 °C for 72 h; the CV assay was then completed as per test plates, and the optical density (OD) values were measured spectrophotometrically.

2.10 Statistical analyses

The effect of pomegranate variety on the capacity of pomegranate peel water extracts to limit biofilm formation was assessed by analysis of variance (ANOVA). When a significant effect was

observed ($P < 0.05$), the differences between means were separated using the Tukey-Kramer multiple comparisons test. Orthogonal projections to latent structures discriminant analysis (OPLS-DA) models were developed to distinguish the different pomegranate varieties from one another according to the chemical composition of their respective PPEs. A preliminary exploratory projection to latent structures discriminant analysis (PLS-DA) model including seven class levels (i.e., all seven pomegranate varieties) was performed to investigate the distances between varieties. Then, in order to select the most discriminatory variables for each variety with high accuracy, models for each of the seven varieties vs. all others were developed (Deiana et al., 2019). The OPLS analysis was used to perform the pairwise models due to its simpler interpretation with respect to PLS analysis. Indeed, OPLS concentrates the predictive information in one component, and the information not correlated to Y is included in further orthogonal components.

Furthermore, PLS-DA models were generated to assess the role of bioactive molecules, both individually and as combinations of molecules, on the antimicrobial activity of PPE. A model was developed for each tested bacterial strain. The antimicrobial activity was expressed according to the results of MIC analyses: MIC values were grouped as "High" (0.09−0.38 mg/mL), "Medium" (0.75−3.00 mg/mL), or "No" (no antimicrobial activity), and these classes were used for the PLS-DA models. In this case, due to the presence of multiple classes in several models and to maintain a uniform method of analysis, only PLS-DA models were performed.

All variables were included in the models at the start, then those with the lowest model relevance, according to the variable influence on projection (VIP) values, were gradually excluded until a statistically significant model was produced according to its performance parameters R2Y and Q2, and the permutation tests used to validate the models (Deiana et al., 2022). The parameter R2Y indicates the percentage of variation explained by the model, whereas Q2 indicates the proportion of variance predictable by the model. Finally, two OPLS regression models, setting the Y variable as DPPH and ABTS, respectively, were performed to identify the pomegranate components with the highest antioxidant activities. R-Studio software (R version 4.1.1, 2021-08-10, ropls package from Bioconductor for OPLS, OPLS-DA and PLS-DA), was used to conduct the statistical analyses.

3. Results

3.1 Chemical composition of PPE and varietal models

Table 1 summarizes the content of PPE obtained at two different temperatures (20 and 40 °C) for seven different cultivars in terms of polar phenols, flavonoids, condensed tannins, and anthocyanins. The data show wide content variability between cultivars, while extraction temperature had only a limited effect on phenolic content. The total phenols (T.Phen) varied from 92.1 mg/g for cultivar AD extracted at 20 °C to 150.7 mg/g for cultivar PS extracted at 40 °C. The PS, together with ME, contained the highest concentration of total flavonoids (T.Flav, 22.2 mg/g in PS; 22.5 mg/g in ME), whereas SS3 and AD contained the lowest (14.3 and 15.4 mg/g, respectively). The values of condensed tannins ranged from 1.6 mg/g (SS2) to 7.9 mg/g (ME).

Eighteen phenolic compounds were identified by HPLC-DAD: six hydrolysable tannins (punicalin α and punicalin β, Punα and Punβ, ellagic acid, and gallic acid), two phenolic acids (4-hydroxybenzoic acid, caffeic acid), chlorogenic acid, two flavan-3-ols (catechin and epicatechin), one flavonoid glycoside (rutin), and six anthocyanins. The level of each phenolic compound in the peel was mostly cultivar-dependent and related to the extraction temperature to a lesser extent. The ellagitannins were the predominant components; with the two punicalagin anomeric structures, Punα and Punβ, being the most abundant phenolics (17.6−125.3 mg/g and 45.0−258.6 mg/g, respectively). Cultivars ME and PS had the highest concentrations of punicalagin in their peel extracts, while WF had the lowest. Both the α and β anomers of punicalin were detected (3.9−29.6 mg/g and 3.0−40.5 mg/g, respectively), with the highest concentrations found in cultivars WF and AD. An unknown peak of great intensity was detected in cultivars WF and AD (190.0 and 118.1 mg/g, respectively). According to the literature data, this peak is sometimes recognized as punicalin (Zhang et al., 2009; Živković et al., 2018) or as an unspecified ellagitannin (Romani et al., 2012). In this work, we recognized the punicalin α and punicalin β peaks based on the retention times for the pure standards. Therefore, we decided to consider the unknown peak as an ellagitannin, to quantify it as an ellagic acid derivative, and to label the peak as "unknown". Cultivar WF contained the highest concentration of ellagic acid (EA, 28.7−30.8 mg/g), whereas the other six cultivars consistently reported lower values (9.8−20.7 mg/g). All other compounds detected were at levels below 5.5 mg/g.

Six major anthocyanin compounds were identified in pomegranate peel extract, namely delphinidin-3,5-diglucoside, cyanidin-3,5- diglucoside, pelargonidin-3,5-diglucoside, delphinidin-3-glucoside, cyanidin-3-glucoside, and pelargonidin-3-glucoside, with large differences in concentration between cultivars. None of these molecules were present in cultivars ME and SS2. Delphinidin-3,5- diglucoside and cyanidin-3,5-diglucoside were only observed in WF peel extracts, which showed the highest concentrations for all the anthocyanins detected.

In order to investigate differences between cultivars, a supervised PLS-DA and OPLS-DA multivariate approach was adopted. According to the preliminary "7-cultivars" PLS-DA model based on the respective peel extract compositions, PS and WF were identified as the varieties that differed from the others the most (Fig. 1a). The remaining varieties clustered into two groups: the first comprised SS2 and ME, probably due to the common absence of anthocyanins, whereas the second consisted of SS1, SS3, and AD. To highlight the varietal-specific features of PPE further, models were generated investigating "single variety vs all others" (Figs. 1b–1h) which achieved both good fit (R2Y > 0.90, Table S1) and predictability (Q2Y > 0.88).

Specifically, AD, SS1, and SS3 stood out for their overall low concentration of phenols, tannins, and flavonoids, with the exception of chlorogenic acid in the case of SS3, and anthocyanins in SS1. On the other hand, SS2 and ME were distinguished by their absence of anthocyanins and good presence of flavonoids and phenolic acids, specifically catechin, rutin, caffeic acid, and 4-hydroxybenzoic acid. The ME and PS varieties presented the highest concentrations of punicalagin isomers. The PS was also characterized as producing the peel with the strongest overall antioxidant activity due to its high concentrations of epicatechin, caffeic acid, rutin, Punα, Punβ, chlorogenic acid, and 4-hydroxybenzoic acid. Finally, WF extracts were distinguished by their high concentrations of anthocyanins, ellagic acid, and punicalin.

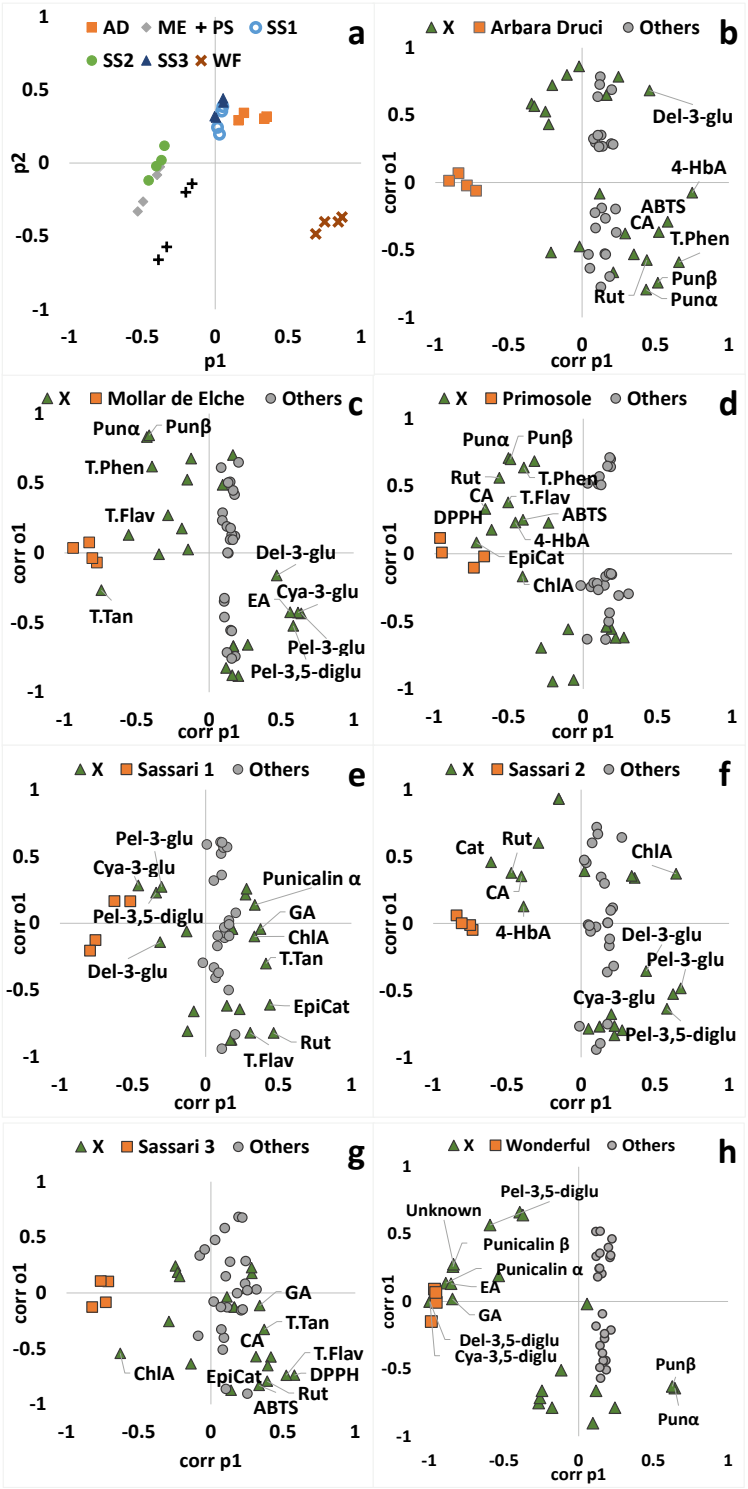

Figure 1: Score plot of projections to latent structures discriminant analysis (PLS-DA) (a, 7-cultivars model) and correlation biplots for orthogonal projections to latent structures discriminant analysis (OPLS-DA) models (b–h) showing both relationships between scores (observations classified by varieties: b, Arbara Druci, c, Mollar de Elche; d, Primosole; e, Sassari 1; f, Sassari 2; g, Sassari 3; h, Wonderful) and loadings (X variables: pomegranate peel extract (PPE) chemical composition). Observations close to X

variables have high concentrations of such variables and low concentrations in variables situated opposite. Labeled variables are those with predictive variable influence on projection (VIP) values above 1.

3.2 Antioxidant activity of PPE

The antioxidant activities of PPEs obtained at the two different extraction temperatures were generally similar. The largest differences in ABTS values were observed for PPEs from ME and PS, being 82.8 and 87.3 mmol per 100 g, respectively, for hot water (40 °C) extraction versus 68.3 and 69.7 mmol per 100 g, for cold water (20 °C) extraction (Table 1).

The differences in values obtained between the two extraction temperatures were even lower for DPPH values, with a maximum difference of 10 units between the two water temperatures. As for ABTS, ME and PS were the two varieties demonstrating the greatest differences, with hot water extraction resulting in higher phenol concentrations.

Table 1: Chemical composition and antioxidant activity of pomegranate peel extracts from seven varieties obtained through cold (20 °C) and hot (40 °C) extraction.

Metabolites	*T*	*Arbara Druci*	*Mollar de Elche*	*Primosole*	*Sassari 1*	*Sassari 2*	*Sassari 3*	*Wonderful*
GA	20	1.0±0.0	0.1±0.0	0.5±0.0	0.0±0.0	0.3±0.0	0.1±0.0	1.9±0.1
	40	0.9±0.2	0.2±0.0	1.0±0.2	0.0±0.0	0.3±0.0	0.1±0.0	1.9±0.0
Unknown	20	63.5±5.3	8.9±0.6	1.1±0.1	1.2±0.1	1.1±0.0	2.4±0.0	143.9±9.7
	40	118.1±8.6	13.5±1.0	1.5±0.0	1.3±0.0	0.8±0.1	2.6±0.1	190.0±3.8
Punicalin α	20	13.8±0.3	9.4±0.3	5.9±0.1	4.5±0.0	5.7±0.3	5.2±0.1	29.6±2.5
	40	17.1±0.7	7.3±0.2	7.9±0.5	3.9±1.1	5.9±0.6	5.9±0.0	26.9±0.0
Punicalin β	20	17.1±0.4	6.5±0.3	5.3±0.3	3.0±0.1	3.5±0.1	4.2±0.1	28.8±1.6
	40	23.2±2	7.3±0.2	5.4±0.0	4.6±0.2	3.6±0.5	4.5±0.0	40.5±0.5
Punα	20	61.5±5.3	97.2±1.0	101.8±0.6	80.4±1.1	84.4±0.4	58.9±2.8	38.0±0.3
	40	27.3±2.2	120.9±0.3	125.3±1.9	85.1±1.6	88.7±3.7	84.9±1.1	17.6±3.0
4-HbA	20	0.01±0.00	0.01±0.00	0.01±0.00	0.01±0.00	0.01±0.00	0.01±0.00	0.01±0.01
	40	0.00±0.00	0.01±0.00	0.01±0.00	0.01±0.00	0.01±0.00	0.01±0.00	0.01±0.00
Punβ	20	110.8±6.7	205.3±3.1	201.4±4.0	156.7±0.4	174.3±3.0	177.8±9.3	81.3±2.5
	40	51.8±5.2	241.3±2.3	258.6±6.1	167.2±8.4	186.7±7.9	179.0±4.0	45.0±4.8
Cat	20	3.5±0.0	3.9±0.2	3.8±0.0	3.4±0.1	4.7±0.1	3.1±0.0	3.9±1.4
	40	3.4±0.0	4.5±0.2	5.5±1.0	3.4±0.1	5.7±0.2	3.5±0.1	2.9±0.1
ChlA	20	1.1±0.0	1.2±0.0	1.2±0.1	1.1±0.1	0.8±0.0	1.5±0.0	1.2±0.1
	40	1.1±0.0	1.4±0.0	1.6±0.1	0.9±0.0	0.8±0.1	1.7±0.1	1.1±0.1

CA	20	0.3±0.0	0.4±0.0	0.6±0.0	0.5±0.0	0.6±0.0	0.3±0.0	0.3±0.1
	40	0.2±0.0	0.4±0.0	0.9±0.0	0.5±0.0	0.7±0.1	0.4±0.0	0.5±0.0
EpiCat	20	0.6±0.0	0.8±0.0	0.8±0.0	0.3±0.0	0.4±0.0	0.3±0.0	0.6±0.0
	40	0.6±0.0	0.8±0.0	1.2±0.0	0.3±0.0	0.3±0.0	0.3±0.0	0.7±0.0
Rut	20	0.4±0.1	0.7±0.0	0.8±0.0	0.4±0.0	0.8±0.0	0.5±0.0	0.7±0.0
	40	0.4±0.0	0.8±0.0	1.0±0.0	0.4±0.0	0.9±0.0	0.5±0.0	0.6±0.0
EA	20	12.6±0.0	9.8±1.0	16.8±0.6	19.0±0.8	16.0±0.1	15.6±0.4	28.7±1.5
	40	16.6±2.1	10.6±0.2	17.9±1.1	20.7±1.2	18.0±1.0	18.6±0.1	30.8±1.6
Cya-3-glu	20	2.3±0.0	0.0±0.0	2.2±0.0	3.4±0.2	0.0±0.0	2.7±0.0	3.3±0.1
	40	2.4±0.0	0.0±0.0	2.3±0.0	3.5±0.0	0.0±0.0	2.8±0.0	3.2±0.1
Del-3-glu	20	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	2±0.0	2.7±0.1
	40	0.0±0.0	0.0±0.0	0.0±0.0	2.1±0.0	0.0±0.0	2±0.0	2.6±0.0
Pel-3-glu	20	2.0±0.0	0.0±0.0	2.0±0.0	2.1±0.0	0.0±0.0	2.0±0.0	2.3±0.1
	40	2.0±0.0	0.0±0.0	2.0±0.0	2.2±0.0	0.0±0.0	2.0±0.0	2.3±0.0
Cya-3,5-diglu	20	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	1.3±0.0
	40	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	1.3±0.0
Del-3,5-diglu	20	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	2.0±0.0
	40	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	2.0±0.0
Pel-3,5-diglu	20	2.1±0.0	0.0±0.0	2.0±0.0	2.9±0.1	0.0±0.0	2.6±0.1	4.0±0.1
	40	2.0±0.0	0.0±0.0	2.1±0.0	3.2±0.1	0.0±0.0	2.7±0.0	3.9±0.0
DPPH	20	48.2±1.4	52.0±1.3	58.0±0.7	46.8±1.9	52.4±3.9	43.0±1.8	58.6±0.8
	40	48.7±1.3	60.3±5.5	68.0±0.3	53.4±2.4	51.9±1.7	45.4±2.0	55.9±1.7
ABTS	20	53.6±1.1	68.3±1.1	69.7±2.7	63.4±2.0	63.9±2.3	59.0±0.1	75.7±1.2
	40	59.7±0.4	82.8±1.5	87.3±0.4	73.7±1.0	72.6±4.5	66.1±2.1	74.5±3.1
T.Flav	20	15.4±1.0	20.8±0.5	20.3±0.2	15.5±0.4	16.9±1.6	14.8±0.5	19.3±1.5
	40	16.0±0.7	22.5±0.0	22.2±0.7	16.4±0.4	18.0±1.1	14.3±0.6	19.1±1.6
T.Tan	20	5.1±0.1	7.5±0.2	5.2±0.2	1.8±0.2	1.6±0.1	1.8±0.1	4.2±0.0
	40	5.0±0.0	7.9±0.1	5.2±0.2	1.9±0.2	2.3±0.3	1.8±0.1	4.4±0.0
T.Phen	20	92.1±3.4	127.0±3.3	127.0±1.4	106.0±0.3	126.0±3.4	105.0±1.0	121.4±4.5
	40	93.0±1.5	149.6±1.3	150.7±2.3	121.4±4.3	137.3±6.2	125.9±2.5	111.3±0.4

Notes: T, temperature; GA, gallic acid; Punα, punicalagin α; 4-HbA, 4-Hydroxybenzoic acid; Punβ, punicalagin β; Cat, catechin; ChlA, chlorogenic acid; CA, caffeic acid; EpiCat, epicatechin; Rut, rutin; EA, ellagic acid; Cya-3-glu, cyaninin-3-glucoside; Del-3-glu, delphinidin-3-glucosid; Pel-3- glu, pelargonidin-3-glucoside; Cya-3,5-diglu, cyanidin-3,5-diglucoside; Del-3,5-diglu, delphinidin-3,5-diglucosid; Pel-3,5-diglu, pelargonidin-3,5- diglucoside; DPPH, 2,2-diphenyl-1-picrylhydrazyl; ABTS, 2,2′-azino-bis-(3-ethylbenzothia zoline-6-sulphonic acid); T.Flav, total flavonoids; T.Tan, total tannins; T.Phen, total phenols.

The AD and SS3 were the varieties which showed the lowest overall antioxidant activities. According to the OPLS models (Fig. 2), total flavonoids, specifically epicatechin and rutin, where the chemical compounds with the highest antioxidant activity. Moreover, the level of phenols such

as caffeic acid and 4-hydroxybenzoic acid in PPE strongly correlated with the DPPH and ABTS values. On the other hand, tannins, such as the punicalagins, contributed only marginally to the antioxidant activity of PPE, whilst anthocyanins, punicalin, and ellagic acid did not make a significant contribution.

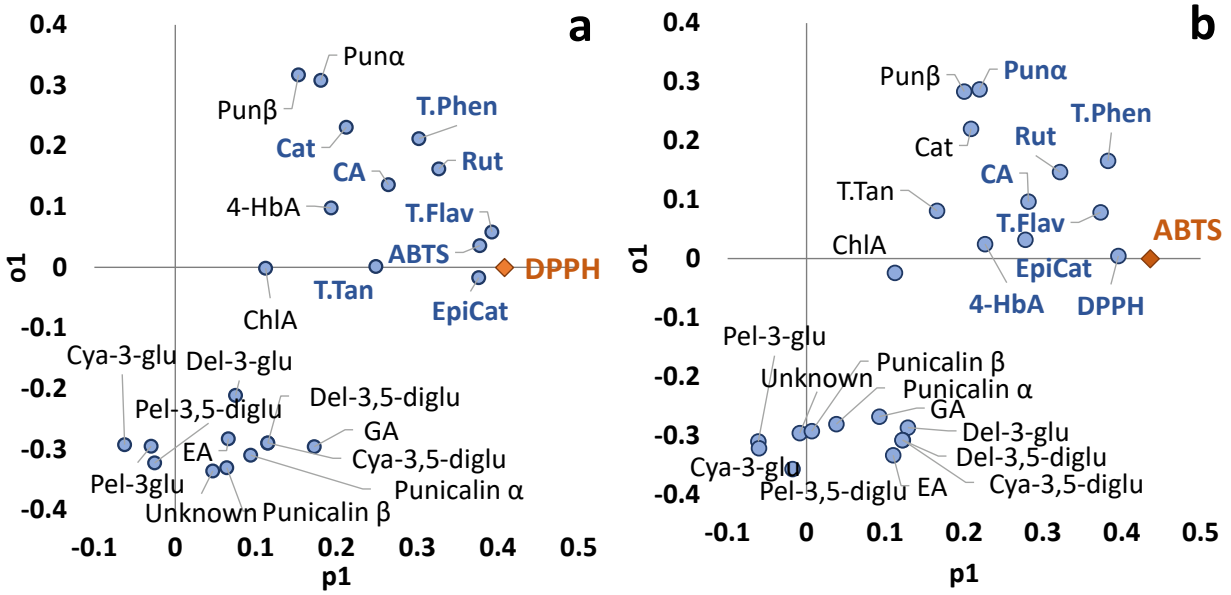

Figure 2: The OPLS loading scatter plots representing relationships between X variables (PPE chemical composition) and Y variable: 2,2-diphenyl-1- picrylhydrazyl (DPPH) (a) and 2,2′-azino-bis-(3-ethylbenzothia zoline-6-sulphonic acid) (ABTS) (b). The variables highlighted in bold blue type are those strongly correlated with Y variable (in terms of variable importance on projection: VIP).

3.3 Antibacterial activity of PPE

The antibacterial activities of the PPEs from the different cultivars were assessed by determining the MIC for each bacteria strain tested (Table 2). Most of the PPEs showed some effectiveness at suppressing microbial growth, with a MIC of the seven extracts from cold and hot water extraction ranging from 0.09 to 3.00 mg/mL, and Gram-negative bacteria notably more resistant than Gram-positive bacteria. A comparison of the antimicrobial activity of all PPEs showed that the most efficacious were those obtained from ME, PS, and SS3; WF extract showed the weakest antimicrobial activity. The *S. aureus* and *L. monocytogenes* strains showed high susceptibility to nearly all the PPEs tested. The *La. casei* Shirota and *Li. reuteri* DSM 17938 showed less susceptibility to the PPEs compared with the other Gram-positive bacteria. In contrast,

S. bongori DSM 13772 showed resistance to all the PPEs tested, whereas the *E. coli* strains showed resistance against most of the PPEs.

Table 2: Minimal inhibitory concentration (mg/mL) of pomegranate peel extracts obtained through cold and hot water extraction against ten bacteria strains.

Metabolites	*T*	*Arbara Druci*	*Mollar de Elche*	*Primosole*	*Sassari 1*	*Sassari 2*	*Sassari 3*	*Wonderful*
S. aureus DSM 20231	20	3	0.75	0.75	ni	0.75	0.75	ni
	40	3	3	0.75	0.75	ni	1.5	ni
S. aureus DSM 2569	20	1.5	0.38	0.75	0.75	0.75	0.75	ni
	40	1.5	0.38	0.38	0.75	ni	ni	1.5
S. aureus DSM 6148	20	1.5	0.75	0.75	ni	0.75	0.75	ni
	40	ni[1]	0.75	1.5	ni	0.75	1.5	ni
L. monocytogenes DSM 15675	20	0.19	3	0.09	ni	0.09	0.19	ni
	40	0.09	1.5	0.75	ni	ni	0.75	ni
L. monocytogenes DSM 20600	20	ni	0.75	ni	ni	ni	0.75	ni
	40	ni	0.38	ni	ni	ni	1.5	ni
E. coli DSM 4415	20	ni	1.5	3	ni	3	3	ni
	40	3	3	ni	ni	3	ni	ni
E. coli DSM 30083	20	ni	ni	ni	ni	ni	ni	ni
	40	ni	ni	ni	ni	ni	0.75	1.5
S. bongori DSM 13772	20	ni	ni	ni	ni	ni	ni	ni
	40	ni	ni	ni	ni	ni	ni	ni
L. casei Shirota Yacult	20	ni	1.5	ni	ni	ni	ni	ni
	40	ni	1.5	3	ni	ni	ni	ni
L. reuteri DSM 17938	20	3	1.5	0.75	3	1.5	3	ni
	40	3	1.5	0.75	3	3	3	3

Notes: ni, no inhibition; T, temperature. *S., Staphyloccocus; L., Listeria; S., Salmonella; E., Escherichia; La., Lacticaseibacillus; Li., Limosilactobacillus.*

The PLS-DA analysis enabled us to identify the bioactive compounds contributing the most to the antimicrobial activities of PPE. Table 3 reports the molecules associated with either "Medium" or "High" antimicrobial activity (according to the highest VIP values) for each microbial strain. Eight PLS-DA models reported significant values of R2Y and Q2Y (see also Table S1 and Fig. S2 for model performance parameters and validation analyses, and S4 for relative correlation biplots showing the relationships between X and Y loadings). No significant model was obtained for *E. coli* DSM 30023 or *S. bongori* DSM 13772. Indeed, a "Medium" level of antimicrobial activity against *E. coli* DSM 30023 was observed for SS3 (MIC=0.75 mg/mL) and WF (1.5 mg/mL) extracts only. On the other hand, *E. coli* DSM 4415 was sensitive to a greater

number of the PPE tested, suggesting a possible inhibitory role of Punα and Punβ, rutin, and catechin. It is worth noting that the two ellagitannin isomers exhibited good activity against all the bacterial species tested. Other relevant species-specific and strain-specific antimicrobial activities could be attributed to total and individual flavonoids. Specifically, epicatechin was relevant against all L. monocytogenes and S. aureus strains tested, *Li. reuteri* DSM 17938, and *La. casei* Shirota, whereas catechin contributed to the inhibition of *E. coli* DSM 4415 and *S. aureus* DSM 6148. Chlorogenic acid exhibited high antimicrobial activity against *L. monocytogenes* and *S. aureus* strains. Finally, high values of total tannins and total phenols were positively correlated with the PPE antimicrobial activity against most of the strains tested.

Table 3: A list of variables (phenolic compounds, tannins, flavonoids, and antioxidant activity) mostly related to antimicrobial activity of pomegranate peel extract (PPE) according to projections to latent structures discriminant analysis (PLS-DA) models performed per each bacterial strain tested.

Strain	***Variables***
S. aureus 20231	Chlorogenic acid, Total Tannins, Punicalagin β, Punicalagin α
S. aureus 2569	Total Tannins, Total Phenols, Epicatechin, Total Flavonoids
S. aureus 6948	Punicalagin β, Punicalagin α, Catechin, Total Flavonoids, Chlorogenic acid
L. monocytogenes 15675	Chlorogenic acid, Total Phenols, Epicatechin, Punicalagin α, Total Tannins, Punicalagin β
L. monocytogenes 20600	Total Tannins, Total Flavonoids, Chlorogenic acid, Punicalagin β, Epicatechin, Punicalagin α
E. coli 4415	Punicalagin α, Punicalagin β, Total Tannins, Rutin, Catechin
***Lac. Paracasei* Shirota**	Total Tannins, Epicatechin, Total Flavonoids, Total Phenols, Punicalagin α, Punicalagin β, Rutin
Lim. Reuteri 17938	Epicatechin, Total Flavonoids, DPPH, Caffeic acid, Punicalagin α, Punicalagin β,

Notes: In the PLS-DA models, antimicrobial activity was classified according to MIC values reported in Table 2 as "High" (MIC=0.094–0.375 mg/mL), "Medium" (0.75–3.00 mg/mL), or "No" (no antimicrobial activity).

3.4 Antibiofilm activity of PPE

Among the bacterial strains tested, *S. aureus* and *L. monocytogenes* showed the highest biofilm formation abilities (strong biofilm producers), whereas S. *bongori* and *E. coli* strains showed the least biofilm formation ability (weak biofilm producers). To test the capacity of PPEs to inhibit biofilm formation, CV assays were performed using three different concentrations of

cold-water extracts (20 °C) from three selected cultivars, for which the MIC values ranged from 3 to 0.09 mg/mL (Fig. 3). The PS extract reported the strongest inhibitory activity, reducing the biofilm development of S. aureus strains by more than 65% at a sub-MIC (1/16) concentration of 0.19 mg/mL (Fig. 3a–3c). Among the two factors considered in the trial-cultivar and MIC concentration, the latter was predominant, accounting for about 50% of the variance in antibiofilm activity against *S. aureus* strains (Table S2, factorial analysis). In this case, the effect of PPE cultivar provenience was significant for the first two *S. aureus* strains, explaining 35% of the model variance. The *L. monocytogenes* strains (Fig. 3e–3f) also exhibited high sensitivity to the PPEs for which more than 70% reduction of biofilm development was observed, once again at a sub-MIC concentration: 0.019 mg/mL. Finally, *S. bongori* DSM 13772 (Fig. 3d) showed the highest resistance to all PPEs tested, and its biofilm formation ability was not significantly affected by sub-lethal concentrations of all PPEs tests.

4. Discussion

4.1. Varietal composition and antioxidant activity

In agreement with Wang et al. (2011), we found extraction with water at 40 °C for 4 h to be an efficient method for the extraction of pomegranate peel antioxidants (phenolics, proanthocyanins, and flavonoids). However, our data also showed that extraction at this higher temperature was not always accompanied by a higher concentration of extracted compounds compared with extraction at room temperature (20 °C). Kharchoufi et al. (2018), on the other hand, showed that a further increase in temperature from 40 to 55 °C had a significant effect on the extraction of phenolic compounds from the peel of the Gabsi variety of pomegranate. Considering the significant impact in terms of cost for large scale and industrial applications, we did not take higher extraction temperatures into consideration. The levels of total phenols, flavonoids, and tannins are in line with the previous literature. Saad et al. (2012) reported a total phenolic content of 134.3−181.0 mg/g in pomegranate peels from Tunisian varieties extracted using a methanolwater (80:20) solution. Turrini et al. (2020) found a total phenolic content of 148 mg GAE per g in the Wonderful cultivar, which was slightly higher than our results, obtained through the decoction of peels in ultrapure water. Similarly, Young et al. (2017) reported a total phenol content equal to 134.16 mg

GAE per g in peel from Wonderful pomegranates grown in California and extracted with aqueous methanol. Some pomegranate peels from Turkish varieties described by Çam and Hışıl (2010) reported total flavonoid and tannin concentrations similar to our findings. By contrast, other varieties from Algeria (Kennas and Amellal-Chibane, 2019) showed higher total phenol and tannin concentrations, but the water extraction process carried out involved longer extraction times. Montefusco et al. (2021) analyzed four different pomegranate cultivars grown in Southern Italy, and the reported total flavonoids (4.0−5.1 mg CE per g) and TEAC values (ABTS 34.5−41.4 mmol per 100 g DW) were very similar to ours.

El-Beltagi et al. (2022) reported a total phenolic content of 513.8 mg of gallic acid per 100 g in water extracts of pomegranate peel, a much lower value than that obtained in the present study. However, the fruits used in their study had been purchased from a supermarket, thus the storage and shelf-life conditions of the fruits were unknown. Conversely Derakhshan et al. (2018) reported a value for the total phenolic content of Iranian pomegranate peel that was two to three-fold that obtained here, but they used a different solvent for extraction and a longer extraction time.

In agreement with our findings, Young et al. (2017) reported Wonderful peel to be poor in punicalagin. Balli et al. (2020) extracted pomegranate peel bioactive molecules from Mollar de Elche and Wonderful varieties through decoction and obtained much lower concentrations of punicalagin isomers in Mollar de Elche compared with our data, but their values for the Wonderful variety were comparable. Gullón et al. (2020) highlighted strong varietal differences in terms of total amounts of hydrolysable tannins. The authors reported a wide range in punicalagin values (98.02−612.80 mg/g) for pomegranate peel methanol: water extracts obtained from Egyptian and Israeli cultivars. Rosas-Burgos et al. (2017), using methanol as extraction solvent, reported ellagic acid concentrations in Spanish pomegranate cultivars, such as Mollar de Elche, that were very similar to those reported here.

Our results are consistent with those found by Gigliobianco et al. (2022), who detected extremely high levels of punicalagin A and B in peel extract of Wonderful and Mollar de Elche pomegranate cultivars grown in the Marche region of Italy. Moreover, as in the present work, they demonstrated higher levels of ellagic acid in peel from the Wonderful variety than from the peel

of any other pomegranate variety. The observed absence of anthocyanins in the peel from the Mollar de Elche variety has also been reported by other authors (Da Silva Veloso et al., 2020, Gigliobianco et al., 2022), as has the high anthocyanin concentration we observed in Wonderful peel (Zhao et al., 2013, Gigliobianco et al., 2022). Previous studies have attributed the main antioxidant activity of PPE to punicalagins, punicalins, and ellagic acids (Rosas-Burgos et al., 2017; Kumar and Neeraj, 2018). In contrast, our findings attributed a negligible role to the latter two compounds, and only a secondary role to punicalagins. Instead, our results indicated overall antioxidant activity as partially related to the presence of flavonoids epicatechin, catechim, and rutin, and principally related to the levels of total flavonoids and total phenols. These results suggested that the antioxidant power of a vegetal matter should be primarily ascribed to the specific mixture of its bioactive molecules and their synergic activity. Moreover, the differences between our data and previous literature might be due to the different number of the considered bioactive compounds and to the different applied methods of statistical analysis.

In general, the differences in our quantitative data compared with those in the literature could also be explained by genetic factors, different ripening stages, or pedoclimatic conditions. All these factors can strongly influence the levels and detection of bioactive compounds in pomegranate peel, making it difficult to compare published works. In our experiment, we could conclude that a significant genotype-dependent variability in content was observed since environmental, agronomic, and analytical conditions were the same for all varieties. Moreover, it is worth noting that, to the best of our knowledge, this study is the first to describe in detail the PPE composition of Primosole, an Italian pomegranate cultivar of international commercial interest, and the Sardinian varieties Arbara Druci, Sassari 1, Sassari 2, and Sassari 3.

With regard to the Sardinian varieties, the results reported in this study highlight the importance of preserving and exploiting local varieties as a source of nutraceutical compounds, which provided producers with more selection possibilities in accordance with the market's demands or production goals. In the case of the four Sardinian varieties investigated, it is worth noting the good punicalagin concentrations, close to the highest values observed in Mollar de Elche and Primosole, in PPEs from Sassari 1, Sassari 2, and Sassari 3.

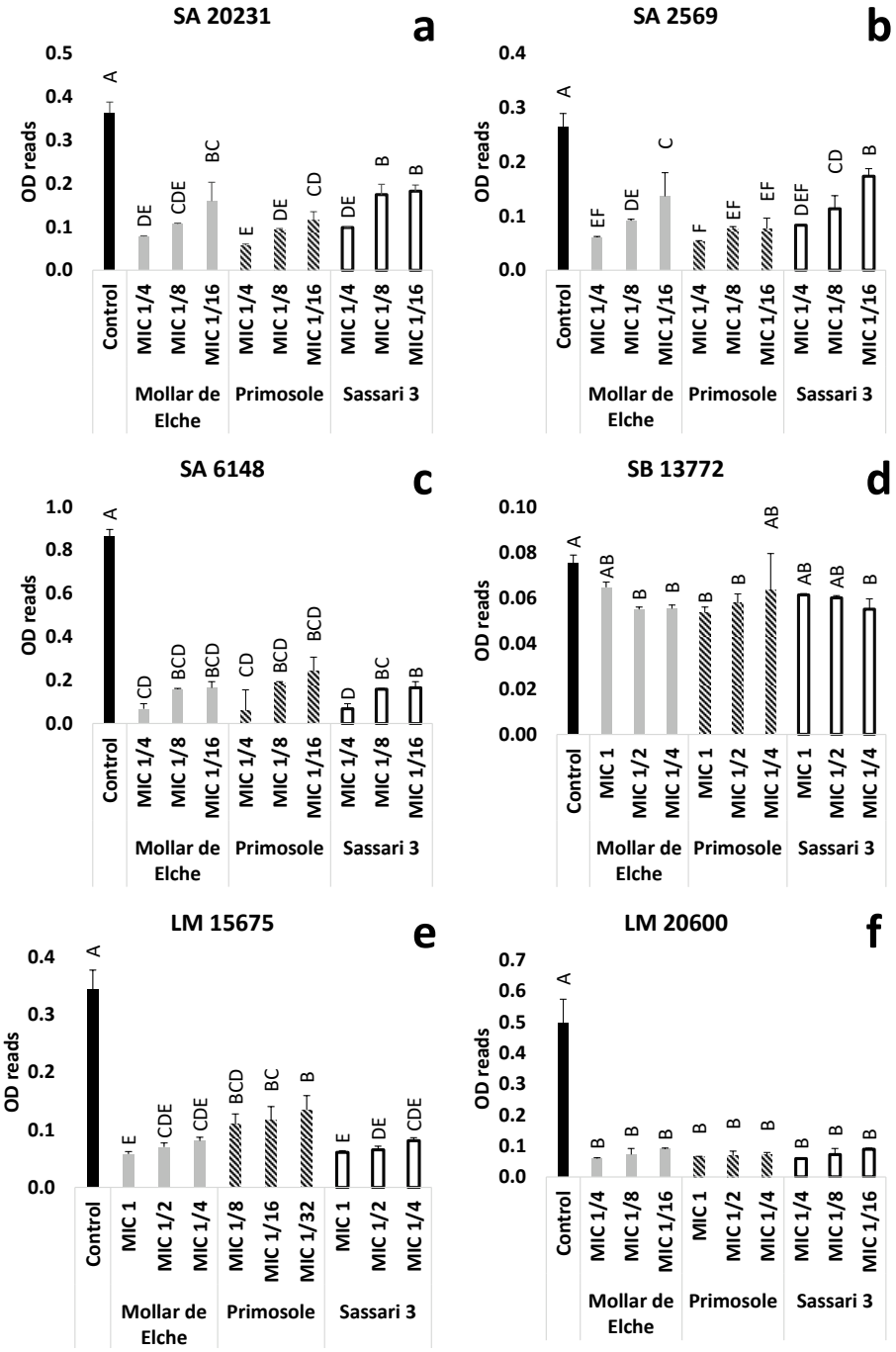

Figure 3. Effect of three cold water PPE (from Mollar de Elche, Primosole, Sassari 3 cultivars), applied at different concentrations (minimum inhibitory concentrations, MIC), on biofilm formation. The letters above the columns indicate statistical differences according to Tukey's test (MIC 1=3 mg/mL; MIC 1/2=1.5 mg/mL; MIC 1/4=0.75 mg/mL; MIC 1/8=0.375 mg/mL; MIC 1/16=0.188 mg/mL; MIC 1/32=0.094 mg/mL). (OD, optical density; SA 20231, Staphyloccocus aureus DSM 20231; SA 2569, Staphyloccocus aureus DSM 2569; SA 26148, Staphyloccocus aureus DSM 6148; SB13772, Salmonella bongori DSM 13772; LM 15675, Listeria monocytogenes DSM 15675; LM 20600, Listeria monocytogenes DSM 20600).

Sassari 1 and Sassari 2 were also good sources of ellagic acid, catechin, and cyanidin-3-glucoside, whereas Arbara Druci can be considered a good source of punicalins and total tannins.

4.2 Antimicrobial and antibiofilm activity

Naturally derived antimicrobials from various plant sources, including *P. granatum*, have been successfully applied as alternatives to synthetic chemicals for suppressing the growth of a number of foodborne bacteria (Kanatt et al., 2010). The antimicrobial properties of plants can probably be attributed to their specific secondary metabolites (Bensilmane et al., 2020; Balaban et al., 2021), which vary due to the variance in the chemical composition and as a result the mechanism of action from one plant extract to another (Hanafy et al., 2021). Noteworthily that *P. granatum* peel extracts' antimicrobial activity was reported in several previous studies against different foodborne pathogens. These studies provide evidence for the presence of polyphenolic bioactive compounds in PPE that was effective against the growth of several microbes including *S. aureus, E. coli, L. monocytogenes*, and *S. enteritidis, Aspergillus niger, Saccharomyces cerevisiae* (Malviya et al., 2014; Assar and Shahate, 2017; Benslimane et al., 2020; Balaban et al., 2021), *Fusarium sambucinum, Penicillium italicum, B. subtilis* (Chen et al., 2020), *S. epidermidis, K. pneumoniae, S. typhi, Yersinia enterocolitica*, and *Candida albicans* (Qahir et al., 2021).

In the current study, we screened the antimicrobial and antibiofilm activities of different locally cultivated (Sardinia) pomegranate cultivars against several food pathogens and probiotic bacteria. The initial screening for antimicrobial activity showed that most of the PPEs were effective in suppressing the microbial growth of the tested bacteria, albeit to different degrees. We also found that the Gram-positive strains *S. aureus* and *L. monocytogenes* were more sensitive than *E. coli* and *S. bongori* Gram-negative strains (Table 2) to the tested extracts. In addition, the MIC values recorded in the present study (0.09 to 3.00 mg/mL) were much lower than those reported in previous studies (Wafa et al., 2017; Nasreddine et al., 2018; Nozohour et al., 2018). Hanafy et al. (2021) reported in a study to evaluate the antimicrobial potentials of different fruit peels that PPEs showed the most significant inhibitory effect against the tested strains *B. cereus, S. aureus* MRSA, *S. aureus, L. monocytogenes*, and *S. typhimurium* where the MIC values ranged between 6.25 and 12.5 mg/mL.

Pagliarulo et al. (2016) showed that pomegranate Phyto-complex extracts had an effective inhibitory effect on the bacterial growth of clinical isolates of *S. aureus* and *E. coli* with a MIC ranging from 20 to 30 mg/mL. Moreover, in several studies, the MIC values were observed to be 0.62−10.00 mg/mL against S. *aureus, E. coli*, and *P. aeruginosa*. Such antimicrobial variances could be a result of the different extraction methods and different used solvents, fruit freshness, time, and region of cultivation (Hany et al., 2011; El-Beltagi et al., 2022). Among the strains investigated by Rahnemoon et al. (2016), the authors reported *S. aureus*, followed by *L. monocytogenes*, showed the highest sensitivity to PPEs, whereas *E. coli* showed the lowest sensitivity. This is in line with the results of the current study, with the addition of *S. bongori* which showed complete resistance to all the tested PPEs. Inconsistently, different levels of inhibition for the different studied pomegranate peel extracts were reported, where the *Salmonella* strain was the most sensitive among the tested pathogens (Abou El-Nour, 2019). This might be due to a strain-specific action of PPE, which could be more or less efficient against strains of the same species. Gram-positive bacteria were reported to be more sensitive to PPEs than Gram-negative bacterial strains, which is consistent with the results obtained in this work. This variation in sensitivity could be ascribed to differences in cell wall composition (Kanatt et al., 2010; Alexandre et al., 2019; Hanani et al., 2019; Benslimane et al., 2020).

Gram-positive bacteria lack an outer membrane which contributes to the easier diffusion of phytochemicals through the cell wall. The outer membrane of Gram-negative bacteria has a lipopolysaccharide layer and periplasmic space in the cell wall that hinders the penetration of antimicrobial substances. However, in Gram-positive bacteria, the plant extracts are able to disrupt the molecular structure of the bacterial cell wall, reacting in several ways and ending with cell death, which may explain the different resistances of Gram-positive and Gram-negative bacteria to the actions of phenolic compounds (Al-Zoreky, 2009; Alexandre et al., 2019; Benslimane et al., 2020; Balaban et al., 2022). Phenolic compounds can react through different mechanisms of action to express their antimicrobial activity against microbes. This can be achieved by inhibiting several virulence factors (e.g., by inhibition of biofilm formation, neutralization of bacterial toxins, and reduction of host ligand adhesion), reducing membrane fluidity, and inhibiting the synthesis of nucleic acids or energy metabolism (Takó et al., 2020).

One potential mechanism to explain the antimicrobial activity of phenolic compounds against foodborne pathogens could be the hyperacidification of the plasma membrane interphase resulting from phenolic acid dissociation. This procedure leads to changes in the cell's membrane potential which would increase its permeability. This mechanism may also explain the differences in the sensitivity of the different pathogenic microorganisms toward phenolic acids (Alexandre et al., 2019). Punicalagin is one of the main ellagitannins (ETs) present in pomegranate peel extract that is found to be responsible for its antimicrobial potentials where it was observed to inhibit the growth of several bacterial strains, such as *S. aureus, S. epidermidis, S. xylosus, S. enteritidis, E. coli., C. albicans, Pseudomonas aeruginosa, B. cereus, Lactobacillus sakei ssp. S. Lactobacillus plantarum, Pediococcus acidilactici,* and *Enterococcus* spp. (Maphetu et al., 2022).

In addition, Rosas-Burgos et al. (2017) suggested punicalagin and ellagic acid be the bioactive molecules principally responsible for the antimicrobial activity of pomegranate peel. This is partially in line with the results of the current study, where the inhibitory effect was primarily correlated with Punα and Punβ, but also with other flavonoid and phenolic molecules. However, the PLS-DA models investigating the relationship between PPE composition and relative antimicrobial activity only partially describe the biological process at play. Indeed, the presence of other broad-spectrum antimicrobial compounds, such as organic acids, could also contribute to the antibacterial activity of PPEs (Devatkal et al., 2013; Malviya et al., 2014; Balaban et al., 2021). In some cases, as in the model for E. coli. DSM 4415, for example, the antimicrobial effect of specific phenolic compounds was not clear, since the model's performance was affected by the presence of other molecules, such as anthocyanins, present in the PPE samples with no antimicrobial activity. This aspect might be related to the relatively low number of PPE samples tested; consequently, the result from PLS-DA should be considered as indicative. Further studies are thus required to confirm these results in which single or mixtures of PPE components are tested. Nonetheless, the recurrent correlation with the "High" or "Medium" PLS-DA classes of certain bioactive compounds, such as the punicalagin isomers, epicatechin, catechin and chlorogenic acids, strongly suggests them to be responsible for the antimicrobial activity of pomegranate peel extracts. The combination of natural compounds with high contents of bioactive molecules in bio-based films is proven to enhance the activity of edible coatings and reinforce food product

conservation. Among these, PPEs have been widely used alone or combined with edible films for food conservation as a source of bioactive compounds.

A study showed that the combination of different PPE concentrations within bio-based films provides a biofunctional edible film for the packaging process. In similar studies, the addition of PPE to edible films or its usage as food preservatives resulted in high antioxidant activity and significant antibacterial ability against several bacterial strains (Yuan et al., 2015; Chen et al., 2020), including *L. monocytogenes* (Takó et al., 2020), *S. aureus, E.coli* (Emam-Djomeh et al., 2015; Ali et al., 2019), *B. cereus*, and *S. Typhimurium* (Hanani et al., 2018). Thus, based on the antimicrobial activity that was observed in the current study, some of the PPEs had high efficiency and potential applications in the food industry as a natural alternative to chemically synthetic antimicrobial agents against food-borne bacteria. Previous studies have confirmed water extracts of pomegranate peel exhibit significant antimicrobial activity against both Gram-positive and Gram-negative bacteria (Chen et al., 2020; El-Beltagi et al., 2022). While the present study similarly revealed water extracts to exert strong antimicrobial activity against *S. aureus* and *L. monocytogenes* strains, very weak antimicrobial activity was observed against *S. bongori* DSM 13772 and *E. coli* strains. This goes along with the results of previous studies where aqueous PPE showed no antimicrobial activity against several gram-negative bacteria like *E. coli*, *P. mirabilia,* and *K. pneumonia* (Chebaibi and Filali, 2013). Sadeghian et al. (2011) reported that both aqueous and methanolic extracts showed good antibacterial activity against *S. aureus* and *P. aeruginosa*, however, the methanolic extract had higher antimicrobial activity against all tested bacteria. The same was observed in a different study where aqueous and methanolic extracts of PPE were proven to be efficient against *P. aeruginosa*, *S. marcescens*, *E. coli*, or *K. pneumoniae* bacteria (El-Beltagi et al., 2022). Another study demonstrated that the aqueous PPE had a significantly higher inhibitory effect on the growth of *Salmonella* when compared to juice powder extract (Wu et al., 2022). Other past studies reported comparatively weaker antimicrobial activities for water extracts compared with those of acetone and/or methanol.

The inconsistencies in findings between studies might be explained by various differences between studies, including pomegranate cultivars, different methods of extraction and extraction solvent polarities, and the different type of bacterial species/strains (Devatkal et al., 2013;

Nozohour et al., 2018; Balaban et al., 2021; Abdel Fattah et al., 2022). Regarding the protechnological strains, both *La. casei* Shirota and *Li. reuteri* 17938 showed less sensitivity to high concentrations or were not inhibited at all by the specific PPEs used in this study. These results agree with those by Alexandre et al. (2019), who reported promising results in relation to LAB, which was not inhibited by any of the tested PPE extracts. Indeed, the resistance of LAB to these compounds could be considered advantageous since these bacteria are potentially beneficial for human health. This finding could be of interest to the dairy industry; for example, the application of PPE could increase the antioxidant activity of dairy products, reducing the risk of contamination by foodborne pathogens, while leaving the stability of naturally present LAB undisturbed. Indeed, Al-Hindi and Abd El Ghani (2020) confirmed that the addition of pomegranate peel polyphenol extracts to fermented dairy products did not cause the LAB density to fall below the functional levels needed for exerting health benefits. Both LAB and phenolic compounds are advantageous for human health, so it might be convenient to develop novel function-enhanced food products that intentionally contain both components.

A further study found that PPE-containing films used for packaging specialty cheeses helped maintain the LAB cultures during storage; in fact, they enhanced their populations increased (Mushtaq et al., 2018). However, in a study conducted by Abd El-Aziz et al. (2013), it was reported that low concentrations of the aqueous PPE stimulated the growth of starter LAB, contrastingly, it was also observed that the gradual increase of the PPE can inhibit the bacterial growth and vitality of starter LAB. Based on this, further in-depth studies would always be required to assess the suitability of these extracts for any practical applications of such technology. Biofilm-associated bacteria are a huge issue where with ease they can resist host defenses, antimicrobial agents, and other stresses in comparison to planktonic cells (Fink et al., 2018). The present study also investigated the susceptibility of microbial biofilms to PPEs. The results showed that PPEs were able to inhibit biofilm development at concentrations below the MIC of the tested isolates.

Benslimane et al. (2020) reported the inhibition of biofilm formation by PPE for all the bacteria strains tested in their study, and the level of inhibition increased by increasing extract concentrations. In the present study, significant reductions were also obtained at much lower concentrations of water-extracted PPE, with more than 70% reduction in biofilm formation

observed at a concentration of 0.75 mg/mL for S. aureus strains and 0.75–3.00 mg/mL for L. monocytogenes strains. A recent study investigated the effect of different extraction solvent types and methods on the antibacterial and antibiofilm activities of PPE and reported that water extract concentrations of 12.5 and 6.25 mg/mL reduced biofilm formation by 94% and 96%, respectively (Nasreddine et al., 2018). More other studies reported the PPE antibiofilm activity against several pathogens, including *B.cereus* strain where biofilm was removed at high ratios ranging between 79% and 83% (Balaban et al., 2021). The *C. albicans* and *Streptococcus parasanguinis* where biofilms were inhabited by 55% and 62%, respectively (De Almeida Rochelle, 2016). In addition, up to 35% of *E. coli* biofilm biomass could be removed by PPE (Fink et al., 2018). The antibiofilm activity of PPEs could be attributed to the presence of phenolic compounds, such as punicalagin and ellagic acid, which might exert their effects through different mechanisms of action (Balaban et al., 2021).

It was observed that methanolic pomegranate extract rich in ellagic acid was able to inhibit the formation of biofilm of *S. aureus*, MRSA, *E. coli*, and *C. albicans*. Phenolic compounds inhibit bacterial biofilm formation by suppressing different regulatory mechanisms: they can alter bacteria performance by reducing its motility, decreasing adhesion, blocking the expression of virulence factors associated with bacteria pathogenicity, and intervening with the mechanism of cell-substratum attachment by modulating the surface charge of bacteria (Dubreuil, 2020; Ebrahimnejad et al., 2020; Takó et al., 2020). Thus, the results of this study indicated PPEs' antibacterial and antibiofilm potentials that might be used as food preservatives in food industries and contribute to food waste reduction. In the context of Italy, the good antimicrobial activities of PPEs obtained in the present study from Sardinian varieties of the fruit support the carrying out of further research into the pomegranate germplasm from local sources and its valorization.

5. Conclusions

The current study contributes to furthering our knowledge of the phenolic composition of pomegranate peel extracts from two internationally known varieties, Wonderful and Mollar de Elche, a commercially relevant Italian variety, Primosole, and four lesserknown Sardinian varieties, Sassari 1, Sassari 2, Sassari 3, and Arbara Druci. Expanding the study to include local

varieties was important from the perspective of Italian plant breeding and the valorization of local biodiversity. Detailed characterization of the bioactive components of peel extracts from specific varieties of pomegranate is necessary in order to explain their antimicrobial activity against some of the most common pathogens such as *S. aureus, L. monocyogenes*, and *E. coli*. Three pomegranate varieties were shown to exhibit strong antimicrobial activity: Mollar de Elche, Primosole, and Sassari 3. The first two varieties were shown to be rich in punicalagin, flavonoids, and chlorogenic acid, the presence of which could account for their antimicrobial activities. On the other hand, the overall low to medium level of phenolic compounds in Sassari 3, with the exception of chlorogenic acid, lead us to hypothesize that this variety's anti-microbial properties are more likely to arise from the combination and synergic action of specific molecules. In conclusion, the results from this study support the valorisation of pomegranate peel, an agro-industrial waste product, in the view of ecological sustainability and circular economies.

6. References

Abou El-Nour, M.M. (2019). Functional properties and medical benefits of pomegranate (*Punica granatum* L.) peels as agro-industrial wastes. Egypt. J. Exp. Biol. 15, 377-392.

Al-Hindi, R.R., Abd El Ghani, S. (2020). Production of functional fermented milk beverages supplemented with pomegranate peel extract and probiotic lactic acid bacteria. J. Food Quality. 2020. https://doi.org/10.1155/2020/4710273

Ali, A., Chen, Y., Liu, H., Yu, L., Baloch, Z., Khalid, S., Zhu, J. and Chen, L. (2019). Starch-based antimicrobial films functionalized by pomegranate peel. Int. J. Biol. Macromol. 129, 1120-1126.

Al-Zoreky, N.S. (2009). Antimicrobial activity of pomegranate (*Punica granatum* L.) fruit peels. Int. J. Food Microbiol. 134, 244-248. https://doi.org/10.1016/j.ijfoodmicro.2009.07.002

Alexandre, E.M., Silva, S., Santos, S.A., Silvestre, A.J., Duarte, M.F., Saraiva, J.A., Pintado, M. (2019). Antimicrobial activity of pomegranate peel extracts performed by high pressure and enzymatic assisted extraction. Food Res. Int. 115, 167-176. https://doi.org/10.1016/j.foodres.2018.08.044

Akhtar, S., Ismail, T., Fraternale, D., Sestili, P. (2015). Pomegranate peel and peel extracts: Chemistry and food features. Food Chem. 174, 417-425. https://doi.org/10.1016/j.foodchem.2014.11.035

Assar, N.H., Shahate, A. (2017). A Study on Biofilms Inhibition and Preservative Activity of Pomegranate Peel Methanol Extract (PPME). J. Adv. Pharm. Res. 1, 110-120.

Bakkiyaraj, D., Nandhini, J.R., Malathy, B., Pandian, S.K. (2013). The anti-biofilm potential of pomegranate (Punica granatum L.) extract against human bacterial and fungal pathogens. Biofouling. 29, 929-937. https://doi.org/10.1080/08927014.2013.820825

Balaban, M., Koc, C., Sar, T., Akbas, M.Y. (2021a). Antibiofilm effects of pomegranate peel extracts against *B. cereus, B. subtilis*, and *E. faecalis*. Int. J. Food Sci. Technol. 56, 4915-4924. https://doi.org/10.1111/ijfs.15221

Balaban, M., Koc, C., Sar, T., Akbas, M.Y. (2021b). Screening for bioactive compound rich pomegranate peel extracts and their antimicrobial activities. Johnson Matthey Tech., 66, 81-89. https://doi.org/10.1595/205651322X16104587974507

Balli, D., Cecchi, L., Khatib, M., Bellumori, M., Cairone, F., Carradori, S., Zengin, G., Cesa, S., Innocenti, M., Mulinacci, N. (2020). Characterization of arils juice and peel decoction of fifteen varieties of *Punica granatum* L.: A focus on anthocyanins, ellagitannins and polysaccharides. Antioxidants, 9(3), 238. https://doi.org/10.3390/antiox9030238

Benslimane, S., Rebai, O., Djibaoui, R., Arabi, A. (2020). Pomegranate Peel Extract Activities as an Antioxidant and Antibiofilm against Bacteria Isolated from Caries and Supragingival Plaque. Jordan J. Biol. Sci., 13, 403-412.

Çam, M., Hışıl, Y. (2010). Pressurised water extraction of polyphenols from pomegranate peels. Food Chem. 123, 878-885. https://doi.org/10.1016/j.foodchem.2010.05.011

Celiksoy, V., Heard, C.M. (2021). Antimicrobial Potential of Pomegranate Extracts In Lagouri, V. (Eds), Pomegranate, IntechOpen. DOI: 10.5772/intechopen.95796

Chandra, R., Babu, K.D., Jadhav, V.T., Jaime, A., Silva, T.D (2010). Origin, history and domestication of pomegranate. Fruit, Vegetable and Cereal Science and Biotechnology, 2, 1-6.

Chebaibi, A., Filali, F.R. (2013). Bactericidal activity and phytochemical screening of Moroccan pomegranate (*Punica granatum* Linn.) peel aqueous extracts. J. Med. Plant Res. 7, 887-891.

Chen, J., Liao, C., Ouyang, X., Kahramanoğlu, I., Gan, Y., Li, M. (2020). Antimicrobial activity of pomegranate peel and its applications on food preservation. J. Food Quality, 2020,1-8. https://doi.org/10.1155/2020/8850339

de Almeida Rochelle, S.L., Sardi, J.D.C.O., Freires, I.A., de Carvalho Galvão, L.C., Lazarini, J.G., de Alencar, S.M., Rosalen, P.L. (2016). The anti-biofilm potential of commonly discarded agro-industrial residues against opportunistic pathogens. Ind Crops Prod. 87, 150-160.

Dubreuil, J.D. (2020). Fruit extracts to control pathogenic *Escherichia coli*: A sweet solution. Heliyon, 6, e03410.

da Silva Veloso, F., Caleja, C., Calhelha, R.C., Pires, T., Alves, M., Barros, L., Genena, A.K., Barreira, J.C., Ferreira, I.C. (2020). Characterization and application of pomegranate epicarp extracts as functional ingredients in a typical Brazilian pastry product. Molecules, 25, 1481. https://doi.org/10.3390/molecules25071481

Daotong, L., Pengpu, W., Yinghua, L., Mnegyao, Z., Fang, C. (2017). Health benefits of anthocyanins and molecular mechanisms: Update from recent decade. Crit Rev Food Sci. 58, 1729-1741. https://doi.org/10.1080/10408398.2015.1030064

Deiana, P., Santona, M., Dettori, S., Culeddu, N., Dore, A., Molinu, M.G. (2019). Multivariate approach to assess the chemical composition of Italian virgin olive oils as a function of variety and harvest period. Food Chem. 300:125243. https://doi.org/10.1016/j.foodchem.2019.125243

Deiana. P., Molinu, M.G., Dore, A., Culeddu, N., Dettori, S., Santona, M. (2022). Evolution of monovarietal virgin olive oils as a function of chemical composition and oxidation status. Nat. Prod. Res. 1-5. https://doi.org/10.1080/14786419.2022.2042813

Derakhshan, Z., Ferrante, M., Tadi, M., Ansari, F., Heydari, A., Hosseini, M.S., Conti, G.O., Sadrabad, E.K. (2018). Antioxidant activity and total phenolic content of ethanolic extract of pomegranate peels, juice and seeds. FCT, 114, 108-111.

Devatkal, S.K., Jaiswal, P., Jha, S.N., Bharadwaj, R., Viswas, K.N. (2013). Antibacterial activity of aqueous extract of pomegranate peel against *Pseudomonas stutzeri* isolated from poultry meat. J. Food Sci. Technol. 50, 555-560. DOI 10.1007/s13197-011-0351-y

El–Aziz, A., El-Gammal, R., Abo-Srea, M.M., Youssf, F.I. (2013). Antioxidant and antimicrobial activity of pomegranate (*Punica granatum* L.) fruit peels extract on some chemical, microbiological and organoleptical properties of yoghurt during storage. JFDS, 4, 387-400.

El-Beltagi, H.S., Eshak, N.S., Mohamed, H.I., Bendary, E.S., Danial, A.W. (2022). Physical characteristics, mineral content, and antioxidant and antibacterial activities of *punica granatum* or citrus sinensis peel extracts and their applications to improve cake quality. Plants, 11, 1740.

Ebrahimnejad, H., Ebadi, M., Mansouri-Najand, L. (2020). The anti-planktonic and anti-biofilm formation activity of Iranian pomegranate peel hydro-extract against *Staphylococcus aureus*. Iran J. Vet. Sci. Technol. 12, 1-9. DOI: 10.22067/veterinary.v12i1.82364

Emam-Djomeh, Z., Moghaddam, A., Yasini Ardakani, S.A. (2015). Antimicrobial activity of pomegranate (Punica granatum L.) peel extract, physical, mechanical, barrier and antimicrobial properties of pomegranate peel extract-incorporated sodium caseinate film and application in packaging for ground beef. Packag. Technol. Sci. 28, 869-881.

Endo, E.H., Costa, G.M., Makimori, R.Y., Ueda-Nakamura, T., Nakamura, C.V., Dias Filho, B.P. (2018). Anti-biofilm activity of Rosmarinus officinalis, *Punica granatum* and Tetradenia riparia against methicillin-resistant *Staphylococcus aureus* (MRSA) and synergic interaction with penicillin. J. Herbal Med. 14, 48-54. https://doi.org/10.1016/j.hermed.2018.07.001

Fattah, A.F.A.A., Kishk, Y.F., El-Beltagy, A.E., El-Sayed, M.E., Khalil, S., Alharthi, S.S. (2022). Antibacterial activity of crude and nano-pomegranate (*Punica granatum* L.) peel powder extracts. Med. Plants - Int. J. Phytomed. Relat. Ind. 14, 323-333.

Fink, R., Kulaš, S., Oder, M. (2018). Efficacy of sodium dodecyl sulphate and natural extracts against *E. coli* biofilm. Int. J. Environ. Health Res. 28, 306-314.

Gigliobianco, M.R., Cortese, M., Nannini, S., Di Nicolantonio, L., Peregrina, D.V., Lupidi, G., Vitali, L.A., Bocchietto, E., Di Martino, P., Censi, R. (2022). Chemical, Antioxidant, and Antimicrobial Properties of the Peel and Male Flower By-Products of Four Varieties of *Punica granatum* L. Cultivated in the Marche Region for Their Use in Cosmetic Products. Antioxidants, 11, 768.

Gullón, P., Astray, G., Gullón, B., Tomasevic, I., Lorenzo, J.M. (2020). Pomegranate peel as suitable source of high-added value bioactives: Tailored functionalized meat products. Molecules, 25, 2859. https://doi.org/10.3390/molecules25122859

Hanafy, S.M., El-Shafea, A., Mohamed, Y., Saleh, W.D., Fathy, H.M. (2021). Chemical profiling, in vitro antimicrobial and antioxidant activities of pomegranate, orange and banana peel-extracts against pathogenic microorganisms. JGEB, 19, 1-10

Hanani, Z.N., Husna, A.A., Syahida, S.N., Khaizura, M.N., Jamilah, B. (2018). Effect of different fruit peels on the functional properties of gelatin/polyethylene bilayer films for active packaging. Food Packag. Shelf Life, 18, 201-211.

Hanani, Z.N., Yee, F.C., Nor-Khaizura, M.A.R. (2019). Effect of pomegranate (Punica granatum L.) peel powder on the antioxidant and antimicrobial properties of fish gelatin films as active packaging. Food Hydrocoll. 89, 253-259.

Hany, M.Y., Manal, F.E., Ahmed, E.A.M. (2011). Antimicrobial activity of pomegranate rind peel extracts. Afr. J. Microbiol. Res. 5, 3664-3668.

Kaderides, K., Kyriakoudi, A., Mourtzinos, I., Goula, A.M. (2021). Potential of pomegranate peel extract as a natural additive in foods. Trends Food Sci. Technol. 115, 380-390. https://doi.org/10.1016/j.tifs.2021.06.050

Kanatt, S.R., Chander, R., Sharma, A. (2010). Antioxidant and antimicrobial activity of pomegranate peel extract improves the shelf life of chicken products. Int. J Food Sci. Technol., 45, 216-222. https://doi.org/10.1111/j.1365-2621.2009.02124.x

Kandylis, P., Kokkinomagoulos, E. (2020). Food applications and potential health benefits of pomegranate and its derivatives. Foods, 9, 122. https://doi.org/10.3390/foods9020122

Kennas, A., Amellal-Chibane, H. (2019). Comparison of five solvents in the extraction of phenolic antioxidants from pomegranate (Punica granatum L.) peel. NAJFNR, 3, 140-147. https://doi.org/10.51745/najfnr.3.5.140-147

Kharchoufi, S., Licciardello, F., Siracusa, L., Muratore, G., Hamdi, M., Restuccia, C. (2018). Antimicrobial and antioxidant features of 'Gabsi' pomegranate peel extracts. Ind. Crop Prod., 111, 345-352. https://doi.org/10.1016/j.indcrop.2017.10.037

Kumar, N., Neeraj Dr. (2018). Study on physico-chemical and antioxidant properties of pomegranate peel. J. Pharm. Phytochem. 7, 2141-2147.

Malviya, S., Jha, A., Hettiarachchy, N. (2014). Antioxidant and antibacterial potential of pomegranate peel extracts. J. Food Sci. Technol. 51, 4132-4137. DOI 10.1007/s13197-013-0956-4

Maphetu, N., Unuofin, J.O., Masuku, N.P., Olisah, C., Lebelo, S.L. (2022). Medicinal uses, pharmacological activities, phytochemistry, and the molecular mechanisms of *Punica granatum* L.(pomegranate) plant extracts: A review. Biomed. Pharmacother. 153, 113256.

Melgarejo-Sánchez, P., Núñez-Gómez, D., Martínez-Nicolás, J.J., Hernández, F., Legua, P., Melgarejo, P. (2021). Pomegranate variety and pomegranate plant part, relevance from bioactive point of view: a review. Bioresources Bioprocess. 8, 1-29. https://doi.org/10.1186/s40643-020-00351-5

Melito, S., La Bella, S., Martinelli, F., Cammalleri, I., Tuttolomondo, T., Leto, C., Fadda, A., Molinu, M. G., Mulas, M. (2016). Morphological, chemical, and genetic diversity of wild myrtle (*Myrtus communis* L.) populations in Sicily. Turkish J. Agric. Forestry. 40, 249-261. doi:10.3906/tar-1505-118

Montefusco, A., Durante, M., Migoni, D., De Caroli, M., Ilahy, R., Pék, Z., Helyes, L., Fanizzi, F.P., Mita, G., Piro, G., Lenucci, M.S. (2021). Analysis of the Phytochemical Composition of Pomegranate Fruit Juices, Peels and Kernels: A Comparative Study on Four Cultivars Grown in Southern Italy. Plants, 10, 2521. https://doi.org/10.3390/plants10112521

Mostafa, A.A., Al-Askar, A.A., Almaary, K.S., Dawoud, T.M., Sholkamy, E.N., Bakri, M.M. (2018). Antimicrobial activity of some plant extracts against bacterial strains causing food poisoning diseases. Saudi J. Biol. Sci. 25, 361-366. https://doi.org/10.1016/j.sjbs.2017.02.004

Mushtaq, M., Gani, A., Gani, A., Punoo, H.A., Masoodi, F.A. (2018). Use of pomegranate peel extract incorporated zein film with improved properties for prolonged shelf life of fresh Himalayan cheese (Kalari/kradi). Innov. Food Sci. Emerg. Technol. 48, 25-32. https://doi.org/10.1016/j.ifset.2018.04.020

Nasreddine, S., Mcheik, A., Al-Seblani, M.A., Shahrour, H., Hammoud, M., Chokr, A. (2018). Influence of the Extractive Method of *Punica granatum* Peels in its Antibacterial and Antibiofilm Activities Against Gram-Negative and Gram-Positive Bacteria. J. Dis. Med. Plants, 4, 133-141. doi: 10.11648/j.jdmp.20180406.11

Nozohour, Y., Golmohammadi, R., Mirnejad, R., Fartashvand, M. (2018). Antibacterial activity of pomegranate (*Punica granatum* L.) seed and peel alcoholic extracts on *Staphylococcus aureus* and *Pseudomonas aeruginosa* isolated from health centers. J. Applied Biotechnol. 5, 32-36. https://doi.org/10.29252/jabr.01.01.06

Nuamsetti, T., Dechayuenyong, P., Tantipaibulvut, S. (2012). Antibacterial activity of pomegranate fruit peels and arils. Sci. Asia. 38, 319-22. doi: 10.2306/scienceasia1513-1874.2012.38.319

Orgil, O., Schwartz, E., Baruch, L., Matityahu, I., Mahajna, J., Amir, R. (2014). The antioxidative and anti-proliferative potential of non-edible organs of the pomegranate fruit and tree. LWT. 58, 571-577. https://doi.org/10.1016/j.lwt.2014.03.030

Oudane, B., Boudemagh, D., Bounekhel, M., Sobhi, W., Vidal, M., Broussy, S. (2018). Isolation, characterization, antioxidant activity, and protein-precipitating capacity of the hydrolyzable tannin punicalagin from pomegranate yellow peel (Punica granatum). J. Mol. Struct. 1156, 390-396. https://doi.org/10.1016/j.molstruc.2017.11.129

Pagliarulo, C., De Vito, V., Picariello, G., Colicchio, R., Pastore, G., Salvatore, P., Volpe, M. G. (2016). Inhibitory effect of pomegranate (*Punica granatum* L.) polyphenol extracts on the bacterial growth and survival of clinical isolates of pathogenic *Staphylococcus aureus* and *Escherichia coli*. Food Chem. 190, 824-831.

Qahir, A., Khan, N., Hakeem, A. a., Kamal, R. (2021). The antioxidant, antimicrobial, and clinical effects with elemental contents of pomegranate (*Punica granatum*) peel extracts: A review. BJ-BABS, 2, 21-28.

Pai, V., Chanu, T.R., Chakraborty, R., Raju, B., Lobo, R., Ballal, M. (2011). Evaluation of the antimicrobial activity of *Punica granatum* peel against the enteric pathogens: an in vitro study. Asian J. Plant Sci. Res. 1, 57-62.

Petretto, G.L., Fancello, F., Bakhy, K., Al Faiz, C., Sibawayh, Z., Chessa, M., Zara, S., Sanna, M.L., Maldini, M., Rourke, J.P., Pintore, G. (2018). Chemical composition and antimicrobial activity of essential oils from *Cuminum cyminum* L. collected in different areas of Morocco. Food Biosci. 22, 50-58. https://doi.org/10.1016/j.fbio.2018.01.004

Piluzza, G., Campesi, G., Molinu, M.G., Re, G.A., Sulas, L. (2020). Bioactive Compounds from Leaves and Twigs of Guayule Grown in a Mediterranean Environment. *Plants*, *9*, 442. https://doi.org/10.3390/plants9040442

Pirzadeh, M., Caporaso, N., Rauf, A., Shariati, M.A., Yessimbekov, Z., Khan, M.U., Imran, M., Mubarak, M.S. (2021). Pomegranate as a source of bioactive constituents: A review on their characterization, properties and applications. Crit. Rev. Food Sci. Nutr. 61, 982-999. https://doi.org/10.1080/10408398.2020.1749825

Puneeth, H.R., Chandra, S.S.P. (2020). A review on potential therapeutic properties of Pomegranate (*Punica granatum* L.). Plant Sci. Today, 7, 9-16. https://doi.org/10.14719/pst.2020.7.1.619

Rahnemoon, P., Jamab, M.S., Dakheli, M.J., Bostan, A. (2016). Phenolic compounds and antimicrobial properties of pomegranate (Punica granatum) peel extracts. Int. J. Agric. Biosys. Eng. 10, 646-651.

Re, G.A., Piluzza, G., Sanna, F., Molinu, M.G., Sulas, L. (2019). Polyphenolic composition and antioxidant capacity of legume-based swards are affected by light intensity in a Mediterranean agroforestry system. J. Sci. Food Agric. 99, 191-198. https://doi.org/10.1002/jsfa.9160

Romani, A., Campo, M., Pinelli, P. (2012). HPLC/DAD/ESI-MS analyses and anti-radical activity of hydrolyzable tannins from different vegetal species. Food Chem. 130, 214-221. https://doi.org/10.1016/j.foodchem.2011.07.009

Rosas-Burgos, E.C., Burgos-Hernández, A., Noguera-Artiaga, L., Kačániová, M., Hernández-García, F., Cárdenas-López, J.L., Carbonell-Barrachina, Á.A. (2017). Antimicrobial activity of pomegranate peel extracts as affected by cultivar. J. Sci. Food Agric., 97, 802-810. https://doi.org/10.1002/jsfa.7799

Roy, R., Tiwari, M., Donelli, G., Tiwari, V. (2018). Strategies for combating bacterial biofilms: A focus on anti-biofilm agents and their mechanisms of action. Virulence. 9, 522-554. https://doi.org/10.1080/21505594.2017.1313372

Russo, M., Fanali, C., Tripodo, G., Dugo, P., Muleo, R., Dugo, L., De Gara, L., Mondello, L. (2018). Analysis of phenolic compounds in different parts of pomegranate (*Punica granatum*) fruit by HPLC-PDA-ESI/MS and evaluation of their antioxidant activity: application to different Italian varieties. Anal. Bioanal. Chem. 410, 3507-3520. https://doi.org/10.1007/s00216-018-0854-8

Saad, H., Charrier-El Bouhtoury, F., Pizzi, A., Rode, K., Charrier, B., Ayed, N. (2012). Characterization of pomegranate peels tannin extractives. Ind. Crops Prod. 40, 239-246. https://doi.org/10.1016/j.indcrop.2012.02.038

Sadeghian, A., Ghorbani, A., Mohamadi-Nejad, A., Rakhshandeh, H. (2011). Antimicrobial activity of aqueous and methanolic extracts of pomegranate fruit skin. AJP, 1, 67-73.

Schwartz, E., Tzulker, R., Glazer, I., Bar-Ya'akov, I., Wiesman, Z., Tripler, E., Bar-Ilan, I., Fromm, I, Borochov-Neori, H., Holland, D., Amir, R. (2009). Environmental conditions affect the color, taste, and antioxidant capacity of 11 pomegranate accessions' fruits. J Agric. Food Chem. 57, 9197-9209. https://doi.org/10.1021/jf901466c

Singh, B., Singh, J.P., Kaur, A., Singh, N. (2018). Phenolic compounds as beneficial phytochemicals in pomegranate (Punica granatum L.) peel: A review. Food Chem. 261, 75-86. https://doi.org/10.1016/j.foodchem.2018.04.039

Singh, B., Singh, J.P., Kaur, A., Singh, N. (2019). Antimicrobial potential of pomegranate peel: a review. Int. J. Food Sci. Technol. 54, 959-965. https://doi.org/10.1111/ijfs.13964

Slobodníková, L., Fialová, S., Rendeková, K., Kováč, J., Mučaji, P. (2016). Antibiofilm activity of plant polyphenols. Molecules, 21, 1717. https://doi.org/10.3390/molecules21121717

Takó, M., Kerekes, E.B., Zambrano, C., Kotogán, A., Papp, T., Krisch, J., Vágvölgyi, C. (2020). Plant phenolics and phenolic-enriched extracts as antimicrobial agents against food-contaminating microorganisms. Antioxidants, 9, 165.

Turrini, F., Malaspina, P., Giordani, P., Catena, S., Zunin, P., Boggia, R. (2020). Traditional decoction and PUAE aqueous extracts of pomegranate peels as potential low-cost anti-tyrosinase ingredients. Appl. Sci. 10, 2795. https://doi.org/10.3390/app10082795

Wafa, B. A., Makni, M., Ammar, S., Khannous, L., Hassana, A.B., Bouaziz, M., Es-Safi, N.E., Gdoura, R. (2017). Antimicrobial effect of the Tunisian Nana variety *Punica granatum* L. extracts against Salmonella enterica (serovars Kentucky and Enteritidis) isolated from chicken meat and phenolic composition of its peel extract. Int. J. Food Microb. 241, 123– 131. https://doi.org/10.1016/j.ijfoodmicro.2016.10.007

Wang, Z., Pan, Z., Ma, H., Atungulu, G.G. (2011). Extract of phenolics from pomegranate peels. The Open Food Sci. J. 5, 17−25. DOI: 10.2174/1874256401105010017

Wu, W., Solval, K.M., Chen, J. (2022). Inhibitory activity of aqueous extracts of pomegranate peel products and juice powder against Salmonella enterica. LWT, 155, 112934.

Wu, X., Venkitasamy, C., McHugh, T., Pan, Z. (2021). Process Development for Antioxidant Extraction from Wet Pomegranate Peel. Trans. ASABE, 64(1), 191-202. doi: 10.13031/trans.14008 Punicalagin

Xu, Z., Liang, Y., Lin, S., Chen, D., Li, B., Li, L., Deng, Y. (2016). Crystal violet and XTT assays on *Staphylococcus aureus* biofilm quantification. Current Microb., 73, 474-482. DOI 10.1007/s00284-016-1081-1

Xu, Y., Shi, C., Wu, Q., Zheng, Z., Liu, P., Li, G., Peng, X., Xia, X. (2017). Antimicrobial activity of punicalagin against *Staphylococcus aureus* and its effect on biofilm formation. Foodborne Pathog. Dis. 14, 282-287. DOI: 10.1089/fpd.2016.2226

Young, J.E., Pan, Z., Teh, H.E., Menon, V., Modereger, B., Pesek, J.J., Matyska, M.T., Dao, L., Takeoka, G. (2017). Phenolic composition of pomegranate peel extracts using an liquid chromatography-mass spectrometry approach with silica hydride columns. J. Sep. Sci. 40, 1449-1456. https://doi.org/10.1002/jssc.201601310

Yuan, G., Lv, H., Yang, B., Chen, X., Sun, H. (2015). Physical properties, antioxidant and antimicrobial activity of chitosan films containing carvacrol and pomegranate peel extract. Molecules, 20, 11034-11045.

Zhang, Y., Wang, D., Lee, R.P., Henning, S.M., Heber, D. (2009). Absence of pomegranate ellagitannins in the majority of commercial pomegranate extracts: implications for standardization and quality control. J. Agric. Food Chem. 57(16), 7395-7400. https://doi.org/10.1021/jf9010017

Zhao, X., Yuan, Z., Fang, Y., Yin, Y., Feng, L. (2013). Characterization and evaluation of major anthocyanins in pomegranate (Punica granatum L.) peel of different cultivars and their development phases. Eur.Food Res. Technol. 236, 109-117. DOI 10.1007/s00217-012-1869-6

Živković, J., Šavikin, K., Janković, T., Ćujić, N., Menković, N. (2018). Optimization of ultrasound-assisted extraction of polyphenolic compounds from pomegranate peel using response surface methodology. Sep. Purif. Technol. 194, 40-47. https://doi.org/10.1016/j.seppur.2017.11.032

CHAPTER II

Bioassay-guided fractionation and identification of the antimicrobial bioactive compounds of *Punica Granatum* peels against *Staphylococcus aureus*

Amira Salim[1], Lewis Marquez[2], Sunmin Woo[3], Cassandra L. Quave[3 4], Francesco Fancello[1], Severino Zara[1]

[1]Department of Agricultural Sciences, University of Sassari, 07100 Sassari, Italy
[2]Molecular and Systems Pharmacology Program, Laney Graduate School, Emory University, USA
[3]Center for the Study of Human Health, Emory University, USA
[4]Department of Dermatology, Emory University School of Medicine, USA

Abstract

The escalating concern over antibiotic resistance has fuelled the exploration of alternative sources for antimicrobial agents, with a growing focus on natural compounds derived from agricultural by-products. Pomegranate peel a by-product of the fruit processing industry, is a rich source of bioactive compounds such as polyphenols, flavonoids, and tannins. The antimicrobial properties of these compounds have been documented in previous studies, prompting the investigation of their efficacy against infectious diseases. This study investigates the potential of pomegranate peel extracts, as a natural antibiotic against *Staphylococcus aureus*, a notorious pathogen implicated in various human infections. A bioassay-guided fractionation approach was employed to isolate and identify the antimicrobial bioactive compounds present in *P. granatum* peels. Subsequent chemical analyses and chromatographic techniques were employed to identify and characterize the specific compounds responsible for the observed antibacterial activity. Beyond its antimicrobial properties, the study also explores the safety profile of pomegranate peel extracts, considering their potential application in pharmaceutical and healthcare settings. This involves assessing cytotoxicity and potential side effects in determining the feasibility of natural extracts as viable alternatives to synthetic antibiotics. The outcomes of this research contribute to the growing body of knowledge on sustainable antimicrobial solutions, leveraging the untapped potential of agricultural by-products. If successful, pomegranate peel extracts could emerge as a novel, cost-effective, and eco-friendly alternative to traditional antibiotics, offering a ray of hope in the battle against antibiotic-resistant pathogens like *S. aureus*.

Keywords: *Staphylococcus aureus*, natural products, *Punica Granatum*, Fractionation, punicalagin

1. *Introduction*

Food-borne diseases caused by pathogens including bacteria, viruses, fungi, prions, and parasites found in contaminated food and water pose significant public health challenges worldwide. There are more than 200 diseases linked to contaminated food (Castro et al., 2018), where contaminated food results in 600 million cases of foodborne diseases and 420,000 deaths worldwide every year (Ene, 2020; Lee and Yoon, 2021). These account for about 23 million cases of illness and 5000 deaths in Europe only every year (Castro et al., 2018; Martin et al., 2022). The fight against foodborne diseases encounters several obstacles due to ever-changing human consumption habits, the globalization of the food industry, and climate change. Consequently, infectious diseases are emerging rapidly, posing significant threats to the global economy and public health (Godwin et al., 2022).

Bacterial pathogens such as *Staphylococcus aureus, Escherichia coli*, *Salmonella, Listeria monocytogenes, Bacillus cereus, Campylobacter,* and *Clostridium* are considered the cause of acute food-borne diseases (Castro et al., 2018; Khan et al., 2023). Despite intensified attempts to improve hygiene standards and raise awareness, foodborne illness outbreaks have not declined. Pathogenic bacteria have the potential to induce foodborne diseases and contaminate food at various stages in the storage and distribution processes, including production, transportation, handling, and packaging. To ensure the safety of the food supply chain, it is crucial to implement effective interventions for both food products and the processing environment (Khan et al., 2023)

Staphylococcal food-borne diseases is a prevalent global foodborne illness caused by the presence of *S. aureus* bacteria in contaminated food. This pathogen poses a significant risk to consumers, leading to severe economic losses and decreased human productivity due to foodborne illnesses (Kadariya et al., 2014). Many strains of *Staphylococcus* bacteria produce enterotoxin, which is responsible for causing staphylococcal food poisoning. Globally, staphylococcal intoxication remains a very common food poisoning (Sousa, 2008). Extensive scientific research has been dedicated to minimizing health hazards and economic damages caused by bacterial contamination from *S. aureus* in food. Efforts have focused on preventive measures such as

maintaining proper temperatures and personal hygiene. Additionally, the use of chemical preservatives has been explored to inhibit the growth of *S. aureus* in food. While conventional antibiotics and antimicrobial agents have been employed to prolong the shelf life of food products, concerns about the potential impact of these preservatives on human health continue to be raised (Xu et al., 2017). Furthermore, the excessive and unjustified use of antibiotics in treating infectious diseases has created favorable conditions for the exposure and dissemination of resistant strains of various pathogens (Song et al., 2022).

S. aureus bacterium has demonstrated a rapid ability to develop resistance against antibiotics (Salam et al., 2021), where Methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Staphylococcus aureus* (VRSA) are good examples of the inappropriate usage of antibiotics against *S. aureus* (Song et al., 2022). MRSA is a major global concern in infectious diseases, causing significant morbidity and mortality. It poses a severe threat to human health due to its continual emergence and rapid dissemination, with the absence of new antibiotics suggesting the arrival of a post-antibiotic era (Salam et al., 2021; Xie et al., 2021). Antibiotic resistance in *S. aureus* can result from various mechanisms, such as enzymatic modification of drugs, changes in drug targets, increased expression of efflux pumps, and modifications in membrane permeability. Intrinsic resistance mechanisms, like biofilm formation and the production of virulence factors that evade the host immune response, also significantly contribute to the persistence and severity of *S. aureus* infections (Tang et al., 2020).

The prevalence of MRSA strains as pathogens and the general rise in antimicrobial resistance is a growing global concern and has become a significant public health issue. Consequently, there is a necessity to explore novel antimicrobial agents including those derived from plants (Machado et al., 2002; Celiksoy and Heard, 2021; Silva et al., 2023). Growing awareness of the benefits of compounds derived from natural sources, which possess health-enhancing properties like antimicrobial and antioxidant effects, has led to an increased number of research papers investigating the functional properties of these compounds, to explore their potential applications in functional foods or preventive medicine (Pirzadeh et al., 2021). Natural compounds especially those derived from plants have been demonstrated as a viable alternative source of novel active

molecules (Foss et al., 2014), where medicinal plants serve as important reservoirs of new molecules due to the wide range of chemical compounds in their metabolites, offering various biological properties.

Among the phytochemical groups with antimicrobial and antioxidant effects, polyphenols, carotenoids, steroids, and terpenes are notable. Phenolic compounds, in particular, have garnered special interest due to numerous reports highlighting their substantial antioxidant, antibacterial, and antifungal properties, even against multidrug-resistant bacteria, both Gram-positive and Gram-negative (de Lima et al., 2021). Hence, there has been a notable rise in interest in plant extracts, particularly those derived from byproducts like fruit peels, which show potential for applications in food preservation and pharmaceutical uses in recent years (Kharchoufi et al., 2018).

Punica granatum L. (Punicaceae family) commonly known as Pomegranate, is originally from the Mediterranean and the Middle East regions whereas it is cultivated and consumed globally today. With a history rooted in traditional herbal medicine since ancient times, this fruit is recognized as an abundant source of components with well-established potent biological activities (Yang et al., 2016; Peršurić et al., 2020; Gosset-Erard et al., 2021; Eid et al., 2022). Pomegranate fruits, beverages, and associated products are often emphasized for their presumed positive impacts on health, targeting conditions such as arthritis, diabetes, cardiovascular disease, prostate cancer, and various other medical conditions (Ma et al., 2015), with activity more recently being attributed to its rich secondary polyphenolic compounds (Celiksoy and Heard, 2021). Pomegranates contain phytochemical elements in different parts of the fruit, with studies confirming that the outer rind, known as the exocarp, contains the highest concentration of polyphenols (Barbieri and Heard, 2019).

The peel of the pomegranate fruit, a by-product resulting from the pomegranate juice industry (Panichayupakarananta et al., 2010), constitutes approximately 30–40% of the fruit's total weight and is typically considered as biological waste (Peršurić et al., 2020). This peel accounts for half of the three million tons of pomegranates processed globally each year. Because of its substantial organic content, disposing of pomegranate peels in landfills is prohibited in the EU due to the potential environmental hazards associated with uncontrolled greenhouse gas emissions and the

risk to watercourses. Consequently, the food industry needs to reassess its waste management practices, moving away from extensive landfill use and adopting more sustainable strategies aimed at minimizing waste disposal. A promising approach involves transforming fruit and vegetable waste into valuable resources by recovering and utilizing their beneficial components in the agricultural, food, cosmetic, and pharmaceutical sectors (Gosset-Erard et al., 2021).

Pomegranate peel is documented to possess a significant number of phytochemicals, including hydrolyzable tannins, phenolic acids, triterpenoids, phytosterols, lignans, and flavonoids (Li et al., 2006; Farag et al., 2015; El-Hamamsy and El-Khamissi, 2020). Among these phytochemicals, polyphenolic compounds have demonstrated a diverse array of bioactivities, including antioxidant, anti-inflammatory, anticancer, antidiabetic, anti-atherogenic, and antifungal properties. Additionally, they exhibit immunomodulatory activities and serve as potent inhibitors against *S. aureus, L. monocytogenes, S. typhi, E. coli, Yersinia enterocolitica, Enterobacter aerogenes*, and *Klebsiella pneumoniae* (Moradi et al., 2019; Nazeam et al., 2020; Sun et al., 2021).

Furthermore, extracts from pomegranate peel were found to exhibit inhibitory effects on biofilms, in addition to its impact on planktonic forms (Celiksoy and Heard, 2021). Biofilm-associated bacteria pose a significant challenge, as they can easily resist against host defenses, antimicrobial agents, and various stresses when compared to individual planktonic cells (Salim et al., 2023). Several studies propose that the ability of pomegranate to inhibit quorum sensing (QS) may contribute to its effectiveness in preventing biofilm formation. QS serves as a communication system among bacteria within a biofilm, facilitating interactions related to nutrients, defence against other microorganisms, virulence, and biofilm development. In another investigation, various compounds derived from herbs, fruits, and plant extracts were examined for their QS-modulating effects, revealing that pomegranate extract exhibited the most potent inhibition activities. The QS-modulating properties of pomegranate have been linked to various polyphenols, including punicalagin and ellagic acid (Li et al., 2014; Yang et al., 2016; Celiksoy and Heard, 2021).

As a result of the health potentials of pomegranate and its peels, PPE serves as a valuable by-product in the food preservation sector thanks to its high content of substantial bioactive substances

(Chen et al., 2020). The majority of bioactive compounds found in PPE have been previously examined as natural additives to enhance the quality of food preservation (Munir et al., 2019). Various natural extracts have been studied for their capacity to extend the shelf life of chicken products (Kanatt et al., 2010), muscle foods (Das et al., 2021), Cheese (Parafati et al., 2021). As a results Pomegranate rind extract could be commercially exploited as a natural preservative in different food products and successfully improves the oxidative stability and storage quality (Mahajan et al., 2015).

Overall and despite the significant attention pomegranate extracts have received lately, the specific compounds responsible for the antimicrobial effects of pomegranate have not been definitively determined. Currently, no two studies have verified the existence of identical antimicrobial compounds in pomegranate, even though the identified compounds are frequently encountered in this fruit. Discrepancies could arise from variations in the employed methodologies, but they may also be associated with diverse phytochemical compositions among plants of distinct varieties and geographical regions (Gosset-Erard et al., 2021).

2. Materials and methods

2.1 Chemicals

Optima® LC–MS grade acetonitrile and water, containing 0.1% formic acid respectively, and methanol were purchased from Fisher Scientific (Pittsburgh, PA, USA) and Supelco (Bellefonte, PA, USA), Punicalagin α and β were purchased from PhytoLab GmbH & Co.KG (Germany).

2.2 Collection of plant materials and extraction

Fruits from the Primo sole (2707) and Mollar de Elche (2702) varieties of *P. granatum* were harvested on October 20th, 2021. The harvest took place in the pomegranate varietal collection field of the University of Sassari's Experimental Station "A. Milella" in Oristano, Sardinia, at coordinates 39°54′12″N, 8°37′19″E. Four pomegranate fruits for each genotype were processed for extraction where the peel and arils were manually separated, and the peel was finely chopped.

Subsequently, the chopped peel underwent a 72-hour drying process at –55 °C using a vacuum freeze dryer. The dried peel was further processed into a fine powder using a laboratory blender (Waring Commercial Blender 7011S).

Plant material was extracted by maceration as previously described (Dettweiler et al., 2020). Briefly, ground, dried peels of *P. granatum* were macerated (ratio of 1:10 w/v) in 80% aqueous ethanol (v/v) at room temperature for 72 h under constant agitation. This process was repeated for a second time using the same plant residue to increase the extract yield. Both extraction products were filtered, and combined, and the alcoholic filtrate was concentrated using a rotary evaporator, shell-frozen, and lyophilized for 24 hours. The resulting extracts were stored at -20˚C in a dry state until needed, at which point they were dissolved in 100% dimethyl sulfoxide (DMSO) at a stock concentration of 10 mg/mL for the assays.

2.3 Isolation of bioactive compounds

P. granatum extracts 2702 and 2707 underwent bioassay-guided fractionation through reversed-phase high-performance liquid chromatography (HPLC), where method development for the crude extract fractionation was performed on preparative HPLC (Prep-HPLC). All subsequent Prep-HPLC were carried out using an Agilent 1260 Infinity system running OpenLab CDS ChemStation (Agilent Technologies, Santa Clara, CA, United States) equipped with a UV-vis detector, auto collector, Agilent XDB-C18 (21.2 mm × 250 mm, 5 µm) column with a compatible guard column at a column temperature of 40°C. Mobile phases HPLC-grade were 0.1% (vol/ vol) formic acid in water (A) and 0.1% (vol/vol) formic acid in acetonitrile (B), at a flow rate of 1 mL/min and monitored for 36 min. 2702 and 2707 crude extracts were dissolved in MeOH and total of 34 injections each with a 1 mL sample injection (30mg/mL in 80:20 H_2O:MeOH) were performed. Chromatograms were monitored at 254 and 314 nm. A custom-built open-bed fraction collector was used for fractions collection (Caputo et al., 2020). Initial conditions were 90:10 (A:B), held for 5.00 min, changing to 85:15 (A:B) for 10.00 mins, changing to 67:33 (A:B) until 30.00 min, then elution 0:100 (A:B), from 30.01 for 36 min before returning to initial conditions to equilibrate the column. A total of 16 “preparative fractions” (PFs) were obtained using this method; 7 for each crude extract 2702 and 2707. Due to their activity against the *S. aureus* tested

strains, only 2707 PF5 and PF6 were chosen to undergo for further fractionation. A second round of prep-HPLC to split 2707 PF5 and PF6 into "subfractions" (SFs) conducted using the method cited above with some change in the gradient elution. A gradient elution consisting of mobile phases (A) 1% formic acid in H_2O and (B) 1.0% formic acid in acetonitrile at 1.0 mL/ min at a flow rate of 1 mL/min and monitored for 35 min were 95:05 (A:B), held for 5.00 mins, changing to 85:15 (A:B) for 20.00 mins, then elution 0:100 (A:B), from 25.01 to 35 mins before returning to initial conditions to equilibrate the column. 5 sequential runs were performed (2 runs for 2707 PF5 and 3 runs for 2707 PF6), each with a 1 mL sample injection (30mg/mL in 80:20 H_2O:MeOH). A total of 39 SFs from both 2707 PF5 and PF6 were obtained using this method.

2.4 LC–MS characterization of sub-fractions

The dried extracts of 2707 and related subfractions were dissolved with MeOH/H_2O (5:5, v/v) by for LC–MS analysis. The LC–MS analysis was performed on an Agilent 1290 Infinity II UHPLC system coupled to an Agilent 6545XT QTOFMS (Agilent Technologies, Santa Clara, CA, USA), which was equipped with a Dual AJS ESI Ion Source (Agilent Technologies). Chromatographic separations were performed on a Zorbax Eclipse XDB-C18 (100 × 2.1 mm, 1.8 μM) column coupled with Zorbax Eclipse XDB-C18 (5 × 2.1 mm, 1.8 μM) guard column. The mobile phase was comprised of H_2O (A) and acetonitrile (B), both of which were acidified with 0.1% formic acid. The column temperature and sample organizer were maintained at 40 °C and 15 °C, respectively. A stepwise gradient method at a constant flow rate of 0.4 mL/min was used to elute the column with the following conditions: 5–5% B (0.0–0.5 min); 5–15% B (0.5–5.0 min); 15–100% B (5.0–9.0 min); and 100–100% B (9.0–10.5 min), followed by a return to the starting conditions at 10.6 min and 1.4 min of reconditioning the column (total runtime of 12.0 min). Analyses of the samples (2.0 μL, injection volume) were performed in the negative ion mode in both profile and centroid mode. The ESI conditions were set as follows: the capillary voltage was 4.0 kV, the nozzle voltage was 2000 V for negative mode, the fragmentor was 100 V, the drying gas temperature and flow were set to 325 °C and 13 L/min, respectively, and sheath gas temperature and flow were 275 °C and 12 L/min, respectively, and the nebulizer was operating at 35 psi. Nitrogen served both as the nebulizer gas and the dry gas. The Auto-MS/MS mode was

used with an MS range of m/z 100–1700 and an MS2 range of m/z 50–1700, at 7 spectra/s and 5 spectra/s, respectively. The narrow isolation (~ 1.3 *m/z*) width was used. The collision energy was set by the formula based on the *m/z* and charge of the precursor (condition 1: slope of 3.8 and an offset of 20, condition 2: slope of 2.0 and an offset of 6). The maximum precursors per cycle are set to 5, with the absolute precursor threshold set to 500 (relative threshold 0.015%) and active exclusion after 3 scans and released after 0.1 min were performed. MassHunter Workstation Acquisition B.10.00 software and MassHunter Qualitative Analysis 10.0 software (Agilent Technologies) were used for acquiring and processing MS data.

2.5 Bacterial strains

The following bacterial strains were used in this study: *S. aureus* AH845, *S. aureus* AH1677 (Quave lab, Emory University, USA), *S. aureus* DSM 20231, and *S. aureus* DSM 25691 (Leibniz Institute DSMZ, Germany). After streaking from freezer stock and overnight incubation at 37 °C, all strains were grown on tryptic soy agar (TSA). Overnight liquid cultures were maintained in tryptic soy broth (TSB) at 37 °C and with continuous shaking at 200 rpm. Appropriate positive controls (antibiotic) and negative controls (vehicle control, sterile media control) were always incorporated into the assays.

2.6 Growth inhibition assays

2702, 2707 crude extract, fractions, and sub-fractions of *P. granatum* were examined by dose-response experiments to obtain the half maximal inhibitory concentration (IC50) and minimum inhibitory concentration (MIC) values against *S. aureus strains.* Growth inhibition was determined by a change in optical density (OD) readings from the start of incubation to the final time point (18 hours) relative to vehicle control (DMSO). All growth inhibition experiments were carried out following the guidelines set by the Clinical Laboratory Standards Institute (CLSI) M100-S23, for broth microdilution testing (Cockerill et al. 2013). Briefly, standardized working cultures were calculated and diluted from TSB overnight cultures in cation-adjusted Müller-Hinton broth (CAMHB) to an OD_{600} of 0.0006, which corresponds to 5×10^5 CFU/mL using a Cytation 3 multimode plate reader (Biotek). Using two-fold serial dilution, extracts, and vehicle control at

concentrations ranging from 8 to 256 µg/mL, and antibiotic (Vancomycin) ranging from 0.5 to 16 µg/mL were included in the plate setup, and the assays were performed in 96-well flat-bottom non-tissue culture-treated plates (Falcon1 35– 3075, Corning, NY), After treatment, plates were statically incubated at 37°C for 18 h. OD_{600} was measured using a BioTek Cytation3 plate reader at initial and final time points, to account for extract color and the percent inhibition was calculated as previously described (Quave et al., 2015). A media blank was included in each experiment to test for contamination and all concentrations were tested in triplicate, and experiments were performed at least twice on different days to account for two biological replicates to confirm the accuracy of the results. MIC was determined as the lowest treatment concentration at which 90% of growth was inhibited compared with vehicle control and the IC_{50} was defined as the lowest concentration at which 50% of growth was inhibited. Dose–response curves were generated using GraphPad Prism version 10.1.0 software.

2.7 Human keratinocyte toxicity assay

In vitro dose-response cytotoxicity of *P. granatum* active fractions to immortalized human keratinocytes (HaCaTs) was assessed following the Lactate Dehydrogenase (LDH) assay manufacturer's instructions (LDH assay kit, G-Biosciences, St. Louis, MO) as previously described (Quave et al., 2015). Briefly, upon reaching suitable cells confluency (70–90%), HaCaT cells were standardized to 4×10^4 cells/mL› and 200 µL of cell culture were added to wells in 96-well tissue culture microtiter plates and incubated for 24 hours to allow for seeding. After incubation, treatments and fresh media were added to HaCaT cells at a concentration range of 16–128 µg/mL via serial dilution. Plates were subsequently incubated at 37C with 5% CO2 for 24 hours and cells were then processed according to the manufacturer's protocol for chemical-induced cytotoxicity. All tests were performed in triplicate and the full experiment was repeated on a separate day using fresh cell stock.

2.8 Quorum sensing inhibition assay

P. granatum fractions and crude extract were examined by dose-response assay for quorum sensing inhibitory activity against *S. aureus* agr using previously described (Salam et al., 2021).

the *agr* reporter strain was grown and maintained on TSA and TSB, supplemented with chloramphenicol (10 µg/mL) at 37 °C while shaking at 200 rpm. The overnight *S. aureus* AH1677 cultures were standardized to an OD_{600} of 0.0006 for working cultures. The fractions and crude extract were tested to escalating concentrations from 4 to 128 µg/mL of each compound in 96-well black plates (Costar 3,603, final well volume: 200 µL), the plates were in a humidified chamber at 37 °C, while shaking at 1,200 rpm (Stuart SI505 incubator. Bibby Scientific, Burlington, NJ). Fluorescence (top reading, 493 nm excitation, 535 nm emission, gain 60) and OD readings at 600nm using a plate reader (BioTek Cytation3) were measured at 0- and 18-hours post-inoculation. Controls included a vehicle control (DMSO) and a positive control (224CF2) were also assessed from 4 to 128 µg/mL. 224CF2c is an QSI-active fraction extracted from the European chestnut (*Castanea sativa*), as reported in a previous study by the authors (Schultz et al., 2020). All tests were performed in triplicate and repeated using a new stock of bacteria on two different days. Data was analyzed using Microsoft Excel and figures were created with GraphPad Prism version 10.1.0.

2.9 In vivo bactericidal activity towards induced S. aureus contamination

2.9.1 Minced meat and cheese Samples Preparation

The in vivo antibacterial activity of PPE against *S. aureus* strains was evaluated as previously described by (Kanatt et al., 2010; Parafati et al., 2021) with some modifications. The minced meat and cheese were obtained from local grocer. Briefly, Bacterial cultures were overnight maintained in BHI broth to prepare a working solution. After incubation, the concentration of the bacterial suspension was centrifuged, and the obtained pellet was washed twice with sterile distilled water and was adjusted in a sterile buffered peptone water solution with final concentration 1×10^6 cells/mL. Subsequently, *S. aureus* cultures inoculated in cheese and meat equally weighed meat samples (25 g meat and 10 g cheese) to simulate contamination before PPE treatment. Minced meat samples were then treated with 200 mg lyophilized powder of PPE and were aseptically homogenized and individually packed in pre-sterilized polyethylene bags and stored at 5 °C. Negative controls were also prepared and subsequently stored with treated ones in a laboratory refrigerator for periodic total bacterial count determination in days 0, 3, and 7. Same procedure was followed with cheese where samples were socked at 25 ml of PPE for 10 minutes at

concentration of 4.5 mg/ml. Next, samples were individually packed in pre-sterilized polyethylene bags and stored at 5 °C. Negative Controls were also prepared and subsequently stored with treated ones in a laboratory refrigerator for periodic total bacterial count determination on days 0, 7, 15, and 30.

2.9.2 Microbiological Analyses

Total viable bacterial counts in stored samples were evaluated by determining the total bacterial count (Plate Count Agar). For both minced meat and cheese samples at each analysis time point, both samples treated and not treated with PPE were aseptically transferred into a Stomacher bag containing 90 ml sterile buffered peptone water solution and homogenized in a stomacher for 2 min. then appropriate serial decimal dilutions ranging from 10^{-1} to 10^{-3} of the obtained homogenate were prepared with the same diluent. Lastly, 100μL of each dilution were plated onto Baird Parker's Agar plates, and plates were then incubated at 37 °C for 24 hr. Each microbiological count was performed in triplicate and expressed as $\log_{10}$ CFU/g.

3. Results

3.1 Bioassay-guided isolation of P. granatum active compounds

P. granatum fruits peels extractions were achieved by mean of maceration in 80% ethanol yielding 2 crude extracts belonging to two different pomegranate varieties. Bioassay-guided fractionation of these organic extracts (named extracts 2702 and 2707) was directed by a set of *S. aureus* strains assays. The fractionation of the crude extracts for compound isolation was done by reverse phase prep-HPLC using a gradient system of water and acetonitrile. A first round of prep-HPLC yielded 17 fractions 2702 (PF1, PF2, PF3, PF4, PF5, PF6, PF7, PF8) and 2707 (PF1, PF2, PF3, PF4, PF5, PF6, PF7, PF8). Subsequently the most bioactive 2 fractions, 2707-PF5 and 2707-PF6, were selected for a second round of prep-HPLC sub-fractionation and chemical analysis because of their strong antibacterial activity both in growth inhibition and because of their lack of toxicity towards human cells. The second round of prep-HPLC led to the generation of 39 subfractions. Out of this 39 SFs only 9 SFs showed good antimicrobial activity and underwent for further structure

determination using liquid chromatography with tandem mass spectrometry (LC-MS). The compounds of the most active sub-fractions were identified by Mass Spectrometry (MS). Putative matches were only obtained for peak number **1** and **2** with an empirical formula of $C_{48}H_{28}O_{30}$, which corresponded to 5 compounds (*α*-punicalagin, *β*-punicalagin, isoterchebulin, terchebulin, punicacortein C) in the database Reaxys. Among them, 3 compounds (*α*-punicalagin, *β*-punicalagin, punicacortein C) were reported from *Punica* genus. By comparison, using pure reference compounds two ellagitannins were identified Punicalagin α and β. Their structures were determined by employing spectroscopic analyses and comparison with literature data Figure 1.

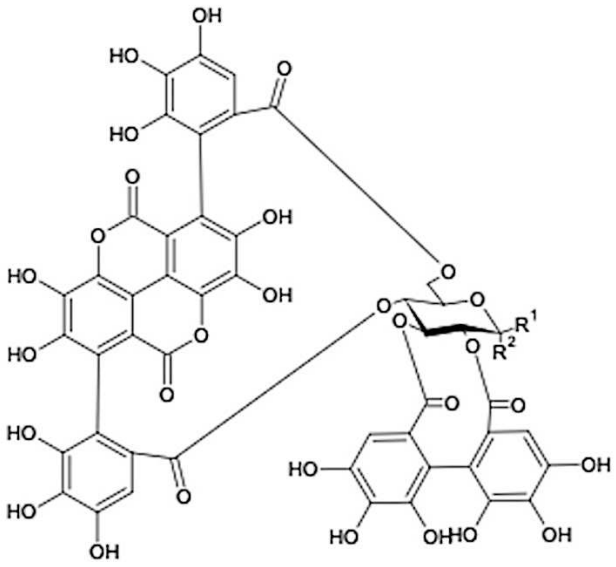

Figure 1: Chemical structure of punicalagin anomers α and β, α-punicalagin, R1 = H and R2 = OH; β-punicalagin, R1 = OH and R2=H. (Salles et al., 2021).

3.2 Growth inhibition

To determine their activity against *S. aureus* strains, a total of 57 samples of *P. granatum* crude extracts, fractions, and sub-fractions were investigated for growth inhibition by dose–response experiments in order to obtain the IC_{50} and MIC (IC_{90}) values. Of the first antimicrobial screening for the crude extracts for both 2702 and 2707 and their fractions, a good number of the tested samples displayed significant activity against the tested *S. aureus* strains with MIC values ranging from 64 to > 256 µg/mL and IC_{50} values ranging from 16 to 128 µg/mL Figure 2.

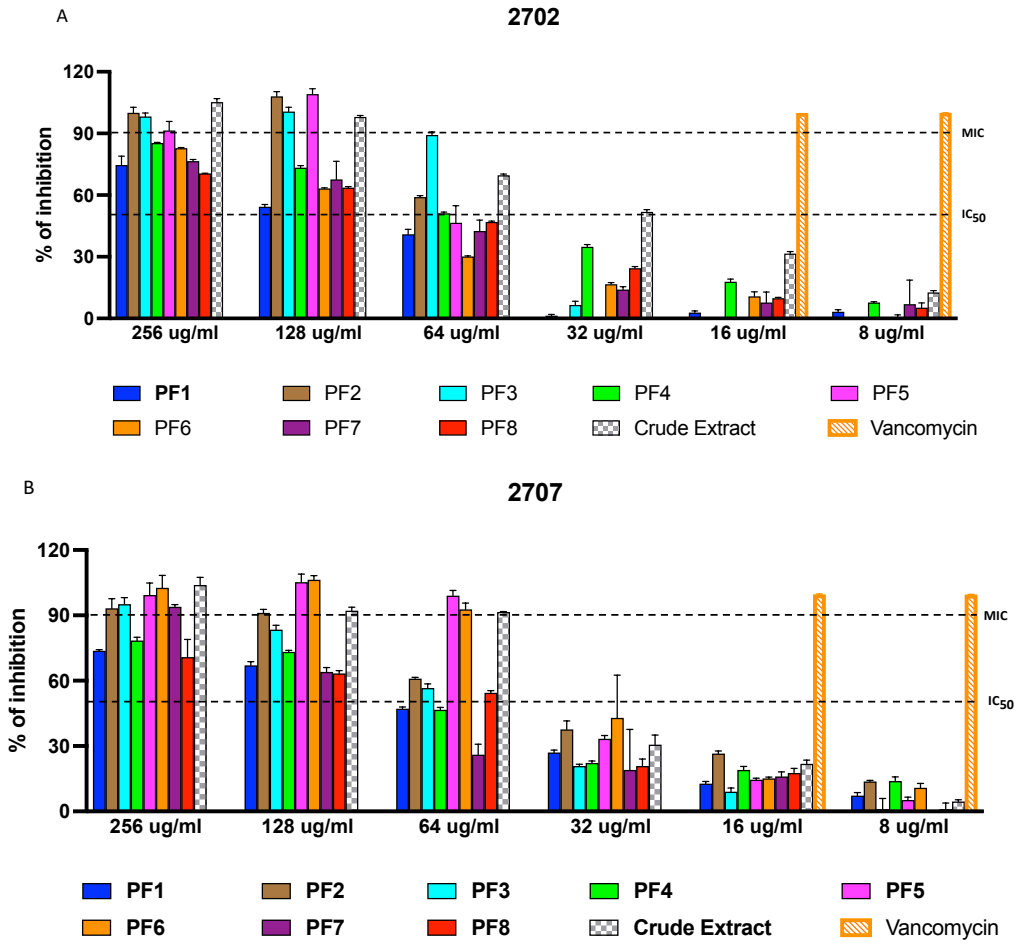

Figure 2: *P. granatum crude extracts (A) 2702 and its fractions* and (B) 2707 and its fractions exert selective concentration-dependent inhibition of *S. aureus* at 18 h post-inoculation. Vancomycin was used as a positive control for growth inhibition.

Based on the MIC observed values only two fractions 2707-PF5 and 2707-PF6 were processed for further fractionation. This step yielded in 39 SFs and were tested for their antimicrobial activity. The tested SFs showed a good antimicrobial activity against the tested *S. aureus* strain, Figure 3 reports the minimum concentrations of the tested samples for both IC_{50} and MIC for the most active SPs that ranging from 32 to 64 µg/mL.

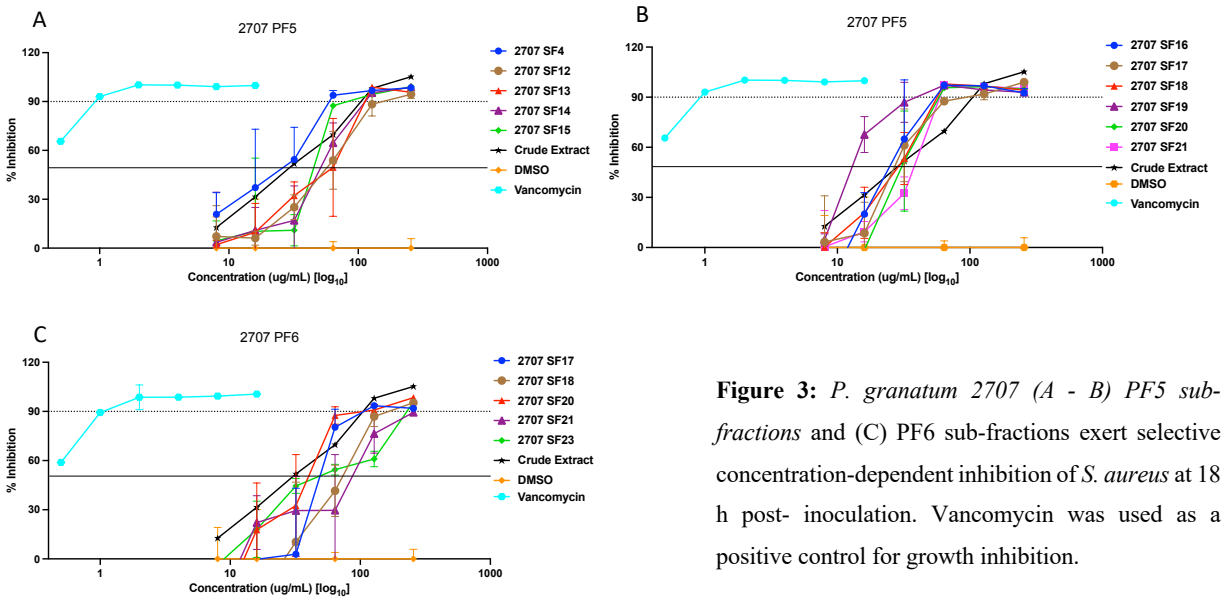

Figure 3: *P. granatum 2707 (A - B) PF5 sub-fractions* and (C) PF6 sub-fractions exert selective concentration-dependent inhibition of *S. aureus* at 18 h post- inoculation. Vancomycin was used as a positive control for growth inhibition.

3.3 Quorum sensing inhibition in S. aureus

The potential of 2702 and 2707 crude extract and fractions to inhabit *S. aureus* quorum-sensing component pathways encoded by the accessory gene regulator (*agr*) system was investigated. The results revealed that all the investigated samples demonstrated no quorum-sensing inhibitory activity against the tested *S. aureus agr* I reporter strain AH-1677 at concentrations up to 128 µg/mL. Moreover, it was observed that the inhibition of the quorum-sensing agr I transcription activity was just a result of the growth inhibition of the tested strain Figure 4.

3.4 Cytotoxicity of P. granatum active fractions

To determine the *P. granatum* fractions potential toxicity to human cells, HaCaTs cells were used in dose-response study using a lactate dehydrogenase assay to assess their cytotoxicity. Of the 16 fractions studied, 3 fractions (2702 PF3 and 2707 PF5 ana PF6) were recognized to have potential antimicrobial activity and were tested for potential cytotoxicity. The fractions were tested at starting concentrations of 128 µg/mL. Figure 5 displays cytotoxicity across the tested samples, demonstrating that none of the fractions tested had high cytotoxicity with IC_{50} greater than 128 µg/mL.

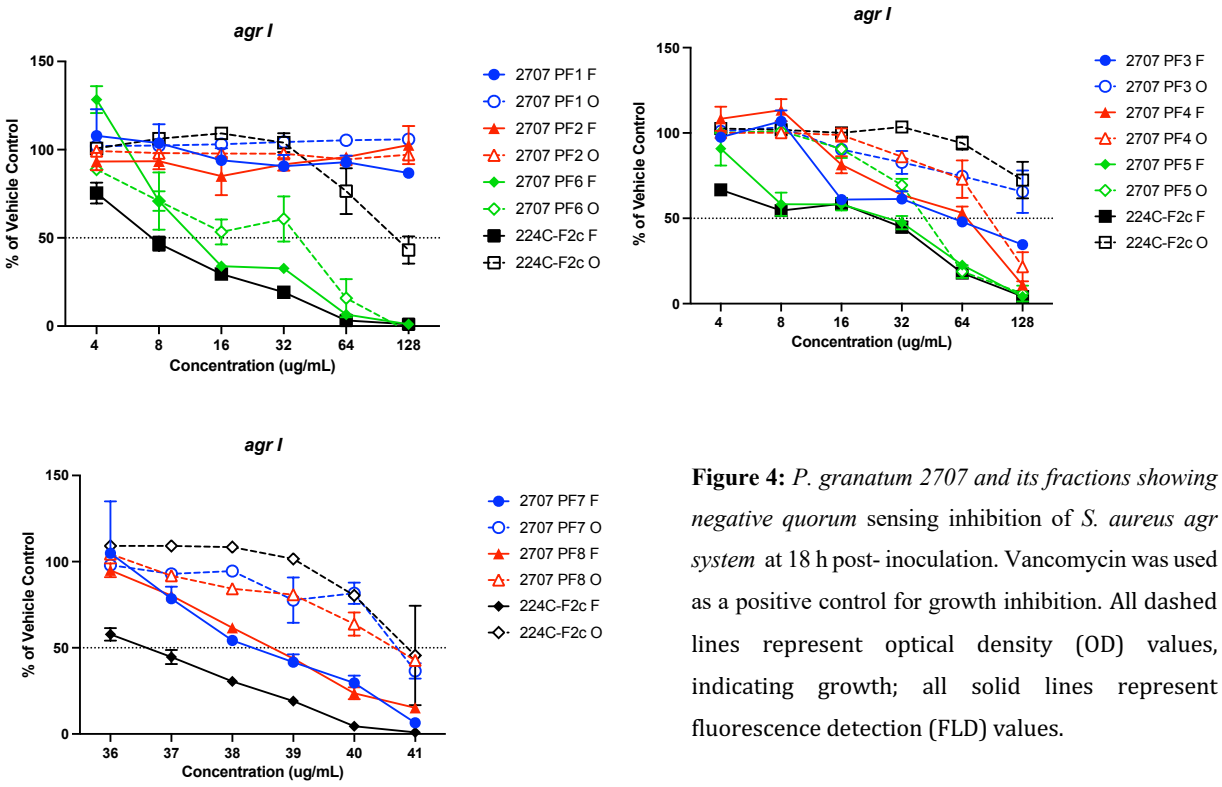

Figure 4: *P. granatum 2707 and its fractions showing negative quorum* sensing inhibition of *S. aureus agr system* at 18 h post- inoculation. Vancomycin was used as a positive control for growth inhibition. All dashed lines represent optical density (OD) values, indicating growth; all solid lines represent fluorescence detection (FLD) values.

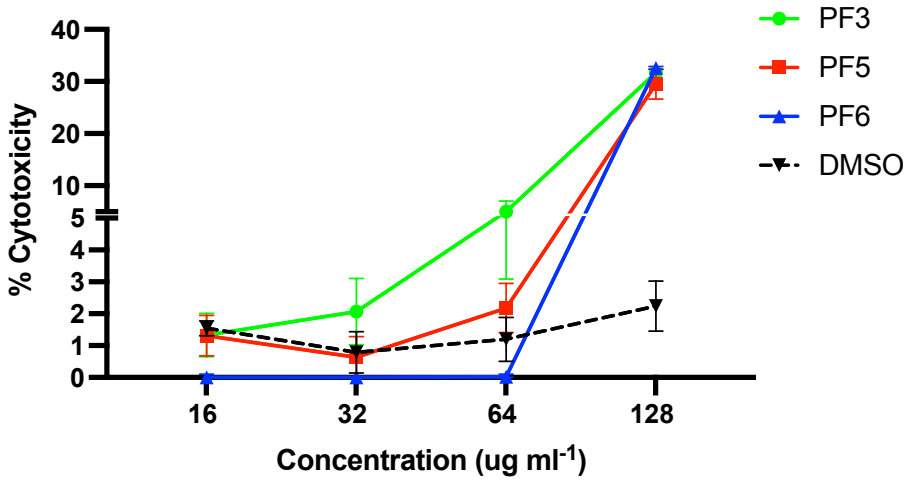

Figure 5: Human keratinocyte cytotoxicity by *P. granatum* most active fractions. Figure made with GraphPad Prism version 10.1.0 for Windows, www.graphpad.com.

3.5 In vivo bactericidal activity towards induced S. aureus contamination

The impact of PPE (at a concentration of 0.8%) on the shelf life of commercially available minced meat and cheese was investigated. A considerable influence of PPE supplementation was identified, where it was observed that the addition of PPE preserved the quality of minced meat and cheese throughout the storage duration. A considerable reduction trend during the storage period in both control and treated samples was observed by the total plate count and the examined food models demonstrated a significant ($p < 0.05$) reduction in *Staphylococcus* spp counts in treated samples when compared to the untreated control Figure 6 – 7. Additionally, mould growth was observed after 10 days of incubation on untreated cheese plates but not on the treated ones. For meat samples colour change was visually observed on day 3 for control samples and on day 5 for treated samples.

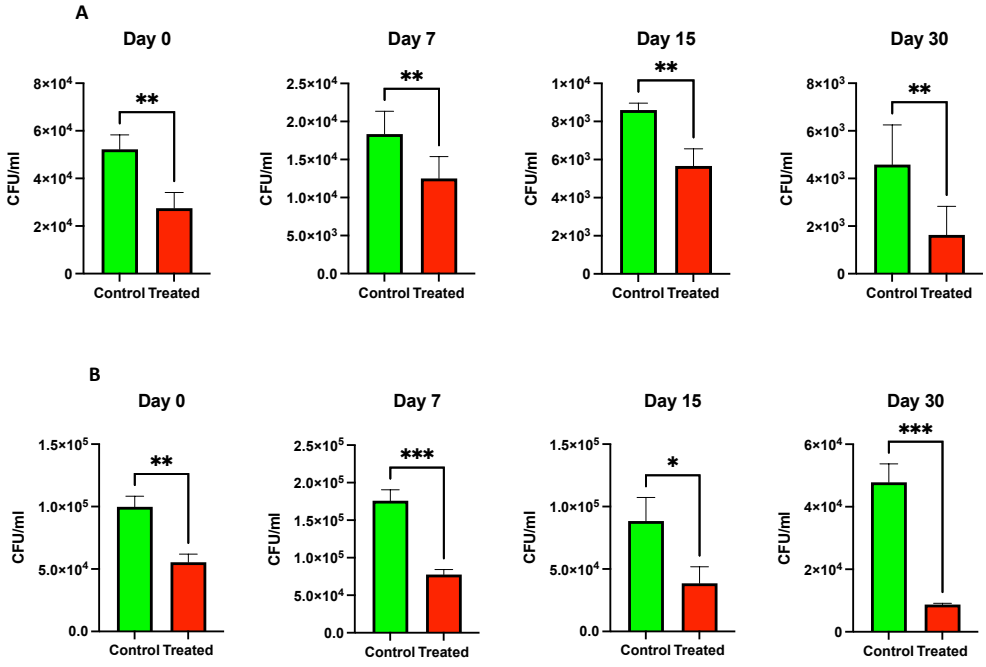

Figure 6: In vivo pomegranate peels extract bactericidal activity towards induced *S. aureus* contamination using cheese food model over 0, 7, 15, and 30 days. A: S. aureus 20231 B: S. aureus 25691

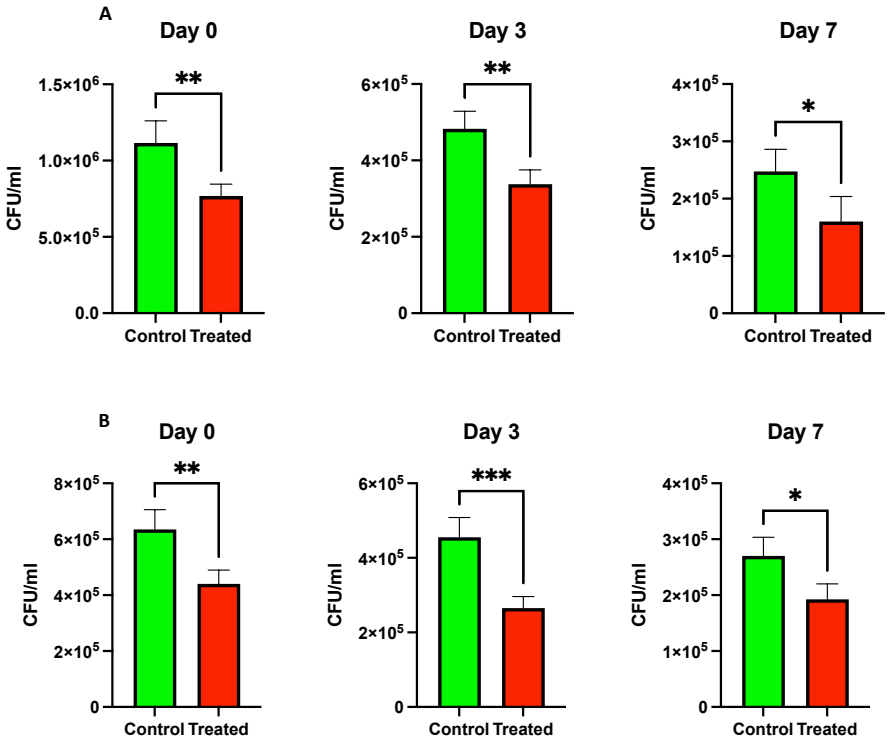

Figure 7: In vivo pomegranate peels extract bactericidal activity towards induced *S. aureus* contamination using miced meet food model over 0, 3, and 7 days. **A**: *S. aureus* 20231 **B**: *S. aureus* 25691.

4. Discussion

4.1 Bioassay-guided isolation of P. granatum active compounds and in vitro assays

Polyphenols found in pomegranates have been shown to exhibit diverse pharmacological and physiological effects, including but not limited to anticancer, antioxidant, antibacterial, and anti-inflammatory properties (Caballero et al., 2022; Ruan et al., 2022; Singh et al., 2023). Specifically, pomegranate peels contain substantial levels of hydrolyzed ellagitannins including punicalins, punicalagins, and pedunculagins. Additionally, aside from ellagitannins, the peel of the pomegranate also contains hydroxybenzoic acids, such as gallic acid and ellagic acid, as well as anthocyanidins and flavonoids (Ali Redha et al., 2018; Singh et al., 2018). As a part of the ongoing efforts to identify natural products as antibiotics, in the current study two ellagitannins, Punicalagin α and β were isolated from Punica granatum peels. Punicalagin is recognized as the primary bioactive compound in pomegranates due to its abundance and biological activity (Kharchoufi et

al., 2018; Read et al., 2019). These compounds were also detected and isolated from the leaves, seeds, and juice of Punica granatum (Machado et al., 2002; Foss et al., 2014; Yan et al., 2017).

The dose-response assay revealed the antimicrobial activity for the isolated Punicalagin α and β against *S. aureus* with MIC 64 µg/mL. The antibacterial activity of the punicalagin α and β anomeric forms both *in vitro* and *in vivo* have been reported by several authors (Barbieri and Heard, 2019; Song et al., 2022; Singh et al., 2023). Per our results, the antibacterial activity for punicalagin MIC was established as 61.5 µg mL^{-1} (Machado et al., 2002). In another study, Xu et al., (2017) reported that punicalagin exhibited an antistaphylococcal effect with a MIC of 250 µg mL^{-1}, however, it showed a moderate inhibitory effect on Salmonella with a MIC of 250 –1000 µg mL^{-1}. Punicalagin demonstrated a significant antimicrobial impact and effectively inhibited the formation of biofilms by *S. aureus*, suggesting potential applications for controlling *S. aureus* contamination in the food industry (Xu et al., 2017)

The antimicrobial effects of pomegranate are linked to polyphenolic tannins, particularly punicalagin and ellagic acid present in the extract, and involve various independent mechanisms. (Celiksoy and Heard, 2021). One proposed antimicrobial mechanism for polyphenolic compounds involves their ability to precipitate with proteins in the bacterial cell membrane, resulting in the lysis of bacterial cells (Akhtar et al., 2015). Moreover, polyphenols may hinder microbial enzymes by interacting with sulfhydryl groups or engaging in nonspecific interactions with proteins (Cowan, 1999). Furthermore, it has been documented that polyphenols can impair the microbial respiratory chain by reducing oxygen consumption, thereby restricting the oxidation of NADH (Haraguchi et al., 1998).

In a study where (Cooper et al., 2018) aimed to gain a comprehensive understanding of punicalagin's inhibitory impact on *S. aureus* growth, the findings revealed that punicalagin disrupts bacterial growth by adversely affecting iron homeostasis and inducing the SOS response, potentially through the inhibition of DNA biosynthesis. These findings will prove valuable to researchers in the fields of antibiotics and Staphylococcus, aiming to safeguard public health and enhance food safety for example punicalagin, with its notable natural properties, could serve as an

effective additive for meat preservation and quality improvement, potentially offering a viable alternative to synthetic antioxidants (Foss et al., 2014; Smaoui et al., 2019)

A quorum-sensing inhibitory activity was also evaluated for the pomegranate fractions however the results revealed that all the investigated samples demonstrated no quorum-sensing inhibitory activity against the tested *S. aureus* agr system. In contrast, several studies have documented the quorum sensing (QS) activity of pomegranate, linking it to the ellagic acid and punicalagin present in pomegranate extract (Celiksoy and Heard, 2021). Another work indicated that punicalagin inhibited the virulence factors of Salmonella and demonstrated anti-quorum-sensing potential when employed at subinhibitory concentrations (Xu et al., 2017)

In cytotoxicity assays with human keratinocytes, the pomegranate fractions showed selectivity for bacterial cells than for mammalian cells, where none of the fractions tested had high cytotoxicity with IC_{50} greater than 128 µg/mL. Such selectivity was also reported by Kilit and Aydemir, (2023) where they observed that punicalagin showed cytotoxicity against several cancer cells but was not cytotoxic against human kidney epithelial cells.

4.2 In vivo bactericidal activity towards induced S. aureus contamination

The widespread resistance of numerous microorganisms to existing antibiotics is a major concern worldwide. This issue, coupled with the increasing consumer focus on "natural food products," has motivated researchers and the food industry to explore novel alternative compounds capable of effectively inhibiting a wide range of microorganisms (Gullón et al., 2020). As a result, the popularity of employing plant extracts as natural antimicrobial agents for food preservation is on the rise (Kanatt et al., 2010). In the present study an aqueous PPE and lyophilized powder were used as natural antimicrobial agents in food preservation and significant reduction in bacterial cell counts were observed for minced meat and cheese after the incorporation of PPE with the tested food models. This aligns with the findings of Shahamirian et al. (2019) and Rasuli et al. (2021) where it was observed that incorporating PPE led to a remarkable reduction in the total bacterial plate count, contributing to the preservation of meat products freshness during refrigerated storage and had a positive effect on colour stabilization. Parafati et al. (2021) conducted a study to

investigate the antimicrobial potential of pomegranate extracts against *S. aureus* when integrated into the cheese matrix during 12 days of refrigerated storage. In addition, Mahajan et al., (2015) observed a significant effect on the microbiological characteristics of cheese when treated with PPE against the different tested strains of bacteria, yeast, and mould where lower count values were recorded when compared to control ones. Employing an alternative food model, it was observed that untreated control samples of chicken spoiled within a week storage period, whereas treated samples exhibited an extension in shelf life, lasting up to 20 days (Kanatt et al., 2010).

As well in the present study, mould growth became apparent after 10 days of incubation in the refrigerated untreated cheese samples, while no such growth was observed in the treated samples. This can be attributed to the antifungal properties of pomegranate peel extracts (PPE), where many studies have already documented the potential antifungal properties of extracts from pomegranate peels and seeds, indicating their potential as natural substitutes for synthetic antifungals (Tehranifar et al., 2011; Li Destri Nicosia et al., 2016). Hence, incorporating pomegranate by-products containing notable bioactive properties not only improves quality and prolongs shelf life by inhibiting oxidative damage to proteins and lipids but also enhances the functional and health-related characteristics of products such as meat, fish, milk, and their derivatives during storage (Das et al., 2021).

In this present study, a concentration of 4.5 mg/ml was employed, exceeding the recorded value of the minimum inhibitory concentration (MIC) for PS, which was 0.75 mg/ml, in order to observe notable inhibition of bacterial growth. The differences between the in vitro and in vivo values could be attributed to many reasons, where Smith-Palmer et al. (2001) determined that the antimicrobial efficacy of specific natural compounds was markedly impacted by the chemical composition of cheese.

Similarly, Gutierrez et al. (2008), observed a decline in the antimicrobial potency of oregano and thyme essential oils against *L. monocytogenes* when exposed to increased lipid levels in a simulated food matrix. Hence, to attain an inhibitory effect comparable to the one observed in vitro, it is necessary to include the same natural compound in foods at higher concentrations (Moro et al., 2013). This was also confirmed by Gammariello et al. (2008), they observed that achieving

a similar antimicrobial effect in cheese necessitated higher concentrations of the examined natural compounds compared to those used in vitro.

5. Conclusions

In conclusion, the investigation into pomegranate peel extracts as a natural antibiotic against *S. aureus* highlights promising avenues in the search for sustainable antimicrobial solutions. Through a bioassay-guided fractionation approach, this study isolated and identified bioactive compounds within pomegranate peel extracts, elucidating their potential as effective antimicrobial agents. Moreover, the exploration of safety profiles underscores the feasibility of utilizing these natural extracts in pharmaceutical, healthcare settings, and the food industry field, offering a safer alternative to synthetic antibiotics and food additives. The findings of this research contribute to the growing body of knowledge on harnessing agricultural by-products for antimicrobial purposes, presenting pomegranate peel extracts as a novel, cost-effective, and eco-friendly solution in combating antibiotic-resistant pathogens like *S. aureus*. As we continue to confront the escalating threat of antibiotic resistance, these findings provide a ray of hope for the future of infectious disease management.

6. *References*

Akhtar, S., Ismail, T., Fraternale, D., & Sestili, P. (2015). Pomegranate peel and peel extracts: Chemistry and food features. *Food chemistry*, *174*, 417-425.

Ali Redha, A., Hasan, A., & Mandeel, Q. (2018). Phytochemical determinations of Pomegranate (Punica granatum) Rind and Aril extracts and their antioxidant, antidiabetic and antibacterial activity. *Nat Prod Chem Res*, *6*(4), 1-9.

Barbieri, M., & Heard, C. M. (2019). Isolation of punicalagin from Punica granatum rind extract using mass-directed semi-preparative ESI-AP single quadrupole LC-MS. *Journal of Pharmaceutical and Biomedical Analysis*, *166*, 90-94.

Caballero, V., Estévez, M., Tomás-Barberán, F. A., Morcuende, D., Martín, I., & Delgado, J. (2022). Biodegradation of punicalagin into ellagic acid by selected probiotic bacteria: a study of the underlying mechanisms by MS-based proteomics. *Journal of Agricultural and Food Chemistry*, *70*(51), 16273-16285.

Castro, A., Silva, J., & Teixeira, P. (2018). Staphylococcus aureus, a food pathogen: Virulence factors and antibiotic resistance. In *Foodborne Diseases* (pp. 213-238). Academic Press.

Celiksoy, V., & Heard, C. M. (2021). Antimicrobial potential of pomegranate extracts. In *Pomegranate*. IntechOpen.

Chen, J., Liao, C., Ouyang, X., Kahramanoğlu, I., Gan, Y., & Li, M. (2020). Antimicrobial activity of pomegranate peel and its applications on food preservation. *Journal of Food Quality*, *2020*, 1-8.

Cockerill, F. et al. Performance Standards for Antimicrobial Susceptibility Testing: Twenty-Third Informational Supplement; M100-S23 (CLSI, Wayne, 2013).

Cooper, B., Islam, N., Xu, Y., Beard, H. S., Garrett, W. M., Gu, G., & Nou, X. (2018). Quantitative proteomic analysis of staphylococcus aureus treated with punicalagin, a natural antibiotic from pomegranate that disrupts iron homeostasis and induces SOS. *Proteomics*, *18*(9), 1700461.

Cowan, M. M. (1999). Plant products as antimicrobial agents. *Clinical microbiology reviews*, *12*(4), 564-582.

Das, A. K., Nanda, P. K., Chowdhury, N. R., Dandapat, P., Gagaoua, M., Chauhan, P., ... & Lorenzo, J. M. (2021). Application of pomegranate by-products in muscle foods: oxidative indices, colour stability, shelf life and health benefits. *Molecules*, *26*(2), 467.

de Lima, L. B., da Silva, W. A. V., Dos Santos, E. C. F., Machado, J. C. B., Procópio, T. F., de Moura, M. C., ... & Soares, L. A. L. (2021). Evaluation of Antioxidant, Antibacterial and Enhancement of Antibiotic Action by Punica granatum Leaves Crude Extract and Enriched Fraction against Multidrug-Resistant Bacteria. *Chemistry & Biodiversity*, *18*(12), e2100538.

Dettweiler, M., Marquez, L., Lin, M., Sweeney-Jones, A. M., Chhetri, B. K., Zurawski, D. V., ... & Quave, C. L. (2020). Pentagalloyl glucose from Schinus terebinthifolia inhibits growth of carbapenem-resistant Acinetobacter baumannii. Scientific reports, 10(1), 15340.

Eid, H. H., El Deeb, K. S., Ali, Z. Y., Metwally, G. F., & El-Fiki, A. M. (2022). Bioassay-guided fractionation and evaluation of anti-diabetic, anti-oxidant and anti-hyperlipidemic potential of punica granatum l. Var nana cultivated in egypt. Journal of medical pharmaceutical and allied sciences, 11, 5440 – 5448. Doi: 10.55522/jmpas.V11I6.2491.

El-Hamamsy, S. M. A., & El-Khamissi, H. A. Z. (2020). Phytochemicals, antioxidant activity and identification of phenolic compounds by HPLC of pomegranate (Punica granatum L.) Peel extracts. *Journal of Agricultural chemistry and biotechnology*, *11*(4), 79-84.

Ene, C. (2020). Food security and food safety: Meanings and connections. *Econ Insights Trends Challs IX (1)*, 59-68.

Farag, M. A., Al-Mahdy, D. A., Salah El Dine, R., Fahmy, S., Yassin, A., Porzel, A., & Brandt, W. (2015). Structure Activity Relationships of Antimicrobial Gallic Acid Derivatives from Pomegranate and Acacia Fruit Extracts against Potato Bacterial Wilt Pathogen. *Chemistry & biodiversity*, *12*(6), 955-962.

Foss, S. R., Nakamura, C. V., Ueda-Nakamura, T., Cortez, D. A., Endo, E. H., & Dias Filho, B. P. (2014). Antifungal activity of pomegranate peel extract and isolated compound punicalagin against dermatophytes. *Annals of clinical microbiology and antimicrobials*, *13*(1), 1-6.

Gammariello, D.; Di Giulio, S.; Conte, A.; Del Nobile, M.A. Effects of natural compounds on microbial safety and sensory quality of Fior di Latte cheese, a typical Italian cheese. *J. Dairy Sci.* 2008, *91*, 4138–4146.

Godwin, E. J. O., Chandrasekaran, V., Smah, A. C., & Faith, E. O. (2022). Emerging Infectious Food System Related Zoonotic Foodborne Disease-A Threat to Global Food Safety and Nutrition Security.

Gosset-Erard, C., Zhao, M., Lordel-Madeleine, S., & Ennahar, S. (2021). Identification of punicalagin as the bioactive compound behind the antimicrobial activity of pomegranate (Punica granatum L.) peels. *Food Chemistry*, *352*, 129396.

Gullón, P., Astray, G., Gullón, B., Tomasevic, I., & Lorenzo, J. M. (2020). Pomegranate peel as suitable source of high-added value bioactives: Tailored functionalized meat products. *Molecules*, *25*(12), 2859.

Gutierrez, J.; Barry-Ryan, C.; Bourke, P. The antimicrobial efficacy of plant essential oil combinations and interactions with food ingredients. *Int. J. Food Microbiol.* 2008, *124*, 91–97.

Haraguchi, H., Tanimoto, K., Tamura, Y., Mizutani, K., & Kinoshita, T. (1998). Mode of antibacterial action of retrochalcones from Glycyrrhiza inflata. *Phytochemistry*, *48*(1), 125-129.

Kadariya, J., Smith, T. C., & Thapaliya, D. (2014). Staphylococcus aureus and staphylococcal food-borne disease: an ongoing challenge in public health. *BioMed research international*, *2014*.

Kanatt, S. R., Chander, R., & Sharma, A. (2010). Antioxidant and antimicrobial activity of pomegranate peel extract improves the shelf life of chicken products. *International journal of food science & technology*, *45*(2), 216-222.

Khan, F. M., Chen, J. H., Zhang, R., & Liu, B. (2023). A comprehensive review of the applications of bacteriophage-derived endolysins for foodborne bacterial pathogens and food safety: recent advances, challenges, and future perspective. *Frontiers in Microbiology*, *14*.

Kharchoufi, S., Licciardello, F., Siracusa, L., Muratore, G., Hamdi, M., & Restuccia, C. (2018). Antimicrobial and antioxidant features of 'Gabsi' pomegranate peel extracts. *Industrial Crops and Products*, *111*, 345-352.

Kharchoufi, S., Licciardello, F., Siracusa, L., Muratore, G., Hamdi, M., & Restuccia, C. (2018). Antimicrobial and antioxidant features of 'Gabsi' pomegranate peel extracts. *Industrial Crops and Products*, *111*, 345-352.

Kilit, A. C., & Aydemir, E. (2023). Cytotoxic Effects of Punicalagin on U87, PANC-1, A549, and 22RV1 Cancer Cell Lines. *HAYDARPAŞA NUMUNE MEDICAL JOURNAL*, *63*(2), 115-119.

Lee, H., & Yoon, Y. (2021). Etiological agents implicated in foodborne illness worldwide. *Food science of animal resources*, *41*(1), 1.

Li Destri Nicosia, M.G.; Pangallo, S.; Raphael, G.; Romeo, F.V.; Strano, M.C.; Rapisarda, P.; Droby, S.; Schena, L. Control of postharvest fungal rots on citrus fruit and sweet cherries using a pomegranate peel extract. *Postharvest Biol. Technol.* 2016, *114*, 54–61.

Li, G., Yan, C., Xu, Y., Feng, Y., Wu, Q., Lv, X., ... & Xia, X. (2014). Punicalagin inhibits Salmonella virulence factors and has anti-quorum-sensing potential. *Applied and environmental microbiology*, *80*(19), 6204-6211.

Li, Y., Guo, C., Yang, J., Wei, J., Xu, J., & Cheng, S. (2006). Evaluation of antioxidant properties of pomegranate peel extract in comparison with pomegranate pulp extract. *Food chemistry*, *96*(2), 254-260.

L. Yan, X. Zhou, L. Shi, D. Shalimu, C. Ma, Y. Liu, Phenolic profiles and antioxidant activities of six Chinese pomegranate (Punica granatum L.) cultivars, Int. J. Food Prop. 20 (2017) S94–S107.

Ma, G. Z., Wang, C. M., Li, L., Ding, N., & Gao, X. L. (2015). Effect of pomegranate peel polyphenols on human prostate cancer PC-3 cells in vivo. *Food science and biotechnology*, *24*, 1887-1892.

Machado, T. D. B., Leal, I. C., Amaral, A. C. F., Santos, K., Silva, M. G. D., & Kuster, R. M. (2002). Antimicrobial ellagitannin of Punica granatum fruits. *Journal of the Brazilian Chemical Society*, *13*, 606-610.

Mahajan, D., Bhat, Z. F., & Kumar, S. (2015). Pomegranate (Punica granatum) rind extract as a novel preservative in cheese. *Food Bioscience*, *12*, 47-53.

Martin, C. S., Jubelin, G., Darsonval, M., Leroy, S., Leneveu-Jenvrin, C., Hmidene, G., ... & Dubois-Brissonnet, F. (2022). Genetic, physiological, and cellular heterogeneities of bacterial pathogens in food matrices: Consequences for food safety. *Comprehensive Reviews in Food Science and Food Safety*, *21*(5), 4294-4326.

Moradi, M. T., Karimi, A., Shahrani, M., Hashemi, L., & Ghaffari-Goosheh, M. S. (2019). Anti-influenza virus activity and phenolic content of pomegranate (Punica granatum L.) peel extract and fractions. *Avicenna journal of medical biotechnology*, *11*(4), 285.

Moro, A.; Librán, C.M.; Berruga, M.I.; Zalacain, A.; Carmona, M. Mycotoxicogenic fungal inhibition by innovative cheese cover with aromatic plants. *J. Sci. Food Agric.* 2013, *93*, 1112–1118.

Munir, S., Hu, Y., Liu, Y., & Xiong, S. (2019). Enhanced properties of silver carp surimi-based edible films incorporated with pomegranate peel and grape seed extracts under acidic condition. *Food Packaging and Shelf Life*, *19*, 114-120.

Nazeam, J. A., Al-Shareef, W. A., Helmy, M. W., & El-Haddad, A. E. (2020). Bioassay-guided isolation of potential bioactive constituents from pomegranate agrifood by-product. *Food chemistry*, *326*, 126993.

Panichayupakarananta, P., Issuriya, A., Sirikatitham, A., & Wang, W. (2010). Antioxidant assay-guided purification and LC determination of ellagic acid in pomegranate peel. *Journal of chromatographic science*, *48*(6), 456-459.

Parafati, L., Pesce, F., Siracusa, L., Fallico, B., Restuccia, C., & Palmeri, R. (2021). Pomegranate byproduct extracts as ingredients for producing experimental cheese with enhanced microbiological, functional, and physical characteristics. *Foods*, *10*(11), 2669.

Peršurić, Ž., Saftić Martinović, L., Malenica, M., Gobin, I., Pedisić, S., Dragović-Uzelac, V., & Kraljević Pavelić, S. (2020). Assessment of the biological activity and phenolic composition of ethanol extracts of pomegranate (Punica granatum L.) peels. *Molecules*, *25*(24), 5916.

Pirzadeh, M., Caporaso, N., Rauf, A., Shariati, M. A., Yessimbekov, Z., Khan, M. U., ... & Mubarak, M. S. (2021). Pomegranate as a source of bioactive constituents: A review on their characterization, properties and applications. *Critical reviews in food science and nutrition*, *61*(6), 982-999.

Quave, C. L., Lyles, J. T., Kavanaugh, J. S., Nelson, K., Parlet, C. P., Crosby, H. A., ... & Horswill, A. R. (2015). Castanea sativa (European Chestnut) leaf extracts rich in ursene and oleanene derivatives block Staphylococcus aureus virulence and pathogenesis without detectable resistance. *PLoS One*, *10*(8), e0136486.

Rasuli, N., Bintoro, V. P., Purnomoadi, A., & Nurwantoro, N. (2021). The shelf life of buffalo meat marinated with pomegranate (Punica granatum) peel extract. *Journal of Advanced Veterinary and Animal Research*, *8*(4), 612.

Read, E., Deseo, M. A., Hawes, M., & Rochfort, S. (2019). Identification of potentially cytotoxic phenolics present in pomegranates (Punica granatum L.). *Animal Feed Science and Technology*, *251*, 187-197.

Ruan, J. H., Li, J., Adili, G., Sun, G. Y., Abuduaini, M., Abdulla, R., ... & Aisa, H. A. (2022). Phenolic compounds and bioactivities from pomegranate (Punica granatum L.) peels. *Journal of Agricultural and Food Chemistry*, *70*(12), 3678-3686.

Salam, A. M., Porras, G., Cho, Y. S. K., Brown, M. M., Risener, C. J., Marquez, L., ... & Quave, C. L. (2021). Castaneroxy A from the leaves of Castanea sativa inhibits virulence in Staphylococcus aureus. *Frontiers in Pharmacology*, *12*, 640179.

Salim, A., Deiana, P., Fancello, F., Molinu, M. G., Santona, M., & Zara, S. (2023). Antimicrobial and antibiofilm activities of pomegranate peel phenolic compounds: Varietal screening through a multivariate approach. *Journal of Bioresources and Bioproducts*, *8*(2), 146-161.

Salles, T. S., Meneses, M. D. F., Caldas, L. A., Sá-Guimarães, T. E., de Oliveira, D. M., Ventura, J. A., ... & Ferreira, D. F. (2021). Virucidal and antiviral activities of pomegranate (Punica granatum) extract against the mosquito-borne Mayaro virus. *Parasites & Vectors*, *14*(1), 1-8.

Schultz, F., Anywar, G., Tang, H., Chassagne, F., Lyles, J. T., Garbe, L. A., & Quave, C. L. (2020). Targeting ESKAPE pathogens with anti-infective medicinal plants from the Greater Mpigi region in Uganda. *Scientific reports*, *10*(1), 11935.

Silva, V., Silva, A., Ribeiro, J., Aires, A., Carvalho, R., Amaral, J. S., ... & Poeta, P. (2023). Screening of Chemical Composition, Antimicrobial and Antioxidant Activities in Pomegranate, Quince, and Persimmon Leaf, Peel, and Seed: Valorization of Autumn Fruits By-Products for a One Health Perspective. *Antibiotics*, *12*(7), 1086.

Singh, J., Prasad, R., Kaur, H. P., Jajoria, K., Chahal, A. S., Verma, A., ... & Bahhou, J. (2023). Bioactive Compounds, Pharmacological Properties, and Utilization of Pomegranate (Punica granatum L.): A Comprehensive Review. *Tropical Journal of Natural Product Research*, *7*(9).

Singh, J., Kaur, H. P., Verma, A., Chahal, A. S., Jajoria, K., Rasane, P., ... & Durul, M. S. (2023). Pomegranate peel phytochemistry, pharmacological properties, methods of extraction, and its application: a comprehensive review. *ACS omega*, *8*(39), 35452-35469.

Singh, B., Singh, J. P., Kaur, A., & Singh, N. (2018). Phenolic compounds as beneficial phytochemicals in pomegranate (Punica granatum L.) peel: A review. *Food chemistry*, *261*, 75-86.

Shahamirian, M.; Eskandari, M.H.; Niakousari, M.; Esteghlal, S.; Hashemi Gahruie, H.; Mousavi Khaneghah, A. Incorporation of pomegranate rind powder extract and pomegranate juice into frozen burgers: Oxidative stability, sensorial and microbiological characteristics. *J. Food Sci. Technol.* 2019, *56*, 1174–1183.

Smaoui, S., Hlima, H. B., Mtibaa, A. C., Fourati, M., Sellem, I., Elhadef, K., ... & Mellouli, L. (2019). Pomegranate peel as phenolic compounds source: Advanced analytical strategies and practical use in meat products. *Meat science*, *158*, 107914.

Smith-Palmer, A.; Stewart, J.; Fyfe, L. The potential application of plant essential oils as natural food preservatives in soft cheese. *Food Microbiol.* 2001, *18*, 463–470.

Song, W., Wang, L., Jin, M., Guo, X., Wang, X., Guan, J., & Zhao, Y. (2022). Punicalagin, an inhibitor of Sortase a, is a promising therapeutic drug to combat methicillin-resistant Staphylococcus aureus infections. *Antimicrobial Agents and Chemotherapy*, *66*(6), e00224-22.

Sousa, C. P. D. (2008). The impact of food manufacturing practices on food borne diseases. *Brazilian Archives of biology and Technology*, *51*, 615-623.

Sun, S., Huang, S., Shi, Y., Shao, Y., Qiu, J., Sedjoah, R. C. A. A., ... & Xin, Z. (2021). Extraction, isolation, characterization and antimicrobial activities of non-extractable polyphenols from pomegranate peel. *Food Chemistry*, *351*, 129232.

Tang, H., Porras, G., Brown, M. M., Chassagne, F., Lyles, J. T., Bacsa, J., ... & Quave, C. L. (2020). Triterpenoid acids isolated from Schinus terebinthifolia fruits reduce Staphylococcus aureus virulence and abate dermonecrosis. *Scientific reports*, *10*(1), 8046.

Tehranifar, A.; Selahvarzi, Y.; Kharrazi, M.; Bakhsh, V.J. High potential of agro-industrial by-products of pomegranate (*Punica granatum* L.) as the powerful antifungal and antioxidant substances. *Ind. Crops Prod.* 2011, *34*, 1523–1527.

Xu, Y., Shi, C., Wu, Q., Zheng, Z., Liu, P., Li, G., ... & Xia, X. (2017). Antimicrobial activity of punicalagin against Staphylococcus aureus and its effect on biofilm formation. *Foodborne pathogens and disease*, *14*(5), 282-287.

Xie, J., Zhou, M., Qian, Y., Cong, Z., Chen, S., Zhang, W., ... & Liu, R. (2021). Addressing MRSA infection and antibacterial resistance with peptoid polymers. *Nature communications*, *12*(1), 5898.

Yang, Q., Wang, L., Gao, J., Liu, X., Feng, Y., Wu, Q., ... & Xia, X. (2016). Tannin-rich fraction from pomegranate rind inhibits quorum sensing in Chromobacterium violaceum and biofilm formation in Escherichia coli. *Foodborne pathogens and disease*, *13*(1), 28-35.

CHAPTER III

Effects of different maturity stages on changes in physicochemical and phytochemical composition of pomegranate peels and juice from different cultivars

Amira Salim[1] Pierfrancesco Deiana[1], Maria Giovanna Molinu[2], Mario Santona[1], Francesco Fancello[1], Severino Zara [1]

[1]Department of Agricultural Sciences, University of Sassari, 07100 Sassari, Italy
[2]National Council of Research, Institute of Food Production Sciences, 07100 Sassari, Italy

Abstract

Pomegranate, recognized for its nutritional and therapeutic attributes, Physiological and biochemical processes occur during fruit ripening, leading to changes in size, color, and flavor, improving the fruit's acceptability for the consumer. This study investigates the impact of varying maturity stages on the physicochemical and phytochemical composition of pomegranate peels and juice across diverse cultivars. Physicochemical parameters, including acidity, total soluble solids, and pH of juice were evaluated, and Phytochemical analysis involving the identification and quantification of bioactive compounds of the peel extract was conducted during three ripening stages. Results revealed distinctive patterns in physicochemical and phytochemical parameters across maturity stages and cultivars. Morphological characteristics and fruit pigmentation exhibited changes throughout the ripening process, reaching peak values in the final stage. The maximum fruit weight was observed at the concluding stage. Qualitative features, including soluble solids, displayed an upward trend until stage III, whereas titratable acidity values decreased across all cultivars during ripening. A decline in total polyphenols up to the III ripening stage was noted in all three cultivars. This research not only enhances our understanding of the intricate interplay between maturity stages and chemical composition but also provides practical guidance for the production of high-quality pomegranate products with enhanced health-promoting properties.

Keywords: Pomegranate, Physicochemical parameters, Maturity, Phenolic compounds, Punicalagin

1. *Introduction*

The pomegranate (*Punica granatum* L.) holds significant global importance as a key fruit primarily situated in tropical and subtropical areas. Its widespread demand in the world market is driven by its multifaceted uses, distinctive health benefits, and nutritional value, making it a crucial component in human diets (Pareek et al., 2015; Zhao et al., 2015; Nikdel et al., 2016; Drogoudi et al., 2021). Various components of pomegranate trees and the fruit itself have been utilized for their therapeutic qualities and for diverse applications, including the production of juice, jams, syrup, and sauce (Nikdel et al., 2016; Khodabakhshian et al., 2017b). Presently more than 1000 pomegranate cultivars are grown in many tropical and sub-tropical countries (Ghosh et al., 2009). Pomegranate fruit consumption has been linked to a lower occurrence of non-communicable diseases like cancer, cardiovascular disease, and diabetes. This is attributed to its substantial antioxidant content, which is beneficial in conditions involving chronic inflammation. The widespread commercialization and increased awareness of pomegranate as a medicinal food and dietary supplement have significantly improved its accessibility and popularity among consumers (Fawoleet al., 2012; Mphahlele et al., 2016; Moga et al., 2021).

Many scientific studies have verified the biological activities and medicinal impacts of various components (such as arils, peels, leaves, and flowers) and derivatives of this substance (Hasnaoui et al., 2014; Magangana et al., 2020). Due to the increasing demand and awareness that 50% of the processed weight results in waste, a substantial amount of peel is generated as output (Hasnaoui et al., 2014). The pomegranate peel serves as a rich source of phenolics, flavonoids, ellagitannins, hydroxybenzoic acids, and various other bioactive molecules. Notably, the extract from pomegranate peel exhibits superior antioxidant properties compared to turmeric and ascorbic acid (vitamin C), both well-known for their antioxidant effects. The diverse bioactive components present in the peel function as anti-inflammatory agents, providing effective prevention against a range of conditions such as heart disease, childhood cerebral ischemia, Alzheimer's disease, diabetes, arthritis, obesity, male infertility, bacterial infections, and radiation-induced tissue damage (Johanningsmeier and Harris, 2011; Montefusco et al., 2021). Considerable focus has been directed towards pomegranate extracts as a natural substitute for addressing a broad spectrum of bacterial and viral infections, given their antimicrobial properties against many bacterial strains

tested (Pagliarulo et al., 2016; Ferrazzano et al., 2017; Salim et al., 2023). A recent study suggests that both pomegranate aril and peel extracts exhibit significant antimicrobial activity, demonstrated by their ability to inhibit the growth of two prominent human pathogens *Staphylococcus aureus* and *Escherichia coli* commonly associated with foodborne illnesses (Pagliarulo et al., 2016).

The chemical profile of pomegranates varies based on factors such as the type of cultivar, the region of cultivation, climate conditions, maturity, cultural practices, and storage conditions (Bar-Ya'akov et al., 2019; Montefusco et al., 2021). Numerous studies indicate that the choice of cultivar significantly impacts antioxidant activity and various physicochemical properties, including peel and juice percentage, dry matter, pH, total soluble solids (TSS), total sugars, titratable acidity (TA), total phenolics, anthocyanins, organic acids, and the composition of water-soluble vitamins. Assessments of morphological and chemical traits, such as pH, TSS, maturity, and harvest index, revealed notable differences among cultivars, underscoring the pivotal role of cultivar selection in influencing physicochemical properties (Nikdel et al., 2016).

Pomegranate, being a non-climacteric fruit, does not undergo further ripening once harvested. Consequently, the acceptability of pomegranate fruit for both consumers and processors relies on a blend of characteristics, encompassing physical attributes like color and size, as well as chemical constituents such as sugar content, acidity, and flavor (Mphahlele et al., 2016). Therefore, it is crucial to comprehend the effects of preharvest and postharvest management practices on flavor and consumer satisfaction for the future expansion of this market (Caleb et al., 2015). Among these factors, the maturity stage during harvesting plays a crucial role in determining both the compositional quality and quantity of bioactive compounds accumulated in fruits. Given that pomegranate is a non-climacteric fruit, it emphasizes the importance of accurately identifying the optimal harvesting stage (Attanayake et al., 2019). Previous studies indicate that harvesting pomegranate fruits too early hinders proper ripening, while delayed harvesting restricts the market lifespan of the fruits. This underscores the significance of identifying the optimal ripening stage for pomegranates to ensure both market viability and consumer acceptance (Babu wt al., 2017).

The aim of this study was to determine the effect of development and ripening stages on the polyphenols content (total phenols, and condensed tannins, total flavonoids, phenolic composition) of pomegranate peel aquas extracts, in addition to studying the change in some physicochemical properties (pH, total soluble solids, total sugars, titratable acidity) of pomegranate juice at three distinct harvest time points maturities.

2. Materials and methods

2.1 Chemicals

The analytical reagent Folin-Ciocalteu was acquired from Sigma-Aldrich Co. (Milan, Italy). High-performance liquid chromatography (HPLC) grade standards for punicalin α and β, punicalagin α (Pun α) and punicalagin β (Pun β), hydroxybenzoic acid, catechin, epicatechin, chlorogenic acid, caffeic acid, rutin, ellagic acid, cyanidin 3,5-diglucoside chloride, and delphinidin 3,5-diglucoside chloride were also obtained from Sigma-Aldrich (Milan, Italy). The standard for cyanidin 3-O-glucoside was purchased from Extrasynthese (Genay, France), and gallic acid was sourced from Carlo Erba Reagenti SpA (Rodano, Milan, Italy). Ultrapure water was prepared using a Milli-Q system (Millipore Corporation, Billerica, USA).

2.2 Pomegranate peel extractions and juice processing

The chemical composition and physicochemical properties of the peel and juice from three pomegranate varieties were evaluated. Fruits were harvested during three different maturations stages. First harvesting was on October 06, 2022, second October 20, 2022, and third and last harvesting on November 02, 2022, from the following *Punica granatum* varieties: Primosole (PS), Sassari 2 (SS2), and Sassari 3 (SS3). All trees are located in the pomegranate varietal collection field of the University of Sassari's Experimental Station "A. Milella" in Oristano, Sardinia (San Quirico – Fenosu, 39° 54′12″ N, 8° 37′19″ E), situated 13 m above sea level. The pomegranate orchard was planted in 2016 according to a 6 x 4.5 m planting distribution. The bioclimate of the study area is classified as thermo-Mediterranean, with annual mean, and maximum and minimum average temperatures of 17.1, 25.4 (July), and 9.6 °C (February), respectively. Precipitation is

concentrated in the autumn and winter seasons, with an annual mean rainfall of 581 mm (data from the Environmental Protection Agency of Sardinia, ARPAS, Supporting information S1).
Five pomegranate fruits per genotype were washed with distilled water. The peel and the arils were manually separated, Aril chemical analyses were performed on centrifuged and filtered juice obtained by squeezing the fruit with a domestic extractor. The analyses were carried out in three replicates of five fruit at harvest during three different maturation stages.

The peel was chopped into small pieces using a sharp knife, then dried using a vacuum freeze dryer (Lio-5P) for 72 hours (-55 °C). The dried peel was ground into a fine powder using a laboratory blender (Waring Commercial Blender 7011S). A 1.5 g sample of pomegranate powder was extracted with 25 ml milli-Q water (solvent/sample ratio 15:1 (w/w)) at room temperature for 4 hours. Then, samples were centrifuged at 5000 rpm for 10 minutes and the filtrate passed through a 0.45 mm hydrophilic nylon membrane. Samples of pomegranate peel extract (PPE) were stored at -20 °C until analysis. During the processing phases described above, the fruit fresh weight and peel, seeds, and juice ratios were calculated.

2.3 Juice chemical analysis; Total soluble solids, titratable acidity, and pH

The following juice parameters were measured: total soluble solids content (TSS), titratable acidity (TA), and pH. TSS content was determined using a digital refractometer Atago PR-101 (Atago, Tokyo, Japan) at 20 °C and results expressed in Brix degrees. Total acidity was quantified by potentiometric titration (pH meter ORION 420A) with 0.1 N NaOH up to pH 8.2, using 5 mL of juice diluted in 50 mL distilled water. The results were expressed as percent citric acid equivalent. The pH was measured by dipping the pH-meter probe (Horion Polyplast) into the juice.

2.4 Determination of total phenolic content

The evaluation of total phenolic content (T.Phen)in pomegranate peel was conducted using the Folin-Ciocalteu assay (Salim et al., 2023). In summary, diluted samples were combined with Folin–Ciocalteu reagent (1:1) and 7.5% sodium carbonate solution in a 25 mL volumetric flask. The mixture was then incubated at room temperature for 2 hours. The spectrophotometric analysis of total phenolic content was performed at 750 nm using a spectrophotometer (8453 UV-Visible

Spectrophotometer, Agilent Technologies, USA). The results were expressed as milligrams of gallic acid equivalents (GAE) per gram of freeze-dried matter (DW), determined through a gallic acid calibration curve (10-100 mg L^{-1}, R^2= 0.989). The analysis was conducted in triplicate.

2.5 Determination of total flavonoid content

The measurement of total flavonoids (T.Flav) was conducted through a colorimetric assay utilizing the AlCl3 method, following established procedures as outlined by Re et al. (2019). Quantification was achieved using a catechin (CE) calibration curve ranging from 2.5 to 20 µg ml^{-1}, with an R2 value of 0.996. The outcomes are presented as milligrams of catechin equivalent (CE) per gram of dry weight (mg CE g^{-1} DW).

2.6 Determination of total tannin content

The assessment of total condensed tannins (T.Tan) was conducted using the vanillin assay following the method described by Melito et al. (2016). The spectrophotometric detection of vanillin-tannin adducts was carried out at 500 nm, and concentrations were determined through a catechin calibration curve ranging from 1 to 6 µg mL^{-1}, with an R^2 value of 0.998. The findings are presented as milligrams of catechin equivalent (CE) per gram of dry weight (mg CE g^{-1} DW).

2.7 HPLC analysis of phenolic compounds

Phenolic compound analysis using reverse-phase HPLC was conducted on an Agilent 1100 Liquid Chromatography (LC) system (Agilent Technologies, Palo Alto, CA, USA). The system was equipped with a quaternary pump (G1311A), degasser, column thermostat, auto-sampler (G1313A), and a diode array detector (G1315 B, DAD). Chromatographic separation utilized a Luna C18 column (250 x 4.6 mm, 5 µm) from Phenomenex (Torrance, CA, USA) with a security guard cartridge (4 × 2 mm). The flow rate was set at 1 mL min^{-1}, and the column temperature was maintained at 30 °C. Elution was performed with a binary mobile phase of solvent A (water and 0.1% trifluoroacetic acid) and solvent B (acetonitrile). The gradient elution program comprised the following: 0 min, 99% A; 5 min, 95% A; 6 min, 93% A; 10 min, 85% A; 15 min, 75% A; 20 min, 10% A; 25 min, 99% A, with a post-time of 2 min. Detection occurred at 280, 360, and 520

nm. Phenolics were identified based on the retention time of a standard mixture and quantified using respective calibration curves. Prior to injection, samples were appropriately diluted. Results are expressed as milligrams per gram of dry weight (mg g^{-1} DW). For anthocyanin detection, 2 mL of pomegranate extract was loaded into C18-Sep-Pak cartridges (Strata C-18-E, 500 mg 6 mL^{-1}, Phenomenex) conditioned with 2 mL methanol, followed by 5 mL of 5 mM H2SO4. After washing with 5 mL of 5 mM H2SO4, anthocyanins were eluted with 5 mL MeOH, followed by 5 mL milli-Q water into a 10 mL calibrated flask.

2.8 Statistical analysis

Influence of the genetic factors (intended as variety), harvest time, and their interaction, on pomegranate peel and juice chemical composition. The effect of harvest time on each individual variety was estimated by one-way ANOVA. Moreover, in order to identify the chemical components that mostly contributed to the variability related to variety and harvest time factors, the pomegranate peel dataset was analyzed to an unsupervised Principal Component Analysis (PCA) using the FactoMineR (Josse and Husson, 2008) package of the R Studio statistical software (R version 4.3.2).

3. Results

3.1 Physical and chemical characteristics of pomegranate fruits and respective juices

The fruit fresh weight increased constantly during the three harvest dates for all the tested cultivars (Figure 1a), values ranged between 255g (SS2 at 1st harvest) and 464g (PS at 3rd harvest), no clear differences between varieties were observed. On the other hand, the fruits of the three varieties differed for the percentage composition of peel, seeds, and juices. The peel percentage values were of SS3 (59-60%, figure 1b) were largely higher than SS2 and PS. This latter variety showed the highest juice yields (from 31 to 38% of fruit weight, Figure 1c). Juice yield achieved the peak values at the second harvest date for all the three varieties. The role of varietal factor was predominant in determining pH and titratable acidity of pomegranate juices (Table 1). Indeed, those parameters evolved differently during harvest period according to the variety. PS reported the highest pH and the lowest acidity levels, which were stable along the three harvests. The total

soluble solids, expressed as °Brix, increased during fruit maturation, similarly for all the tested varieties.

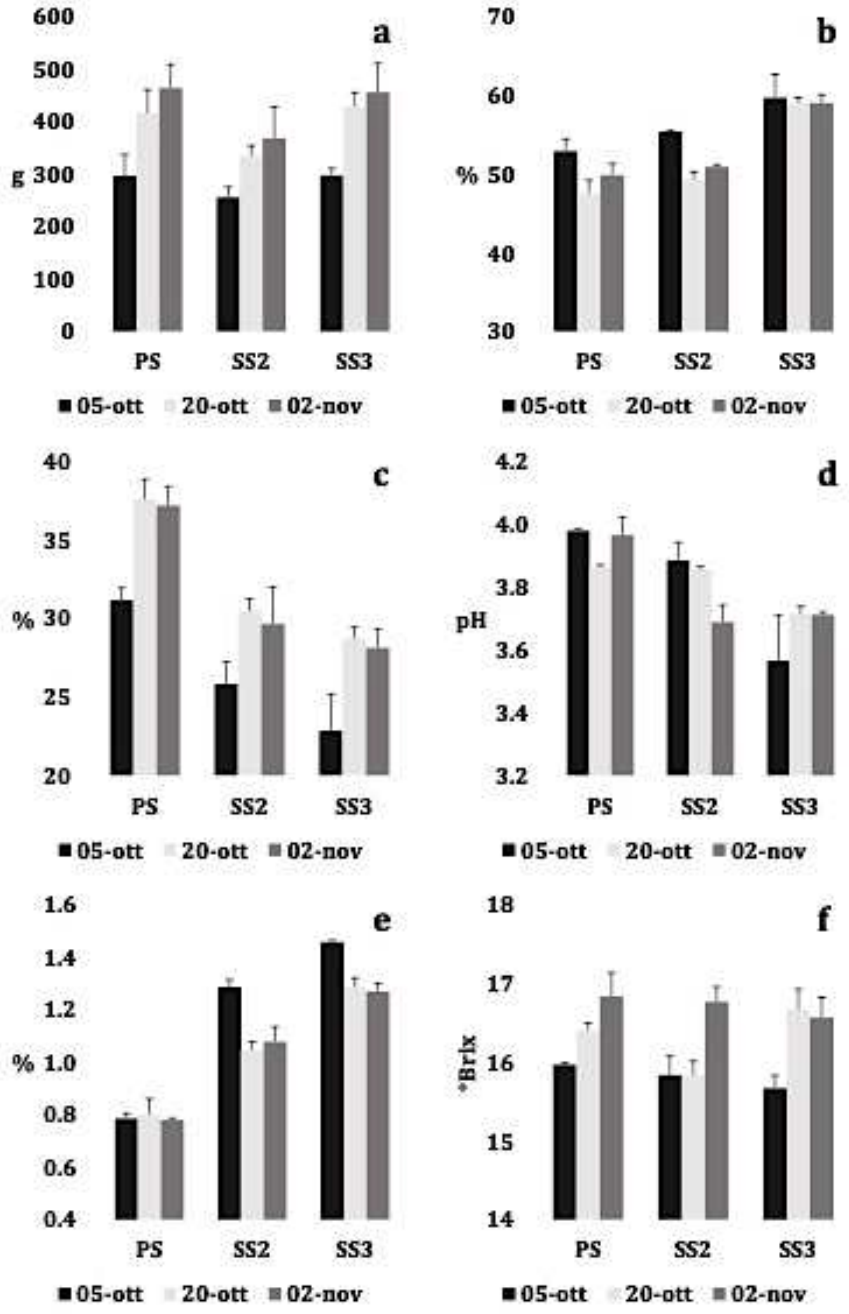

Figure 1. Changes during three harvest dates of physical characteristics of pomegranate fruits (fruit weight, a; peel percentage, b; juice percentage, c) and respective juice chemical parameters (pH, d; titratable acidity, e; total soluble solids, f) from three cultivars: Primosole (PS), Sassari 2 (SS2), and Sassari 3 (SS3).

Table 1. Significance values and eta squared values (η2) for the two-way ANOVA for all the variables analysed.

	Cultivar		Harvest time		Interaction	
Variable	p-value	η^2	p-value	η^2	p-value	η^2
Fruit weight	n.s.	0.12	**	0.39	n.s.	0.01
Peel	***	0.61	**	0.11	n.s.	0.04
Juice	***	0.52	**	0.24	n.s.	0.01
pH	***	0.45	n.s.	0	*	0.18
Acidity	***	0.84	**	0.07	*	0.04
SST	n.s.	0.04	**	0.43	**	0.12
TPC	***	0.13	***	0.71	***	0.11
Tflav	***	0.15	***	0.71	n.s.	0.04
Tann	***	0.39	**	0.24	**	0.11
GA						
PunicalinA	**	0.16	***	0.53	n.s.	0.09
PunicalinB	***	0.18	***	0.72	n.s.	0.03
PunA	***	0.26	***	0.53	n.s.	0.03
PunB	**	0.02	***	0.72	*	0.08
ac.4-Hydb	***	0.37	***	0.48	***	0.13
Cat	***	0.55	***	0.31	n.s.	0.02
ChlAc	***	0.49	***	0.34	***	0.14
CA	***	0.04	***	0.81	***	0.1
EpiCat	***	0.1	***	0.69	***	0.16
Rut	***	0.42	***	0.3	n.s.	0.09
EA	***	0.23	***	0.57	**	0.13
Cya.3.glu	***	0.23	***	0.44	***	0.32
Del.3.glu						
Pel.3.glu	***	0.16	***	0.42	***	0.42
Cya.3,5.diglu	***	0.15	***	0.68	***	0.16
Del.3,5.diglu	***	0.14	***	0.61	***	0.25
Pel.3,5.diglu	***	0.15	***	0.73	***	0.11

3.2 Chemical profile of the PPE

The chemical characteristics (total phenolic compounds, flavonoids, and condensed tannins) of three peel pomegranate cultivars analysed are described in Table 2 and Table 3. The data showed a considerable variation in the chemical properties of studied pomegranate cultivars,

with maturation stages distinctly influencing the levels of phenolic compounds, flavonoids, and condensed tannins. Throughout the maturation period, the polyphenol content across all examined cultivars peaked during the initial harvesting time point, gradually decreasing as the final harvesting time point approached. The total phenols ranged from 166.38 to 122.03 mg g-1 DW, total flavonoids from 16.45 to 11.20 mg g-1 DW, and condensed tannins from 4.33 to 2.59 mg g^{-1} DW for the various cultivars extracted. Among these, SS2 showed the highest content of total flavonoids and total phenols, while PS exhibited the highest concentration of condensed tannins. Conversely, SS3 displayed the lowest polyphenol content.

Eighteen phenolic compounds, including six anthocyanins were identified and quantified through the HPLC-DAD analysis (Table 3). Among the phenolic compounds identified, the ellagitannins derivatives punicalagin (both α and β isomers) were the most representative and showed values within the range 75.87 – 237.40 mg g^{-1}. The SS3 sample at the first harvest reported the highest values of most of the detected molecules, except for anthocyanins, suggesting a later maturation process for this variety. A decreasing trend in the content of quantified phenolic compounds was observed throughout the maturation stages, however, this trend did not apply to anthocyanins, which exhibited an increasing trend during the maturation period, suggesting higher levels in the fully matured pomegranate stage.

According to both two-way ANOVA analysis (Table 2) and PCA analysis (Figure 2) the phenolic composition was strongly affected by the maturation factor and in a lesser extent by variety. Indeed, looking at the PCA summarized results in Figure 2, the PWE samples clearly separated by harvest dates and all the phenolic compounds, specifically the spectrophotometric values of total phenolic content and total flavonoids together with punicalagin β, were positively related to the earliest harvest date, whilst the presence of higher anthocyanins concentrations strongly characterized the later harvest dates. On the other hand, PCA analysis highlighted that the genetic factor was partially masked by maturation. Notwithstanding that, SS2 highlighted clear differences from the other varieties, due to higher total phenols, total flavonoid values, and anthocyanidin content.

Table 2. Changes during three harvest dates of chemical characteristics of pomegranate peel from three cultivars: Primosole (PS), Sassari 2 (SS2), and Sassari 3 (SS3).

Cultivar	Time	Total Phenols	Total Flavonoids	Condensed Tannins
		mg GAE g^{-1} DW	mg CE g^{-1} DW	mg CE g^{-1} DW
PS	1	166.38 ± 2.17	16.45 ± 1.34	4.33 ± 0.46
	2	131.85 ± 7.18	12.23 ± 1.71	4.26 ± 0.10
	3	131.14 ± 1.61	11.20 ± 0.49	3.17 ± 0.22
SS 2	1	189.19 ± 5.43	18.19 ± 0.31	3.70 ± 0.93
	2	160.86 ± 7.95	15.87 ± 0.93	3.10 ± 0.11
	3	126.97 ± 5.07	12.47 ± 1.11	3.12 ± 0.39
SS 3	1	157.69 ± 6.16	15.91 ± 0.77	3.40 ± 0.32
	2	147.01 ± 8.95	13.41 ± 0.43	2.59 ± 0.22
	3	122.03 ± 7.77	11.59 ± 0.90	2.69 ± 0.39

Table 3. Changes during fruit maturation of phenolic (mg g1) and anthocyanin (mg per 100g) composition, determined by HPLC-DAD, of pomegranate peel from three cultivars: Primosole (PS), Sassari 2 (SS2), and Sassari 3 (SS3).

	PS			SS2			SS3		
Variable	**06 oct**	**20 oct**	**02 nov**	**06 oct**	**20 oct**	**02 nov**	**06 oct**	**20 oct**	**02 nov**
Gallic acid	0.29±0.04	0±0	0±0	0.13±0.03	0±0	0±0	0.79±0.06	0±0	0±0
Punicalin α	5.56±0.17	4.21±0.46	3.59±0.13	5.64±0.36	5.34±0.53	4.75±0.51	5.66±0.42	4.28±0.42	4.26±0.15
Punicalin β	4.7±0.42	2.79±0.12	1.73±0.2	5.94±0.59	4.27±0.14	3.48±0.23	6.38±0.63	4.05±0.69	2.42±0.21
Punicalagin α	122.81±7.65	93.92±3.42	90.43±3.81	99.66±6.6	85.51±3.35	75.87±4.13	132.14±8.62	104.45±15.13	92.73±9.83
Punicalagin β	202.14±4.35	164.38±8.65	159.54±9.76	211.98±8.19	182.82±10.31	150.46±10.26	237.4±14.64	169.63±25.06	150.52±18.31
4-Hydroxybenzoic acid	0.65±0.11	0.4±0.06	0.29±0.04	1.34±0.06	0.98±0.05	0.52±0.08	1.99±0.23	0.95±0.03	0.62±0.06
Catechin	3±0.44	2.59±0.14	2.07±0.16	2.2±0.06	1.78±0.22	1.5±0.05	3.72±0.3	3.06±0.29	2.49±0.2
Chlorogenic acid	1.68±0.12	1.58±0.04	1.56±0.07	1.3±0.03	1.18±0.09	0.77±0.05	1.49±0.09	1.03±0.06	0.57±0.02
Caffeic acid	0.44±0.02	0.25±0.01	0.2±0	0.45±0.03	0.29±0.02	0.21±0.01	0.64±0.06	0.23±0.04	0.23±0.01
Epicatechin	0.97±0.08	0.73±0.04	0.36±0.04	1.29±0.07	0.96±0.08	0.64±0.05	1.81±0.21	0.7±0.07	0.47±0.07
Rutin	0.94±0.05	0.86±0.04	0.84±0.05	0.76±0.15	0.67±0.07	0.56±0.03	1.1±0.09	0.8±0.04	0.76±0.02
Ellagic acid	16.55±1.55	10.7±0.5	12.96±0.85	20.9±0.29	15.69±1.52	13.26±0.45	26.99±2.61	16.41±1.02	13.83±1.58
Cyanidin-3-glucoside	2.03±0.01	2.19±0.01	2.42±0.02	2.02±0	2.3±0.04	2.78±0.02	0±0	2.16±0	2.43±0.31
Delphinidin-3-glucoside	0±0	1.96±0.01	2.01±0.01	0±0	1.96±0.01	2.04±0.01	0±0	1.97±0.01	2.04±0.01
Pelargonidin-3-glucoside	2.01±0	2.05±0.01	2.14±0.03	2.04±0	2.11±0.01	2.79±0.02	0±0	2.11±0.01	2.65±0.02
Cyanidin-3,5-diglucoside	0±0	1.58±0.02	1.83±0.07	1.5±0.02	1.6±0.01	2.12±0.03	0±0	1.42±0.01	1.92±0.02
Delphinidin-3,5-diglucoside	0±0	1.97±0	2.15±0.01	1.94±0.01	1.99±0.01	2.16±0.01	0±0	1.94±0	2.11±0.02
Pelargonidin-3,5-diglucoside	1.96±0.01	2.01±0.01	2.14±0.02	1.99±0.02	2.17±0.01	2.48±0.04	1.93±0	2.13±0	2.46±0.02

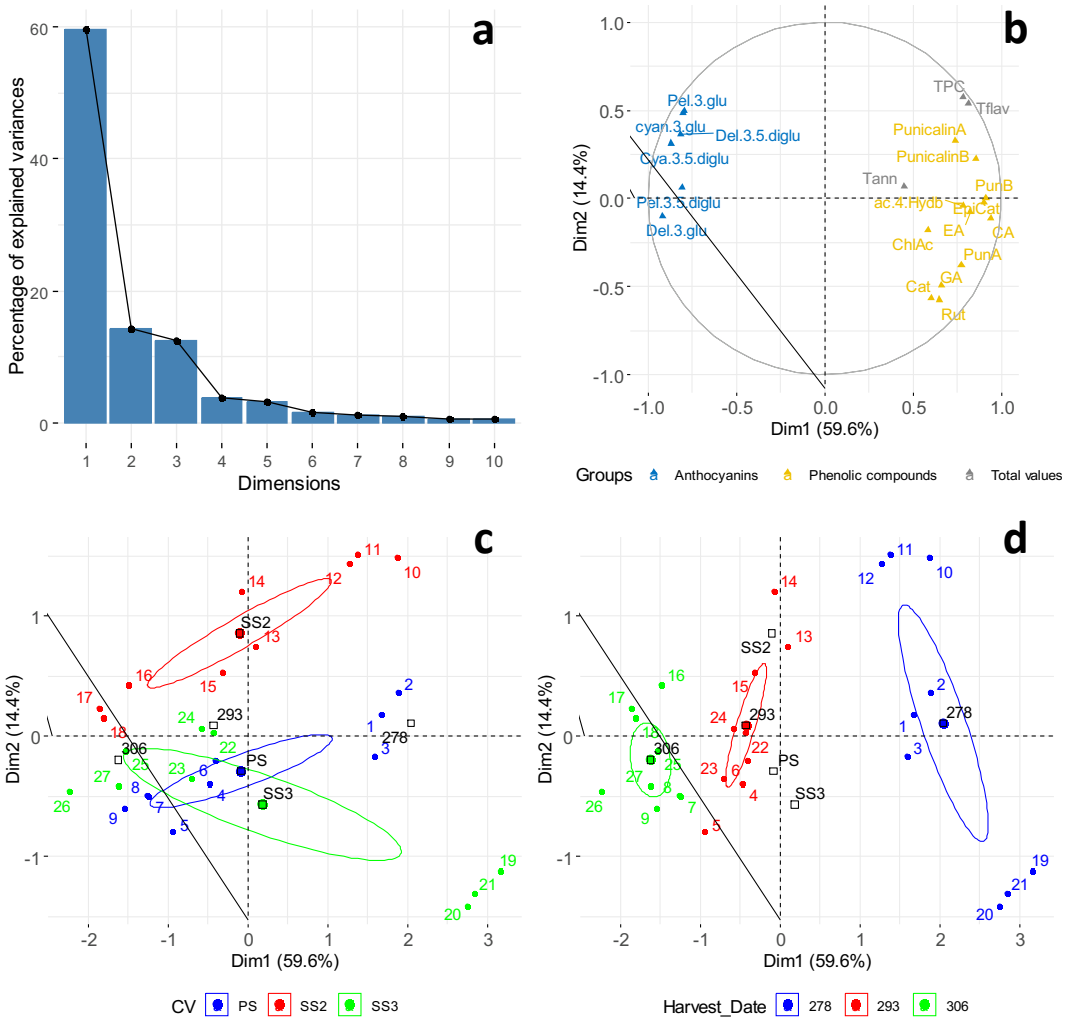

Figure 2. Summary plots of PCA analysis: a) scree plot representing the percentage of explained variance related to each PCA components; b) scatter plot representing the variables distribution on the first two PCA dimensions; c) score plot coloured by CV factor; d) score plot colored by harvest date factor.

4. Discussion

The pomegranate fruit peel contains significant quantities of potentially valuable components such as phenolic acids, flavonoids, and hydrolysable tannins where Most of the therapeutic effects of the pomegranate fruit were attributed to these secondary metabolites (Amri et al., 2017; Singh et al., 2018; Magangana et al., 2021). In a study where it compared the phytochemical content across various parts of the pomegranate fruit, the peel extracts displayed the greatest levels of total

polyphenolic compounds. In fact, the peel extracts exhibited an average twenty-eight-fold higher total polyphenolic content compared to the extracts from the arils across the five tested varieties (Mekni et al., 2018). The findings from the present study indicated notable variations in the chemical properties of the analyzed pomegranate varieties, with maturation stages notably impacting the levels of phenolic compounds, flavonoids, and condensed tannins. Similar to our present findings, a reduction in total polyphenol levels has been observed as pomegranate fruit matures in various cultivars. In several studies, total phenolics, total flavonoid content, and total condensed tannins exhibited considerable changes throughout the process of fruit maturation (Al-Maiman and Ahmad, 2002; Mirdehghan and Rahemi, 2007; Saad et al., 2012; Fawole and Opara, 2013a; Han et al., 2015). Different studies attributed the decline in overall polyphenol content to the oxidation reactions facilitated by polyphenol oxidase, a characteristic feature of these stages of maturity (Amiot et al., 1995; Fawole and Opara, 2013a; Magangana et al., 2021). In other studies, it was observed that the acidic phenolic and a majority of the neutral phenolic content in pomegranate peel reached their lowest levels during the fruit's red stage, confirming a negative correlation between phenolics and peel color (Robbins et al., 1998; Li et al., 2004; Kulkami and Aradhya, 2005; Nian et al., 2011). Moreover, Wang et al. (2003) suggested that the rise in fruit weight could be a primary factor contributing to the decrease in polyphenol content during the progression of fruit development.

We reported a total phenolic content (TPC) ranged between 189.19 to 122.03 for all the tested cultivars during different maturation stages. The TPC levels are in line with the previous literature, where it was reported that TPC in pomegranate peel from different cultivars ranged from 134.3 mg GAE g^{-1} DW to 181.0 mg GAE g^{-1} DW (Saad et al., 2012). TPC in PPE of nine Persian cultivars ranged from 98.24 to 226.56 mg GAE g^{-1} (Ardekani et al., 2011). Close levels of TPC were reported by Orak et al. (2012) when compared TPC in peel, juice, and seed, the highest TPC content was for peel and varied between 126.11 and 212.48 µg GAE mg^{-1}. In addition, in a study where they compared TPC of different fruits the PPE contained the highest TPC of 1639.7 mg (GAE) 100 g^{-1} among the tested fruits (Singh et al., 2016). Lower levels of TPC, 84.89 to 109.79 mg GAE g^{-1} DW were found in PPE of six Tunisian pomegranate cultivars and was much higher than the content reported in their juices (6.89–13.70 mg GAE g^{-1} DW) (Singh et al., 2018).

A reduction in the TPC between the first and the last compared maturation stages was 18.7%, 32.88%, and 22.61% for PS, SS2, and SS3 respectively. In agreement with the present results Li et al. (2016) found that at the unripe maturity stage, the highest TPC was observed at 390 mg GAE g^{-1} DW compared to when the fruits were ripe at 250 mg GAE g^{-1} DW. Similarly, Tehranifar et al. (2010) found a significant difference in TPC among the twenty varieties of pomegranate 295.79 to 985.32 mg GAE /100g. In addition, Fawole and Opara (2013a) reported a reduction in the TPC as fruit maturity progressed, where TPC decreased from 1051.60 to 483.31 mg GAE 100 ml^{-1}. During the maturation of 'Malas Yazdi' pomegranate fruit (Mirdehghan and Rahemi, 2007) recorded an increase in TPC of the fruit peel in the early stages of maturation at almost 120 mg g^{-1} DW, however, it declined to 50.2 mg g^{-1} DW at harvest maturity stage.

The present study reported total flavonoid content (TFC) between 18.19 mg CE g^{-1} DW 11.20 mg CE g^{-1} DW, during the pomegranate maturation stages. Indeed, and regardless of cultivars, Mekni et al. (2018) reported maximum levels of TFC obtained in peels of the Garsi variety (15.13 mg g^{-1} DM). Montefusco et al. (2021) reported TFC of peels (4.0–5.1 mg C g^{-1} fw) and juice (0.88–1.44 mg CE g^{-1} fw). TFC in water and methanol extracts were reported as 21.03 and 51.52 mg RE g^{-1}, respectively in PPE of the Tunisian cultivar (Elfalleh et al., 2012). In another study, TFC in PPE of nine Iranian cultivars was reported to in the range of 18.61–36.40mg CE g^{-1} (Ardekani et al., 2011). Stojanović et al. (2017) found close levels to the level of our study where they reported TFC of 5.83 mg CE g^{-1} in the peel of Serbian PPE. The differences in TFC between cultivars may be related to fruit type (sweet and sour) and geographical area of origin (Mekni et al., 2018). During the maturation we observed also reduction in the TFC content, this was also reported by (Fawole and Opara, (2013a) where they found that as fruit maturity progressed, TFC from 752.18 to 397.27 mg CAE 100 ml^{-1}.

Condensed tannin content (CTC) in the current study varied from 4.33 mg CE g^{-1} DW to 2.59 mg CE g^{-1} DW. Closely to these, Saad et al. (2012) found CTC levels in PPE of four Tunisian cultivars ranged from 3.2 to 7.7 mg CE g^{-1} DW. Peels of two different Tunisian varieties were reported to have CTC of 6.96 and 6.19 mg g^{-1} DM (Mekni et al., 2018). Sharma and Thakur (2018) observed a low amount of tannins in the pulp of four pomegranate cultivars varied from 169.4 to 336.2 mg $100g^{-1}$). Interestingly for the CTC in the present study no huge reduction were observed.

Nevertheless, Kaur et al. (2018) found in their research that green fruits exhibited a higher tannin content (123.55 mg TAE g^{-1} of extracts) compared to red fruits (95.67 mg TAE g^{-1}). This observation is attributed to the significant decrease in total tannins during the ripening process, resulting in lower tannin content in red fruits. Similarly, Fawole and Opara (2013a) found that while pomegranate fruit maturation GTC decreased from 64.80 to 29.07 mg GAE 100 ml^{-1}.

Pomegranate peels are rich source of ellagitannins and ellagic acid derivatives such as punicalagin, punicalin, ellagic acid, ellagic acid and many other phenolics compounds (Singh et al., 2018). In the present study during the maturation for the pomegranate fruits reduction in the quantified phenolics compounds was noticed. These findings align with the study conducted by Han et al. (2015), which reported higher levels of phenolic compounds such as punicalagin, epicatechin, catechin, ellagic acid, protocatechuic acid, chlorogenic acid, ferulic acid, and gallic acid in young pomegranate peel and as the fruit underwent rapid growth and increased in weight, the content of these compounds gradually decreased. Likewise, Bar-Ya'akov et al. (2019) measured gallagic acid, punicalin isomers, and punicalagins in water extracts from maturing fruits. In both the tested cultivars, there was a reduction in the accumulation of all three hydrolysable tannins in the developing fruit peels. On the other hand, Fawole and Opara (2013a) quantified several phenolic compounds where they noticed that the concentration changed between decrease and increase during fruit development. In the present study the most dominated phenolics both Pun α and Pun β showed decreased accumulation in the peels during the maturation. Similar kind of results were also observed by Attanayake et al. (2019) who noted a gradual reduction in α punicalagin content in both peel and arils from flowering to maturity however, β punicalagin content did not change.

Anthocyanins are water-soluble pigments, play a key role in imparting the appealing pink-reddish to various fruits, including pomegranates also they are are well known for their antioxidant properties (Seeram and Nair, 2002; Sharma and Thakur, 2018). In the present work, it was observed that anthocyanins exhibited an increasing trend during the maturation period, recoding higher levels in the fully matured pomegranate stage for the quantified anthocyanins. This observation is also supported by previous studies where, the same quantified anthocyanins showed

that major compositional changes in fruit are developmentally regulated and continual significant increase in anthocyanins composition was recorded (Fawole and Opara, 2013a; Zhaoet al., 2015). Similarly, Pareek et al. (2015) found with advancement of ripening, anthocyanins increased rapidly at ripening.

Being a non-climacteric fruit, pomegranate does not undergo ripening post-harvest and the acceptability of pomegranate fruit among consumers is predominantly determined by preharvest factors, including maturity status, climatic conditions, genotype, and season (Mphahlele et al., 2016). Hence, research has been undertaken to devise techniques for assessing the overall quality of pomegranate fruit, aiming to meet the minimum acceptability standards among consumers (Khodabakhshian et al., 2017a). Maturation mainly aligns with a set of interconnected physiological and biochemical characteristics, including firmness, total soluble solids (TSS), pH, and acidity (Khodabakhshian et al., 2017b). In the current study. The maturity state and quality of pomegranate fruit were studied based on physiological parameters such as total soluble solids (TSS), pH, dry weight (DW) content, and acidity.

Many physical and chemical transformations take place as pomegranate fruit undergoes maturation and ripening (Zarei et al., 2010). According to our data, the fruit weight increased progressively during fruit ripening, similarly, this was reported for wonderful cultivar or other accessions (Shwartz et al., 2009; Fawole, et al., 2013b; Cirillo et al., 2022). The same authors reported that the peel percentage declined significantly due to an increase in the fruit growth rate, as found in PS and SS2 cultivars, while the decrease is less evident and not significant in SS3. The juice percentage increased significantly during fruit maturity (Zarei et al., 2010) and this increase was very evident in all the varieties studied, especially between the first and subsequent samplings. During the ripening of fruit, a notable process involves the breakdown of accumulated starch into simple sugars, occurring in the early stages of fruit development. Both starch and sucrose transform into glucose as part of this process, leading to an elevation in total soluble solids (TSS) and total sugars (Shwartz et al., 2009; Zarei et al., 2010). As it was reported in the current study, during ripening the Significant increases in TSS and pH values coupled with a significant decline in titratable acidity (TA). This was supported with the results from (Fawole, et al., 2013c;

Khodabakhshian et al., 2017a; Cirillo et al., 2022). The decrease in titratable acidity (TA) serves as a standard indicator for identifying maturation, similar to total soluble solids (TSS). The makeup and quantity of organic acids are crucial elements influencing how consumers perceive both the sweetness and sourness of various pomegranate fruit cultivars (Fawole and Opara, 2013b). Concerning our data, the local cultivars SS2 and SS3 showed similar behavior during maturation time, with higher acidity than PS and slightly lower TSS. However, looking at the TSS dynamics SS3 achieved the peak earlier than SS2, which showed a TSS significant increase only at the last harvest day. These findings suggest that further studies, enlarging the harvest period to investigate, are necessary to better clarify the optimal harvesting period.

5. Conclusions

In conclusion, this study sheds light on the dynamic relationship between varying maturity stages and the physicochemical and phytochemical composition of pomegranate peels and juice across diverse cultivars. Through comprehensive evaluation of morphological, physicochemical, and phytochemical parameters, we have uncovered distinct patterns that elucidate the ripening process and its impact on fruit quality. Our findings underscore the importance of considering maturity stages in optimizing pomegranate production for both consumer acceptability and health-promoting properties. By providing valuable insights into the interplay between fruit ripening and chemical composition, this research contributes to the development of strategies for producing high-quality pomegranate products with enhanced nutritional benefits. Further exploration in this area holds promise for advancing our understanding of fruit development and improving agricultural practices for the cultivation of nutritious and marketable pomegranates.

6. *References*

Al-Maiman, S. A., & Ahmad, D. (2002). Changes in physical and chemical properties during pomegranate (Punica granatum L.) fruit maturation. Food Chemistry, 76(4), 437-441.

Amiot, M. J., Tacchini, M., Aubert, S. Y., & Oleszek, W. (1995). Influence of cultivar, maturity stage, and storage conditions on phenolic composition and enzymic browning of pear fruits. *Journal of Agricultural and Food Chemistry*, *43*(5), 1132-1137.

Amri, Z., Zaouay, F., Lazreg-Aref, H., Soltana, H., Mneri, A., Mars, M., & Hammami, M. (2017). Phytochemical content, Fatty acids composition and antioxidant potential of different pomegranate parts: Comparison between edible and non edible varieties grown in Tunisia. International Journal of Biological Macromolecules, 104, 274–280.

Ardekani, M. R. S., Hajimahmoodi, M., Oveisi, M. R., Sadeghi, N., Jannat, B., Ranjbar, A. M., ... Moridi, T. (2011). Comparative antioxidant activity and total flavonoid content of Persian pomegranate (Punica granatum L.) cultivars. Iranian Journal of Pharmaceutical Research: IJPR, 10(3), 519.

Attanayake, R., Rajapaksha, R., Weerakkody, P., & Bandaranayake, P. C. (2019). The effect of maturity status on biochemical composition, antioxidant activity, and anthocyanin biosynthesis gene expression in a pomegranate (Punica granatum L.) cultivar with red flowers, yellow peel, and pinkish arils. *Journal of Plant Growth Regulation*, *38*, 992-1006.

Bar-Ya'akov, I., Tian, L., Amir, R., & Holland, D. (2019). Primary metabolites, anthocyanins, and hydrolyzable tannins in the pomegranate fruit. *Frontiers in plant science*, *10*, 620.

Babu, K. D., Singh, N. V., Gaikwad, N., Maity, A., Suryavanshi, S. K., & Pal, R. K. (2017). Determination of maturity indices for harvesting of pomegranate (Punica granatum). *Indian Journal of Agricultural Sciences*, *87*(9), 1225-30.

Caleb, O. J., Fawole, O. A., Mphahlele, R. R., & Opara, U. L. (2015). Impact of preharvest and postharvest factors on changes in volatile compounds of pomegranate fruit and minimally processed arils–Review. *Scientia Horticulturae*, *188*, 106-114.

Cirillo, A., Magri, A., Scognamiglio, M., D'Abrosca, B., Fiorentino, A., Petriccione, M., & Di Vaio, C. (2022). Evaluation of morphological, qualitative, and metabolomic traits during fruit ripening in pomegranate (Punica granatum L.). *Horticulturae*, *8*(5), 384.

Deiana, P., Santona, M., Dettori, S., Culeddu, N., Dore, A., Molinu, M.G., 2019. Multivariate approach to assess the chemical composition of Italian virgin olive oils as a function of variety and harvest period. Food Chem. 300:125243. https://doi.org/10.1016/j.foodchem.2019.125243

Drogoudi, P., Pantelidis, G. E., & Vekiari, S. A. (2021). Physiological disorders and fruit quality attributes in pomegranate: Effects of meteorological parameters, canopy position and acetylsalicylic acid foliar sprays. *Frontiers in Plant Science*, *12*, 645547.

Elfalleh, W., Hannachi, H., Tlili, N., Yahia, Y., Nasri, N., & Ferchichi, A. (2012). Total phenolic contents and antioxidant activities of pomegranate peel, seed, leaf and flower. Journal of Medicinal Plants Research, 6(32), 4724–4730.

Fawole, O. A., Opara, U. L., & Theron, K. I. (2012). Chemical and phytochemical properties and antioxidant activities of three pomegranate cultivars grown in South Africa. *Food and Bioprocess Technology*, *5*, 2934-2940.

Fawole, O. A., & Opara, U. L. (2013a). Changes in physical properties, chemical and elemental composition and antioxidant capacity of pomegranate (cv. Ruby) fruit at five maturity stages. *Scientia Horticulturae*, *150*, 37-46.

Fawole, O. A., and Opara, U. L. (2013b). Developmental changes in maturity indices of pomegranate fruit: A descriptive review. Scientia Horticulturae, 159, 152-161.

Fawole, O. A., & Opara, U. L. (2013c). Seasonal variation in chemical composition, aroma volatiles and antioxidant capacity of pomegranate during fruit development. African Journal of Biotechnology, 12(25).

Ferrazzano, G. F., Scioscia, E., Sateriale, D., Pastore, G., Colicchio, R., Pagliuca, C., ... & Pagliarulo, C. (2017). In vitro antibacterial activity of pomegranate juice and peel extracts on cariogenic bacteria. *BioMed research international*, *2017*.

Ghosh SN, Bera B, Roy S, Kundu A (2009) Effect of plant growth regulators in yield and fruit quality in pomegranate cv. Ruby. J Hortic Sci Biotechnol 4:158–160

Han, L., Yuan, Z., Feng, L., & Yin, Y. (2015). Changes in the composition and contents of pomegranate polyphenols during fruit development. *Acta Hortic*, *1089*, 53-61.

Hasnaoui, N., Wathelet, B., & Jiménez-Araujo, A. (2014). Valorization of pomegranate peel from 12 cultivars: Dietary fibre composition, antioxidant capacity and functional properties. *Food Chemistry*, *160*, 196-203.

Johanningsmeier, S. D., & Harris, G. K. (2011). Pomegranate as a functional food and nutraceutical source. *Annual review of food science and technology*, *2*, 181-201.

S, Josse J, Husson F (2008). "FactoMineR: A Package for Multivariate Analysis." *Journal of Statistical Software*, **25**(1), 1–18. doi:10.18637/jss.v025.i01.

Kaur, R., Aslam, L., Kapoor, N., & Mahajan, R. (2018). Phytochemical analysis and antioxidant activity of wild pomegranate collected from patnitop, Jammu and Kashmir. *Biosciences Biotechnology Research Asia*, *15*(2), 335-341.

Khodabakhshian, R., Emadi, B., Khojastehpour, M., Golzarian, M. R., & Sazgarnia, A. (2017a). Non-destructive evaluation of maturity and quality parameters of pomegranate fruit by visible/near infrared spectroscopy. International Journal of Food Properties, 20(1), 41-52.

Khodabakhshian, R., Emadi, B., Khojastehpour, M., & Golzarian, M. R. (2017b). Determining quality and maturity of pomegranates using multispectral imaging. *Journal of the Saudi Society of Agricultural Sciences*, *16*(4), 322-331.

Kulkami, A.P., Aradhya, S.M., 2005. Chemical changes and antioxidant activity in pomegranate arils during fruit development. Food Chem. 93, 319–324.

Li, H.W., Feng, S.Q. and Zhao, Y.M. 2004. Studies on correlation between the color and phenol content in the pericarp in "brumal jujube". Journal of Beijing Agricultural College 19(4):63-65.

Li, R., Chen, X. G., Jia, K., Liu, Z. P., & Peng, H. Y. (2016). A systematic determination of polyphenols constituents and cytotoxic ability in fruit parts of pomegranates derived from five Chinese cultivars. *SpringerPlus*, *5*(1), 1-9.

Magangana, T. P., Makunga, N. P., Fawole, O. A., & Opara, U. L. (2020). Processing factors affecting the phytochemical and nutritional properties of pomegranate (Punica granatum L.) peel waste: A review. *Molecules*, *25*(20), 4690.

Magangana, T. P., Makunga, N. P., la Grange, C., Stander, M. A., Fawole, O. A., & Opara, U. L. (2021). Blanching pre-treatment promotes high yields, bioactive compounds, antioxidants, enzyme inactivation and antibacterial activity of 'Wonderful'pomegranate peel extracts at three different harvest maturities. *Antioxidants*, *10*(7), 1119.

Mekni, M., Kharroubi, W., Flamimi, G., Garrab, M., Mastouri, M., & Hammami, M. (2018). Comparative study between extracts of different pomegranate parts issued from five tunisian cultivars (Punica granatum L.): Phytochemical content, volatile composition and biological activity. *Int. J. Curr. Microbiol. Appl. Sci*, *7*(5), 1663-1682.

Melito, S., La Bella, S., Martinelli, F., Cammalleri, I., Tuttolomondo, T., Leto, C., Fadda, A., Molinu, M. G., Mulas, M., 2016. Morphological, chemical, and genetic diversity of wild myrtle (Myrtus communis L.) populations in Sicily. Turkish J. Agric. Forestry. 40, 249-261. doi:10.3906/tar-1505-118

Mirdehghan, S. H., & Rahemi, M. (2007). Seasonal changes of mineral nutrients and phenolics in pomegranate (Punica granatum L.) fruit. *Scientia Horticulturae*, *111*(2), 120-127.

Moga, M. A., Dimienescu, O. G., Bălan, A., Dima, L., Toma, S. I., Bîgiu, N. F., & Blidaru, A. (2021). Pharmacological and therapeutic properties of Punica granatum phytochemicals: possible roles in breast cancer. *Molecules*, *26*(4), 1054.

Montefusco, A., Durante, M., Migoni, D., De Caroli, M., Ilahy, R., Pék, Z., ... & Lenucci, M. S. (2021). Analysis of the phytochemical composition of pomegranate fruit juices, peels and kernels: A comparative study on four cultivars grown in southern Italy. *Plants*, *10*(11), 2521.

Mphahlele, R. R., Caleb, O. J., Fawole, O. A., & Opara, U. L. (2016). Effects of different maturity stages and growing locations on changes in chemical, biochemical and aroma volatile composition of 'Wonderful'pomegranate juice. *Journal of the Science of Food and Agriculture*, *96*(3), 1002-1009.

Nian, H.L., Li, H., Cao, D.D., Cao, J.K. and Jiang, W.B. 2011. Determination of phenolic compounds in jujube peels at different maturity stages by high performance liquid chromatography. Journal of Beijing Forestry University 33(1):139-143.

Nikdel, K., SEIFI, E., Babaie, H., Sharifani, M., & Hemmati, K. (2016). Physicochemical properties and antioxidant activities of five Iranian pomegranate cultivars (Punica granatum L.) in maturation stage. *Acta agriculturae Slovenica*, *107*(2), 277-286.

Orak, H. H., Yagar, H., & Isbilir, S. S. (2012). Comparison of antioxidant activities of juice, peel, and seed of pomegranate (Punica granatum L.) and inter-relationships with total phenolic, Tannin, anthocyanin, and flavonoid contents. *Food Science and Biotechnology*, *21*, 373-387.

Pagliarulo, C., De Vito, V., Picariello, G., Colicchio, R., Pastore, G., Salvatore, P., & Volpe, M. G. (2016). Inhibitory effect of pomegranate (Punica granatum L.) polyphenol extracts on the bacterial growth and survival of clinical isolates of pathogenic Staphylococcus aureus and Escherichia coli. *Food chemistry*, *190*, 824-831.

Pareek, S., Valero, D., & Serrano, M. (2015). Postharvest biology and technology of pomegranate. *Journal of the Science of Food and Agriculture*, *95*(12), 2360-2379.

Re, G.A., Piluzza, G., Sanna, F., Molinu, M.G., Sulas, L., 2019. Polyphenolic composition and antioxidant capacity of legume-based swards are affected by light intensity in a Mediterranean agroforestry system. J. Sci. Food Agric. 99, 191-198. https://doi.org/10.1002/jsfa.9160

Robbins, M. P., Bavage, A. D., Strudwicke, C., & Morris, P. (1998). Genetic manipulation of condensed tannins in higher plants: II. Analysis of birdsfoot trefoil plants harboring antisense dihydroflavonol reductase constructs. Plant Physiology, 116(3), 1133-1144.

Saad, H., Charrier-El Bouhtoury, F., Pizzi, A., Rode, K., Charrier, B., & Ayed, N. (2012). Characterization of pomegranate peels tannin extractives. *Industrial crops and Products*, *40*, 239-246.

Salim, A., Deiana, P., Fancello, F., Molinu, M. G., Santona, M., & Zara, S. (2023). Antimicrobial and antibiofilm activities of pomegranate peel phenolic compounds: Varietal screening through a multivariate approach. *Journal of Bioresources and Bioproducts*, *8*(2), 146-161.

Schwartz, E., Glazer, I., Bar-Ya'akov, I., Matityahu, I., Bar-Ilan, I., Holland, D., and Amir, R. (2009). Changes in chemical constituents during the maturation and ripening of two commercially important pomegranate accessions. Food Chemistry, 115(3), 965-973.

Seeram, N. P., & Nair, M. G. (2002). Inhibition of lipid peroxidation and structure– activity-related studies of the dietary constituents anthocyanins, anthocyanidins, and catechins. Journal Agriculture Food Chemistry, 50, 5308–5312.

Sharma, A., & Thakur, N. S. (2018). Wild pomegranate (Punica granatum L.): A review on physical and chemical attributes of himalayan wild pomegranate fruit. *Journal of Pharmacognosy and Phytochemistry*, *7*(4), 1518-1524.

Singh, B., Singh, J. P., Kaur, A., & Singh, N. (2018). Phenolic compounds as beneficial phytochemicals in pomegranate (Punica granatum L.) peel: A review. *Food chemistry*, *261*, 75-86.

Singh, J. P., Kaur, A., Shevkani, K., & Singh, N. (2016). Composition, bioactive com- pounds and antioxidant activity of common Indian fruits and vegetables. Journal of Food Science and Technology, 53(11), 4056–4066.

Stojanović, I., Šavikin, K., Đedović, N., Živković, J., Saksida, T., Momčilović, M., ... Menković, N. (2017). Pomegranate peel extract ameliorates autoimmunity in animal models of multiple sclerosis and type 1 diabetes. Journal of Functional Foods, 35, 522–530.

Tehranifar A., Zarei M., Nemati Z., Esfandiyari B., Vazifeshenas M.R. 2010. Investigation of physico- chemical properties and antioxidant activity of twenty Iranian pomegranate (*Punica granatum* L.) cultivars. Scientia Horticulturae, 126: 180-185. Doi: 10.1016/j.scienta.2010.07.001

Wang, S.X., Liu, J.C., Jiao, Z.G., Zhang, S.N. and Yang, L. 2003. Changes of polyphenols during fruit development in apples. Journal of Fruit Science 20(6):427-431.

Zarei, M., Azizi, M., and Bashir-Sadr, Z. (2011). Evaluation of physicochemical characteristics of pomegranate (Punica granatum L.) fruit during ripening. Fruits, 66(2), 121-129.

Zhao, X., Yuan, Z., Yin, Y., & Feng, L. (2015). Patterns of pigment changes in pomegranate (Punica granatum L.) peel during fruit ripening. *Acta Hortic*, *1089*, 83-89.