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Chieti-Pescara**



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***“Innovative species-specific formulations for the balance of the
skin microbiota”***

Department of Pharmacy

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PhD student:

Sara D'Arcangelo

Coordinator:

Prof.ssa Assunta Pandolfi

Tutor:

Prof.ssa Mara Di Giulio

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1. INTRODUCTION

1.1 Skin microbiota

1.1.1 Skin: an overview

Skin is the largest organ of the human body that represents an interface with the outside environment. It can be considered as the first defensive line against external invasions that include chemical and biological factors, like environmental pollutants, chemicals and microorganisms. Skin is a dynamic organ composed by different type of cells and structures that work together to maintain the skin barrier function and to respond to environmental challenges. It is composed by 3 layers that, starting from outside to inside, are the epidermis (epithelial tissue, directly in contact with environment), dermis (connective tissue, responsible for the maintenance of skin structure) and hypodermis (adipose tissue that serves as a shock absorber and energy reserve for the body), as shown in **Figure 1** [1].

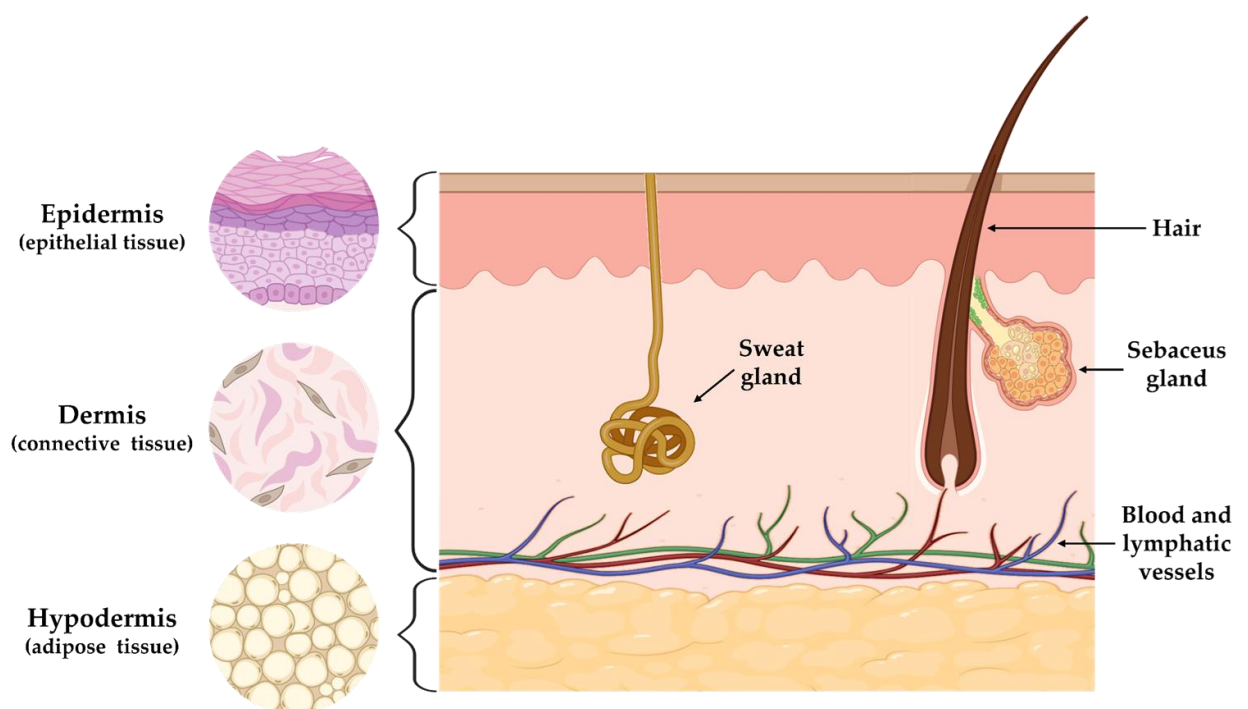


Figure 1. Skin layers (epidermis, dermis and hypodermis) and skin appendages (hair, sweat and sebaceous glands).

In details, the epidermis is made of epithelial tissue, characterized by the process of keratinization of keratinocytes born from stem cells present in the lowest part of epidermis. These cells begin their ascent from the basal layer to the stratum corneum, and in the meantime they proliferate and differentiate through a process of cytomorphosis that leads to the loss of the nucleus and the flattening of the cells, which once they arrive at the surface are eliminated by detachment. In addition to keratinocytes, in this layer are present melanocytes, responsible for skin pigmentation, and other types of cells that contribute to the skin protection such as Langerhans cells and Merkel cells. The second layer of the skin is the dermis that consists of connective tissue that led nutrients and oxygen to the epidermis through the circulatory and lymphatic stream. Here, it is possible to find the glomerulus and the glandular lobes of the sweat and sebaceous glands respectively, which secrete their excreta on the body surface. The third stratum is the hypodermis made of adipose tissue, which performs the function of body thermoregulation, energy stock and connects the skin to the muscle below [2] .

It is normally considered that the surface of the skin has an extension of about 2 m², but it was miscalculated. In fact, the epithelial area is not flat surface and it increases to 25 m² if it is considered all the skin appendages (about 5 million), that include sweat, sebaceous ducts and hair follicles [3], with specific functions: sebaceous glands can produce lipids that are able to retain intra-cell water, limiting the transepidermal water loss (TEWL), while sweat glands secrete antimicrobial peptides (AMPs) to limit the proliferation of pathogenic microorganisms [4,5].

1.1.2 Skin microbiota composition

Human is defined as a holobiont, which is a complex organism characterized by the symbiotic coexistence of organisms that do not share the same DNA but cooperate in a mutualistic manner [6]. Human body hosts about 3.8×10^{13} prokaryotic cells compared to 3.0×10^{13} estimated eukaryotic cells (considering a reference man of 70 kg), with protein-coding gene numbers 150-fold higher than the human genome (3.3 million prokaryotic genes vs about 19000 eukaryotic genes) [7]. Anyway, the mass of prokaryotic cells accounts for about 0.2 kg of the human weight, representing only 0.3% of the total body biomass [8]. Our skin hosts more than 200 genera of bacteria, representing the second organ of our body with the highest bacterial density (second to the gut), with a microbial density of 10^4 - 10^6 cells/cm² depending on the body site [9].

On the skin, there are four main dominant phyla represented by Actinobacteria (51.8%), Firmicutes (24.4%), Proteobacteria (16.5%) and Bacteroidetes (6.3%) [10].

Although the majority of the microbiome consists of generally stable-resident microorganisms, a smaller percentage of them are transient and can opportunistically colonize niches when the skin is compromised [11].

Peculiarities of the skin are the low amount of nutrients and the hostile conditions represented by exposure to ultraviolet radiation, high salinity, oxygen availability, temperature, low pH and sebum content. So, the limited resources and the unfavourable growth environment have made the skin microbiota specialized to thrive on the stratum corneum and at the level of sweat glands, sebaceous glands and hair ducts. This allows colonization that is strongly influenced by the physiology of the skin district, with the formation of peculiar and diverse microbial populations that thrive in niches with different characteristics [12], as it will be analyzed in chapter 1.1.5.

In **Table 1** and in **Figure 2**, it is possible to observe the different colonization of each body sites.

Table 1. Main abundant bacterial, eukaryotic and viral species that are present in healthy skin in different body sites [13].

Dry*	Moist‡	Sebaceous§	Foot
Bacteria			
<i>Propionibacterium acnes</i>	<i>Corynebacterium tuberculostearicum</i>	<i>Propionibacterium acnes</i>	<i>Corynebacterium tuberculostearicum</i>
<i>Corynebacterium tuberculostearicum</i>	<i>Staphylococcus hominis</i>	<i>Staphylococcus epidermidis</i>	<i>Staphylococcus hominis</i>
<i>Streptococcus mitis</i>	<i>Propionibacterium acnes</i>	<i>Corynebacterium tuberculostearicum</i>	<i>Staphylococcus warneri</i>
<i>Streptococcus oralis</i>	<i>Staphylococcus epidermidis</i>	<i>Staphylococcus capitis</i>	<i>Staphylococcus epidermidis</i>
<i>Streptococcus pseudopneumoniae</i>	<i>Staphylococcus capitis</i>	<i>Corynebacterium simulans</i>	<i>Staphylococcus capitis</i>
<i>Streptococcus sanguinis</i>	<i>Corynebacterium fastidiosum</i>	<i>Streptococcus mitis</i>	<i>Staphylococcus haemolyticus</i>
<i>Micrococcus luteus</i>	<i>Corynebacterium afermentans</i>	<i>Staphylococcus hominis</i>	<i>Micrococcus luteus</i>
<i>Staphylococcus epidermidis</i>	<i>Micrococcus luteus</i>	<i>Corynebacterium aurimucosum</i>	<i>Corynebacterium afermentans</i>
<i>Staphylococcus capitis</i>	<i>Enhydrobacter aerosaccus</i>	<i>Corynebacterium kroppenstedtii</i>	<i>Corynebacterium simulans</i>
<i>Veillonella parvula</i>	<i>Corynebacterium simulans</i>	<i>Corynebacterium amycolatum</i>	<i>Corynebacterium resistens</i>
Eukarya			
<i>Malassezia restricta</i>	<i>Malassezia globosa</i>	<i>Malassezia restricta</i>	<i>Malassezia restricta</i>
<i>Malassezia globosa</i>	<i>Malassezia restricta</i>	<i>Malassezia globosa</i>	<i>Trichophyton rubrum</i>
<i>Aspergillus tubingensis</i>	<i>Tilletia walkeri</i>	<i>Malassezia sympodialis</i>	<i>Malassezia globosa</i>
<i>Candida parapsilosis</i>	<i>Malassezia sympodialis</i>	<i>Aureoumbra lagunensis</i>	<i>Pyramimonas parkeae</i>
<i>Zymoseptoria tritici</i>	<i>Pyramimonas parkeae</i>	<i>Tilletia walkeri</i>	<i>Trichophyton mentagrophytes</i>
<i>Malassezia sympodialis</i>	<i>Parachlorella kessleri</i>	<i>Pycnococcus provasolii</i>	<i>Parachlorella kessleri</i>
<i>Epidermophyton floccosum</i>	<i>Aspergillus tubingensis</i>	<i>Gracilaria tenuistipitata</i>	<i>Aspergillus tubingensis</i>
<i>Pyramimonas parkeae</i>	<i>Zymoseptoria tritici</i>	<i>Pyramimonas parkeae</i>	<i>Zymoseptoria tritici</i>
<i>Nannizzia nana</i>	<i>Nephroselmis olivacea</i>	<i>Parachlorella kessleri</i>	<i>Gracilaria tenuistipitata</i>
<i>Parachlorella kessleri</i>	<i>Cyanophora paradoxa</i>	<i>Leucocytozoon majoris</i>	<i>Nephroselmis olivacea</i>
Viruses			
<i>Molluscum contagiosum virus</i>	<i>Molluscum contagiosum virus</i>	<i>Propionibacterium phage</i>	<i>Propionibacterium phage</i>
<i>Propionibacterium phage</i>	<i>Propionibacterium phage</i>	<i>Molluscum contagiosum virus</i>	<i>Merkel cell polyomavirus</i>
<i>Merkel cell polyomavirus</i>	<i>Polyomavirus HPyV6</i>	<i>Merkel cell polyomavirus</i>	<i>Alphapapillomavirus</i>
<i>Polyomavirus HPyV7</i>	<i>Merkel cell polyomavirus</i>	<i>Polyomavirus HPyV6</i>	<i>Human papillomavirus (μ)</i>
<i>Acheta domestica densovirus</i>	<i>Polyomavirus HPyV7</i>	<i>Human papillomavirus (γ)</i>	<i>Human papillomavirus (β)</i>
<i>Human papillomavirus (β)</i>	<i>Human papillomavirus (β)</i>	<i>Human papillomavirus (β)</i>	<i>Pseudomonas phage</i>
<i>Actinomyces phage</i>	<i>Acheta domestica densovirus</i>	<i>Acheta domestica densovirus</i>	<i>Staphylococcus phage</i>
<i>Simian virus</i>	<i>Human papillomavirus (γ)</i>	<i>Staphylococcus phage</i>	<i>RD114 retrovirus</i>
<i>Streptococcus phage</i>	<i>Staphylococcus phage</i>	<i>Gammapapillomavirus HPV127</i>	<i>Molluscum contagiosum virus</i>
<i>Stenotrophomonas phage</i>	<i>Actinomyces phage</i>	<i>Enterobacteria phage</i>	<i>Stenotrophomonas phage</i>

*Hypothenar palm, volar forearm. ‡Nare, antecubital fossa, inguinal crease, interdigital web, popliteal fossa. §Alar crease, cheek, glabella, external auditory canal, manubrium, retroauricular crease, occiput, back. ||Toe web space, toenail, plantar heel.

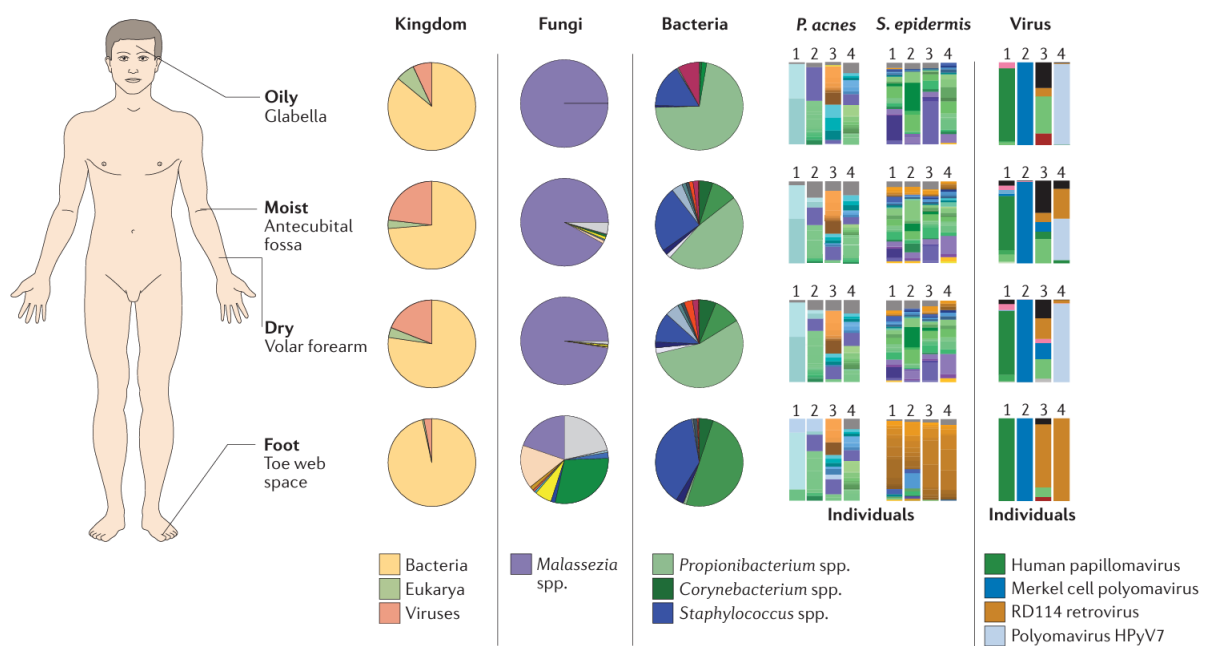


Figure 2. Main skin microbial communities influenced by skin physiological characteristics (oily, moist, dry, foot). Their relative abundances are represented by pie charts. Bar charts displayed *C. acnes* and *S. epidermidis* eukaryotic DNA viruses [13].

1.1.3 Factors influencing diversity

The skin microbiome evolves throughout life in response to the body's physiological changes and external factors. In fact, skin microbial diversity is influenced by a multitude of intricate factors, including both intrinsic (internal) and extrinsic (external) factors [14].

Anyway, it should be considered that the birth represents the most significant environmental transition in life, as the newborn exits from the protective amniotic sac and coming to contact a vast array of microorganisms for the first time [15]. Therefore, this phase represents a crucial point in the formation of everyone's microbiota.

- Birth: vaginal delivery vs c-section

Undoubtedly, the crosstalk among maternal immune cells, dietary antigens, antibodies and metabolites produced by the maternal microbiota, play a pivotal role in equipping the newborn's immune system. In fact, the immune system's development is closely influenced

by pregnancy, birth and breastfeeding, conditions that interact with the microbiota of both mother and infant. These early microbial community can influence the infant's immune system development and may have long-term health implications in terms of disease susceptibility, potentially increasing the risk for conditions such as metabolic syndrome, childhood asthma, susceptibility to allergies and autoimmune type 1 diabetes [15].

Babies born via vaginal delivery are exposed and colonized by their mother's vaginal and faecal microorganisms, resulting in a gut microbiota that is rich in *Bifidobacteria*, *Lactobacillus* and *Bacteroides* species. Conversely, infants delivered by caesarean section primarily acquire microorganisms from the mother's skin and the hospital environment, leading to a skin microbiota dominated by skin-associated bacteria such as *Staphylococcus*, *Streptococcus*, *Clostridium* and *Corynebacterium* [16,17]. The initial colonization sets the stage for the skin microbiome's evolution, which continues to change and diversify with intrinsic and extrinsic factors.

- Intrinsic factors

Within intrinsic factors there are: age, sex and skin type.

1. **Age:** Age-related changes also influence skin microbial diversity; in particular hormonal changes during puberty can alter sebum production and skin physiology, influencing microbial colonization patterns [18]. Specifically, excessive sebum production has been associated with an overgrowth of the commensal bacteria *Cutibacterium acnes* associated with reduction in strain diversity; it could cause an unbalance in the skin microbiota, which can lead to acne, an inflammatory condition of the pilosebaceous unit [19]. In elderly people, changes in skin structure, reduced sebum production and alterations in immune function can lead to shifts in microbial diversity and composition [20]. In fact, an analysis of sebocytes, natural moisturizing factors, skin lipids, and antimicrobial peptides

revealed age-related changes. Specifically, sebocyte area decreased with age, while antimicrobial peptides, natural moisturizing factors and skin lipids showed an increase [21]. These changes are correlated with shifts in specific bacterial genera. The analyses revealed also that bacterial diversity increased across all sites as age advanced [21].

2. **Sex:** There are a lot of biological, physiological and behavioural sex-related factors that influence skin microbiota composition, especially regarding hormonal fluctuations. For example, the female skin microbiome is characterized by a higher species diversity than males [20]. Recent evidence indicates that androgens, like testosterone, may have a dual role in the sebaceous gland, not only by stimulating sebum production to support *C. acnes* colonization but also by directly promoting inflammation [22]. Sebocytes, which have functional androgen receptors, can undergo lipogenic differentiation in response to androgens. This process increases both sebum output and the production of pro-inflammatory cytokines within sebocytes, linking androgens more closely to inflammatory pathways in the skin [23]. Estrogen and testosterone influence also immune cell activity, cytokine production, and antimicrobial peptide secretion, all of which can impact the composition and resilience of the skin microbiota [24]. In a recent study on Korean healthy patients, a lot of differences in facial skin microbiota composition emerged. In particular, it was found that *Staphylococcus* and *Corynebacterium* species were more prevalent in males, while *Lactobacillus* showed greater abundance in females [25]. The interactions between the immune system, hormonal balance and the skin microbiome are intricate and complex, and it was invented the word “microgenderome” to indicate this sexual dimorphism in microbiome [26,27].

3. **Skin Type:** The properties of different skin types (oily, dry, combination, sensitive) create distinct environments that support specific microbial communities. Sebum

production, hydration levels, and pH can all vary with skin type [28]. For example, Staphylococci, like *S. aureus* and *S. epidermidis*, are well-adapted to the acidic pH of oily skin and secrete lipases to metabolize the lipid-rich environment found in these areas. This adaptation allows them to thrive in sebaceous regions, where they utilize skin lipids as a nutrient source [29].

- Extrinsic factors

There is an increasing number of researches that analyze the effects of extrinsic factors such as hygiene practices, diet, environment, lifestyle and medical treatment, on the composition of healthy subjects [30].

1. **Hygiene Practices:** Hygiene practices, including for example the frequency of washing, the use of moisturizing products and cosmetics, can influence microbial diversity. Generally, different studies demonstrated that acute exposure to antiseptic, such as alcohol-based hand sanitizers, ethanol and povidone-iodine, can cause rapid but short-term depletion of the resident microbial community [31,32]. However, in a recent study regarding the role of everyday cosmetics in the alteration of the skin microbiome, it was underlined that high microbial diversity is associated with healthy skin and the excessive use of skin cleaning products can negatively affect the hydration, the TEWL, as well as the skin microbial composition, detecting a correlation between synthetic ingredients of topical formulations and microbial diversity [33]. So, while regular hygiene is essential for personal cleanliness, excessive use of synthetic agents can disrupt the natural balance of the skin microbiota, potentially leading to cutaneous alterations.

2. **Diet:** Since there is a correlation between skin and gut microbiome (skin-gut axis), is easy to understand that dietary habits can indirectly affect the skin microbiota. In recent research by Min et al, it was underlined how a well-balanced plant-based diet can carry out

beneficial effects on inflammatory skin conditions and in wound healing, thanks to anti-inflammatory, probiotic, and antioxidant properties probably due to skin microbiome modulation [34]. On the opposite, it was demonstrated that an high-fat diet in mice led to an alteration of sebum composition and production that is associated with an increase in *Corynebacterium* [35]. Moreover, recent findings highlighted that omega-3 fatty acids like alpha-linolenic, eicosapentaenoic and docosahexaenoic acids can influence the skin barrier by immune modulation, filaggrin expression increase and by improving keratinocyte differentiation [36].

3. **Environment:** Environmental factors such as climate, exposure to pollutants and to UV radiation (UVR) can play a pivotal role in the modulation of skin microbiome [37]. In particular, temperature and humidity are recognized for increasing skin bacteria growth overall. In particular, an *in vivo* study of the United States population, showed an increase in MRSA (a major cause of skin and soft tissue infections) infection in September, suggesting that the high temperature promotes bacterial growth [38]. On the opposite, a temperature decrease has been associated with an advantage of CoNS, in particular of *S. epidermidis*, over *S. aureus* growth, suggesting that environmental temperature are correlated to bacterial competition and balance [39]. Interestingly, also a seasonal variation in acne vulgaris was detected in an old study, as an increase of 1% in temperature was associated with an increase of 10% in sebum production and consequently to major colonization of lipophilic bacteria such as *C. acnes* and *Malassezia spp.* [40]. Like temperature, also the increase of humidity was positively correlated to an increase in bacterial growth since wetted skin promotes microbial colonization compared to dry skin [37].

The effects of UV radiations, both UVA and UVB, on the skin microbiome could occur through both direct and indirect processes. In fact, UVR may act indirectly by altering the microbial habitat of exposed skin, consequently leading to a change in the skin microbiota composition [41]. A wide variation was found immediately and 24 h after UV radiation, specifically an increase in *Cyanobacteria* and a decrease in the *Lactobacillaceae* and *Pseudomonadaceae* families were detected, qualitatively and quantitatively affecting the composition of the skin microbiome [42].

Pollutants present in higher concentrations in urban environment are mainly composed by polycyclic aromatic hydrocarbons can be absorbed through the skin (as well as inhaled and ingested), penetrating in the human body. Long-term exposure to these substances favour changes in the metabolic capacity of skin microbiome, potentially increasing the pathogenicity [43]. Also other pollutant, like nitrogen and ozone, can cause oxidative stress and can create skin dysbiosis due to their capability to affect skin commensal, such as *S. capitis* and *C. tuberculosaricum*, while *S. aureus* is less sensitive to these molecules inducing barrier dysfunction [44].

4. **Lifestyle:** Activities that expose the skin to different environments and substances can affect the microbial communities. Physical activity and sweating can also alter the skin environment so much so that the concept of “athletic microbiome” has been introduced. In fact, several exercise-related variables, such as training volume and intensity, have been found to impact the athlete microbiome (both gut and skin microbiome) in terms of composition and diversity [45]. In particular contact sports, can influence the presence of some species; for example, in wrestlers, it was detected an increase in skin bacterial colonization, associated also the presence of haemolytic forms of opportunistic bacteria in cutaneous microbiota [46].

It's important to take into account that also the surrounding environments, including the cohabitation with other individuals, is a factor influencing diversity, considering both family members [47] and also the presence of animals like dogs living inside the house [48]. In this study, it was highlighted that dog ownership was characterized by a significant increase in the shared skin microbiota in cohabiting adults and, moreover, humans shared some skin strains with their dog in respect to other dogs.

5. **Treatments:** Since antibiotic therapies often use broad-spectrum drugs, in addition to pathogenic bacteria that cause a specific condition, there is a reduction of beneficial and protective bacteria for our body. Unfortunately, the misuse of antibiotics is becoming increasingly spread. For example, in a recent study about systemic acne treatment. There was detected a correlation between the therapy and changes in the composition and diversity of skin population [33].

1.1.4 Interaction within the microbial community

The microbial communities on the skin are shaped by continuous interactions, including competition for resources, cooperative behaviours and intricate communication networks [49]. These dynamic relationships are fundamental to form a balanced ecosystem that protects against disease and supports the health and functions of the skin.

- Competition

Competition among skin microorganisms is a critical factor that shapes microbial community structure. Different microorganisms fight for limited resources such as nutrients, space and access to host tissues, leading to the exclusion of less competitive species and the dominance of others [50]. Some dominant skin commensals are able to occupy specific ecological niches, through self-adaptation and microbe–microbe interactions [51].

1. **Nutrient Competition:** Due to skin is a poor-nutrient habitat and it is continuously exposed to the transient microbiota coming from the external environment, resident skin microorganisms undergo to a high nutrient competition to utilize the limited nutrients available on the skin, which include proteins, lipids, and other host-derived molecules, to support their growth and supplement microbial metabolism [13,52]. Bacteria compete for nutrients, like sebum and sweat. For instance, thanks to its ability to produce degradative enzymes such as lipases, sialidases, and hyaluronidase, that allow to metabolize lipids in sebum, *C. acnes* thrives in sebaceous areas, outcompeting other bacteria that cannot utilize these resources as efficiently [53].
2. **Antimicrobial Production:** Between commensal skin bacteria, different CoNS contribute to host defence by producing bioactive molecules, that selectively target harmful microorganisms, disrupting their cellular integrity or inhibiting essential functions, preventing the overgrowth of pathogens. In particular, *S. epidermidis*, produces bacteriocins like Epidermin and Epilacin K7, that affect the growth of MRSA [54] and also different SCFAs, as fermentation metabolites, such as acetic, butyric, lactic and succinic acid that can suppress the growth of pathogenic *S. aureus* [55] and *C. acnes* [56]. Moreover, different studies demonstrated that these antimicrobial molecules act also against bacterial sessile phase, represented by the biofilm structure [57]. The commensal bacterium *S. hominis* produces a bacteriocin called MP1 that influence the growth of MRSA and different novel AIPs which can inhibit Agr systems of other species, including *S. aureus*, leading to reductions in virulence factor gene expression and pathogenesis [52,58]. In addition, another CoNS, *S. capitis*, is able to produce bacteriocins (Capidermicin, Nisin J) and phenol soluble modulins (PSMs) that display inhibitory activity against a wide range of bacterial targets, like *S. aureus* and *C. acnes* strains [59–61]. Also the commensal *S. lugdunensis*

produce a bactericidal agent, lugdunin, that is a novel thiazolidine-containing cyclic peptide antibiotic that inhibit colonization by *S. aureus* [62]. These competitive strategies are essential for preserving microbial balance by limiting the overgrowth of potentially harmful pathogens, thereby supporting a stable and healthy skin microbiome.

3. **Physical Competition:** Microorganisms also compete for physical space on the skin surface [63]. They are able to synthesize substances that regulate aggregation with each other. In particular, extracellular polymeric substances (EPS) play a pivotal role in the microorganisms adhesion and cohesion, establishing interactions within microbial community and influencing spatial organization. EPS stabilize these microbial clusters through mechanical interactions like hydrogen bonds, dispersion forces and electrostatic bonding, forming a tri-dimensional structure that allow the microorganisms to be retained near each other [64]. Moreover, they also undergo to contact-dependent growth inhibition (CDI), a microbial growth mechanical inhibition system designed to achieve direct physical contact of a bacterial cell with other cells in close proximity through receptor-mediated toxin delivery [65].

- Cooperation

Cooperation among skin microorganisms includes different forms of mutualistic interactions where they work together to enhance their survival and colonization [66].

1. **Metabolic Cooperation:** Metabolic interactions among microorganisms are essential ecological processes in which different species exchange nutrients, metabolites or by-products to support each other's growth. These exchanges can be unidirectional, if only one microorganism uses another's metabolic by-products, to bidirectional/cooperative exchanges in which both partners share resources and essential compounds [67]. Such cross-feeding promotes interdependence and stability in microbial communities, helping them to

survive in different environments, creating a cooperative microbial network. For example, *C. acnes* promote lipid synthesis within human skin keratinocytes, important for the skin's structural integrity and protective function; moreover, it metabolize sebum free fatty acids decreasing in this way the skin pH, creating an acidic environment that helps to inhibit pathogenic bacteria such as *S. aureus* while favouring commensal bacteria also limiting an overabundance of beneficial ones [68,69].

2. **Biofilm Formation:** A biofilm is a structured community of microorganisms embedded in a self-produced extracellular matrix called extracellular polymeric matrix (EPS), composed by polysaccharides, lipids, proteins and extracellular DNA, which helps them to survive under unfavourable conditions [70]. Biofilm formation occurs in four stages, as displayed in **Figure 3**. First, free microorganisms attach to a surface through weak, reversible interactions. Next, they form irreversible attachments, anchoring firmly and beginning to produce an extracellular matrix. Finally, the biofilm matures, growing in size and complexity, developing channels that allow nutrients and wastes to flow through the microbial community. Finally, in the dispersal stage, single cells or cell aggregates break away to colonize new areas, further spreading the biofilm community [71].

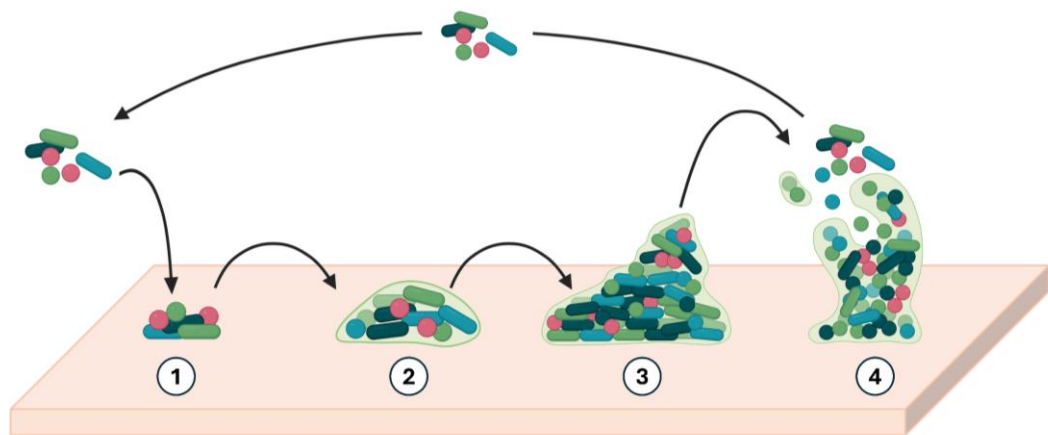


Figure 3. Biofilm formation stages. 1: Adhesion; 2: Formation; 3: Maturation; 4: Dispersion.

The transition from planktonic cells to sessile phase starts with the attachment of microorganisms to a biotic or abiotic surface [72]. Within biofilms, microbes can exchange genetic material, enhancing their adaptability and resilience, protecting them from external threats, including antibiotics and host immune system [73,74]. Different bacterial species isolated from human skin have demonstrated their ability to form biofilms both *in vitro* and *in vivo* and, in this condition, they may be involved in the etiology and exacerbation of skin disorders [75]. For example, it was demonstrated that there is a correlation between microbial biofilm and skin disease. In particular, in atopic dermatitis flares *S. aureus*-associated biofilm, represents the major colonizer of the skin lesions, with a direct association with disease severity [76]. Also *C. acnes*, that is an opportunistic pathogen of the skin, can cause the *acne vulgaris*, a common puberty-related skin inflammatory disease. This bacterium can form biofilm inside hair and sweat ducts, inducing inflammation and skin disorders [77,78].

- Communication

Microbial communication is essential for coordinating complex behaviours and maintaining the stability of the community. This communication can occur mainly through chemical signalling of quorum sensing and through gene exchange.

1. **Quorum Sensing:** Quorum sensing is a cell-to-cell communication mechanism that enables bacteria to respond to changes in population density. This process consists in the production, release and detection of signalling molecules called autoinducers [79]. Each microbial species produces specific types of autoinducers and as microbial density increases, these molecules accumulate in the external environment. When the concentration of autoinducers reaches a critical threshold, a signalling cascade is triggered that leads to changes in gene expression by promoting coordinated behaviours [80]. This synchronized

response allows bacteria to perform functions that would be ineffective if performed by individual cells. For example, quorum sensing is critical for the formation of biofilms, in which bacteria work together to produce an extracellular matrix, creating a protected community structure [81]. In pathogenic bacteria, quorum sensing controls the expression of virulence factors, allowing the population to launch a collective infection strategy only when their numbers are sufficient to overwhelm host defences [82].

2. **Gene Exchange:** Horizontal gene transfer (HGT) is a communication and cooperation process in which microorganisms acquire genetic material from non-progenitor cells, allowing to the rapid spread of new traits between species, such as antibiotic resistance, metabolic pathways and virulence factors [83].

There are three main mechanisms of HGT: transformation, transduction and conjugation. In transformation, bacteria take up free DNA from the environment, often released from dead cells. This DNA can integrate into the genome of the recipient, conferring new characteristics. In transduction, bacteriophages, viruses that specifically infect bacteria, transport DNA from one bacterial cell to another, introducing new genes. Conjugation involves direct cell-to-cell contact, typically facilitated by the formation of a pilus bridge, through which plasmids, small circular DNA molecules, are transferred. Plasmids often carry genes for antibiotic resistance or other survival-enhancing functions, allowing these traits to spread rapidly within bacterial communities [84].

1.1.5 Localization and distribution

Skin microbiota varies significantly across different microenvironments of the body, influenced by specific physical and chemical conditions that create distinct habitats for microbial communities [85].

One of the primary factors influencing the microbial ecology of the skin is the pilosebaceous unit, comprising the hair follicle and the sebaceous gland which is connected to it [86]. These glands secrete sebum, a waxy, oily substance that softens the skin and encourages the growth of microbes that can utilize its nutrients and thrive in lipid-rich environments.

The sebaceous areas are mainly represented by the face (nose, forehead and chin) and upper chest, are characterized by low oxygen levels that provides a suitable habitat for *Cutibacterium acnes*, a facultative anaerobe that, thanks to its ability to metabolize sebum lipids, producing fatty acids and glycerol, can thrive in this environment. This acidic environment is hostile for a lot of pathogenic bacteria, thus preventing their colonization and maintaining a balanced microbial ecosystem. [87,88].

Moist areas, such as the armpits, groin and between the toes, host a microbial community that is different from other regions, due to the higher humidity and temperature, which create a favourable environment for species like *Corynebacterium* and *Staphylococcus spp.* [89]. Fungi, such as *Malassezia spp.*, are also prevalent in these areas contributing to the skin ecosystem by metabolizing sebum and other lipids. While these fungi are usually harmless, when there is a skin microbial imbalance, they can cause conditions like seborrheic dermatitis or fungal infections [90].

Dry areas such as the forearms, legs and hands, are characterized by lower humidity and fewer sebaceous glands, creating a specific environment that show lower variability compared to moist or sebaceous regions. The dominant bacteria in these areas are often Gram-positive species such as *Staphylococcus* and *Micrococcus* [91]. *Staphylococcus epidermidis* is a common resident belonging to the group of Coagulase Negative Staphylococci (CoNS) that plays a pivotal role in protecting the skin by outcompeting

potential pathogens, producing antimicrobial peptides and contributing to skin barrier homeostasis by generating protective ceramides [92,93].

The unique conditions of each skin region influence microbial colonization and the disruptions of these microbial community, can lead to dysbiosis and potentially contribute to complex skin issues.

In dermatological research and clinical practice, targeting the microbiota specific to each skin microenvironment offers potential advantages for developing specific treatments that promote microbial balance and enhance skin health.

1.1.6 Role in health and skin integrity

The skin microbiota represents an external shield that is in close contact with the upper layers of epidermis. All the interactions between microorganisms and human cells, play a pivotal role in the maintenance of the integrity and the health of the skin through several functions. Different data suggest that the resident microbiota can influence skin permeability and therefore physical barrier, through different interactions involving mainly gene expression and metabolic activity.

- Keratinocyte differentiation and cell junctions modulation

The outermost layer of the epidermis is represented by stratum corneum (SC), consisting in corneocytes, programmed-manner dead cells resulting as the terminal differentiation process of keratinocytes. Living keratinocytes below, are strictly connected to each other by different type of intracellular junctions such as desmosomes, gap, adherent junctions and tight junctions (TJs). Since SC and TJ are represent the first interaction for drug delivery and in skin absorption, their regulation is fundamental to the maintenance of the integrity and functions of the skin [94,95]. TJs consist of about 40 different proteins, which mainly have the functions of transmitter or adaptor and belong to the claudin (CLDN),

occludin and junctional adhesion molecule families of proteins, and their composition can change dynamically depending on different factors [96]. Microorganisms, for example, can affect the CLDN content of TJs, probably caused by toll-like receptors (TRL) activation. A recent study showed that *C. acnes* can change the expression of the different type of CLDN [97]. In addition, an investigation regarding the effect of *Staphylococcal* strains on tight junctions showed underlined that the pathogenic *S. aureus* time and concentration dependent manner downregulation of TJ, adherent junctions, desmosomal proteins and protein kinase C, that represents an essential player in TJ formation, while the non-pathogenic commensal *S. epidermidis* showed minor effects [98].

The skin integrity depends also by the maturations and desquamations of epithelial cells, process that are regulated by different genes mainly belonging to epidermal differentiation complex (EDC) including filaggrin, involucrin and loricrin, and microbiota can influence their expression [99]. *In vitro* and *ex vivo* studies on keratinocyte cultures reported that *C. acnes*, a member of balanced skin microbiota, increase the expression of several differentiation marker, playing an important role in maintaining an intact skin barrier [100,101]. At the opposite, the pathogenic *S. aureus*, was found to inhibit keratinocyte differentiation by interfering with p63 functions through lipoteichoic acid [102].

- Metabolic process

In addition to gene expression regulation, metabolic activities of skin microbiota are crucial for maintaining skin health. Upper layers are composed by skin surface lipids (SSL), a three-dimensional structure called lipid lamellae, which support permeability, antimicrobial and antioxidant barrier. They are mainly composed from ceramides, that can be synthesized *de novo* by lipolytic action of some skin commensals. *Staphylococcus epidermidis* express a sphingomyelinase, an enzyme able to catalyze ceramides formation,

through breaking sphingomyelin (a lipid found on the surface of cells), into ceramides and phosphocholine (PC), a nutrient source for the bacteria [92]. Another component of the SSL is represented by the sebum, that is made of triglycerides or triacylglycerol lipids and is produced in sebaceous glands. Sebum can be used as nutrient sources by lipases-producing microorganisms, that are able to hydrolyse triglycerides in free fatty acids, mono and diacylglycerols, and free glycerol. *Cutibacterium acnes*, as well as *Corynebacterium* spp., is capable of this degradation thanks to different enzymes such as lipases, proteases, hyaluronidases, acid phosphatases and sphingomyelinases [103]. Free fatty acids maintain a low pH, which has a dual function: on one hand it creates a chemical environment that is hostile to pathogens, and on the other hand it enhances skin immunity by stimulating the expression of one of the most abundant skin antimicrobial peptides (AMPs), the human-defensin 2 (hBD-2) [104]. Moreover, some microorganisms are directly inhibited by the product of microbial lipidic metabolism, such as sapienic acid that can inhibit pathogenic *S. aureus*, suggesting a role of this cutaneous antimicrobial lipid in the selection of cutaneous staphylococci [105].

- Wound healing

Wound healing is an elaborate and dynamic process that can be divided into four coordinated overlapping phases: haemostasis, inflammation, proliferation and remodelling [106]. The skin microbiome has a critical role in modifying these steps, through the interplay with the immune system and with the surrounding tissue. In fact, interactions between commensal microbiota and different skin cell types such as keratinocytes, fibroblasts and immune cells, involved in cutaneous wound healing, thanks to the modulation of immune response and supporting the barrier repair [107].

Recent studies showed that wounds healing is supported by beneficial microorganisms, by interaction with host immune system; healthy skin-associated commensals show high diversity, and they are involved in local T cell-mediated immune response, which promotes tissue repair [108]. They activate neutrophils which express the chemokine CXCL10, that recruits interferon-producing plasmacytoid dendritic cells (pDC) and kills the exposed bacteria, and they induce IFNs type representing playing a pivotal role in the activation of fibroblasts and macrophages, producing tissue growth factors [109,110]. In particular, the commensal *S. epidermidis* is able to induce CD8⁺ T cells and consequently induce re-epithelialization and tissue granulation after skin damage [111]. Moreover, it is able to limit inflammation after skin injury, thanks to TLR signalling and lipoteichoic acid production, promoting wound healing by favouring the transition from the inflammation phase into the proliferation phase [112]. *Staphylococcus epidermidis* can also protect the host from invasion of other pathogenic bacteria, by inducing the keratinocytes expression of AMPs [113].

On the opposite, bacterial colonization of wounds often leads to the formation of biofilms, which interfere with healing by triggering the activation of pro-inflammatory neutrophils and macrophages. This process leads to the release of pro-inflammatory cytokines, cytolytic enzymes and reactive oxygen species, creating a prolonged inflammatory state [114]. The dysregulated immune response in chronic wounds promotes bacterial proliferation, perpetuating biofilm growth and prolonged inflammation [115]. The main bacterial genera involved include the Gram-negative *Acinetobacter*, *Enterobacter* and *Pseudomonas* and *Staphylococcus*, *Streptococcus* and *Enterococcus* as Gram-positive [116].

Pathogenic strains associated with skin alterations such as *S. aureus*, can produce pore-forming toxins, including alpha toxin and the leukotoxins, which damage immune

cells, inducing a local pro-inflammatory response, increasing vascular permeability and facilitating the bacterial migration in wounds where they form biofilm, affecting healing outcomes [117].

Recent findings indicate that sustaining a healthy skin microbiome, or its restoration, can lead to improved wound healing process outcomes. Different strategies, including local administration of probiotics, prebiotics, postbiotics or natural extracts, appear as promising contributions for commensal skin microorganisms, limiting pathogenic overgrowth [109]. This topic will be discussed in the Chapter 1.2.

1.1.7 Eubiosis vs Dysbiosis

The community of microorganisms that constitute the skin microbiome, plays an essential role in maintaining skin health by interacting with the immune system, protecting against pathogenic colonization and supporting barrier integrity.

These microorganisms interplay with the human body through different type of interactions that can be neutral, beneficial or harmful for the host. Neutralism is expressed by saprophytism, in which both microorganisms and the human body coexist without influencing each other. Instead, in commensalism, while bacteria benefit from these interactions, the host stays neutral from them; when both take advantages from these interactions, the relation is defined mutualism. Finally, parasitism represents a harmful relationship in which microbial community thrive at the expense of the host, utilizing its biological systems and eventually causing cells damage [118].

The skin microbiota can maintain a state of equilibrium, called eubiosis, in which the microbial community can synergistically interact with the host, supporting a homeostatic condition in terms of immune and barrier integrity [119]. Commensals can contribute to this balance by producing antimicrobial peptides, modulating immune responses and

metabolizing lipids to reinforce the skin structural barrier, preventing the overgrowth of pathogenic species and maintaining a protective and stable environment. In particular *C. acnes*, a skin commensal widely distributed across different skin sites, is able to produce cutimycin, a thiopeptide antibiotic that can limit Staphylococcal colonization of hair follicle, significantly contributing to bacterial community composition [120]. As well as *S. epidermidis*, different CoNS play a pivotal role in the eubiosis maintenance. It is important to mention *S. lugdunensis* that produce a novel thiazolidine-containing cyclic peptide antibiotic named lugdunin, that inhibit the colonization by *S. aureus*, without leading to development of bacterium resistance [62]. A recent study highlights that *S. hominis* secrete a strain-specific AMP that selectively killed *S. aureus*, also synergizing with the human AMP LL-37 [121]. In addition, during skin colonization, *S. capitis* and *S. caprae* provided protection competing with *S. aureus* by inhibiting agr-mediated quorum sensing, necessary for the pathogenicity of this strain [122]. These data suggest that the bacterial-derived AMPs should be considered as a component of the balanced skin immune defence system.

In the opposite hand, dysbiosis represents a disruption in the balanced composition and functionality of microbiota, generally resulting in decreasing in microbial diversity and increasing in microbial load, with the proliferation of pathogenic strains, the depletion of beneficial species and the loss of microbiome site-specificity [123]. This state can be triggered by factors such as environmental threats, antibiotic misuse, immune dysfunction or systemic conditions, resulting in association with various inflammatory skin disorders, that will be discussed in Chapter 1.1.7.

The consequences of dysbiosis are extend beyond local effects, as it influences also immune activation and systemic inflammation [124]. Infections become more frequent, because pathogens take advantages of disrupted microbial barriers, while the ability of the

skin to repair itself is compromised due to chronic inflammation and impaired wound healing process. Moreover, pathogens often show high virulence through biofilm formation, which protect themselves from host immune responses and antimicrobial treatments, leading to complex clinical outcomes [114].

For these reasons, restoring microbial balance has become a focus in dermatological research, searching for novel and alternatives therapeutical strategies such as probiotics, prebiotics, postbiotics, natural compounds, targeted antimicrobials and microbiome-friendly skincare formulations aim to reestablish microbial diversity and functionality. Also advanced treatments, like bacteriophage therapy and microbiome transplantation, have been explored for their potential to restore dysbiotic states. The distinction between eubiosis and dysbiosis highlights the pivotal role of the skin microbiota in health and disease, underscoring its importance as a target for innovative therapies that promote cutaneous and systemic well-being.

- *Staphylococcus epidermidis* vs *Staphylococcus aureus*

Staphylococcus epidermidis and *S. aureus* are two significant bacterial species commonly founded on human skin; despite sharing the same genus, these bacteria have markedly different interactions with the host and with the skin environment. The interaction between commensals, such as *S. epidermidis* and pathogenic strains, like *S. aureus* on the skin is a key factor in determining the overall health of the skin microbiome [121].

Staphylococcus epidermidis is a predominant and beneficial inhabitant of dry skin areas, thriving thanks to its symbiotic relationship with the human host, playing a pivotal role in maintaining skin health and integrity. As previously analyzed in Chapter 1.1.3.1, it produces a variety of antimicrobial peptides and interfere with *S. aureus agr* system, counteracting the colonization and growth of pathogenic microorganisms. In particular, *S.*

epidermidis produce phenol soluble modulins γ and δ that cause membrane damages in *S. aureus* cells and it produces specific serine proteases that interfere with *S. aureus* biofilm formation [125].

In addition to its antimicrobial properties, *S. epidermidis* helps modulate the skin's immune responses by the activation of gamma delta (GD) T cells, the most expressed T cell type in epithelial tissues and it upregulates the production of perforin-2 (P-2), an antimicrobial protein able to kill intracellular bacteria, increasing the ability of skin cells to counteract *S. aureus* colonization [126]. It interacts with keratinocytes, the predominant cell type in the outermost layer of the skin, to promote the production of cytokines and other signalling molecules that help to maintain a balanced immune environment. This modulation is essential for preventing excessive inflammation, which can damage skin tissues and disrupt the microbiota. Moreover, this bacterium is essential for the reduction of TEWL and in the enhance of stratum corneum ceramide content, fundamental factors for the maintenance of a healthy skin [92]. Finally, to these functions, *S. epidermidis* can metabolize specific substrates, like glycerol, to produce short-chain fatty acids, such as butyric acid, which has anti-inflammatory activity and inhibits the growth of *S. aureus* both *in vitro* and *in vivo*, thereby contributing to the maintenance of microbial eubiosis [55,127].

Staphylococcus aureus is less commonly found as a stable resident of healthy skin but is a well-known opportunistic pathogen. When present, it can cause significant health issues, particularly in individuals with compromised skin barriers. This is due to different virulence factors of this strain that enable it to colonize and infect the skin. They include surface proteins that facilitate adhesion to skin cells, enzymes that degrade host tissues and toxins like hemolysins and leukotoxins, that can kill host cells and evade immune responses. For example, Protein A (SpA) is a multifunctional surface protein, important for the bond with

IgG and other ligands, such as TNF receptor 1; this protein has different immunosuppressive traits, that establish mechanisms of immune evasion of *S. aureus* [128]. One of the most well-known toxins produced by *S. aureus* is Pantone-Valentine Leukocidin (PVL), a β -pore-forming toxins (PFT), leading to cell lysis and skin and soft tissue damage. Moreover, among PFT, it is important to mention α -Hemolysin (α -Toxin or Hla), which expression is under control of the quorum-sensing agr system, that in specific conditions oligomerizes forming pores in host cell membranes, forming a hydrophilic transmembrane channel [129].

Also, Phenol-Soluble Modulins (PSMs) represents one of the most aggressive virulence factors of this bacterium with cytolytic activity versus different cell type [130].

Staphylococcus aureus has also the ability to evade the radical action associated with ROS thanks to the production of staphyloxanthin, an orange pigment (that attributes the name “aureus”, golden, to the bacterium) which contains a series of conjugated double bonds with an antioxidant activity [131].

In addition to producing antimicrobial peptides, commensals protect against pathogens growth by competing for resources such as nutrients and space on the skin surface, fundamental in maintaining a balanced microbiota and preventing the overgrowth of pathogenic bacteria.

However, when the population of commensals is reduced, for example due to external factors, harmful strains can exploit the weakening of defences and proliferate, leading to infections. This dynamic underscore the importance of maintaining a healthy and balanced skin microbiota for preventing pathogenic invasions.

1.1.8 Skin alterations

The skin microbiota, a complex community of microorganisms living on the skin, plays a vital role in maintaining skin health. However, alterations in this microbial ecosystem, can lead to different skin conditions. The balance between beneficial and pathogenic microorganisms is crucial for skin homeostasis and its disruptions can trigger or exacerbate skin alterations. This chapter explores the role of skin microbiota, main features and etiological factors that trigger skin conditions, mainly focusing on atopic dermatitis.

- Atopic dermatitis

Atopic dermatitis (AD), commonly known as eczema, is a chronic inflammatory skin condition characterized by itchy, red, and dry skin [132,133]. Many factors contribute to its development and exacerbation, including genetic predisposition, environmental factors, immune dysregulation and skin barrier dysfunction [133]. Within skin inflammatory conditions, it is important to distinguish AD from seborrheic dermatitis (SD) is a common inflammatory skin condition that, unlike AD, occurs mainly on the sebum-rich skin of the scalp and face and is distinguished by a lack of excoriation; it occurs particularly during early childhood and affects males and females equally [134]. This condition is characterized by skin barrier dysfunction due to a deficit in ceramide and fatty acid production, an altered differentiation of keratinocyte proteins (such as filaggrin), a decrease in the production of antimicrobial peptides from skin cells and a persistent pro-inflammatory cytokine release [135].

Atopic dermatitis affects millions of people worldwide, predominantly children, but can persist into adulthood or even begin later in life; anyway, the epidemiological trends are different across the different parts of the world [136]. For example, two recent studies showed that the children incidence and prevalence of AD in Germany was 10.35% while

among adults is 3.67% [137,138]. Only 25% of patients with AD diagnosed in childhood will be affected by this condition in adulthood [139].

Among etiologic factors that contribute to the exacerbation of this skin conditions, it is important to mention filaggrin (FLG) deficiency, since this protein is necessary to maintain the structural integrity of the SC. A lack of FLG was associated with an early onset of AD and the mutation of the gene associated with this protein, represent a causative of AD since it follows a compromised skin barrier, increasing susceptibility to environmental allergens, irritants and microbial invasion. [140].

Several other factors have been associated with the presence of this skin condition. For example, areas with higher average temperatures, higher UV indices, less precipitation and less indoor heating were negatively correlated with AD. Conversely, exposure to airborne pollutants such as nitrogen oxide and carbon monoxide promote the presence of this inflammatory condition. In addition, certain foods, such as soy, eggs, cod, peanuts, and cow's milk, are also a trigger in 20-30% of moderate-to-severe AD cases [136].

Immune dysregulation is also central to AD, characterized by a downstream systemic production of type 2 cytokines by keratinocytes due to the expression of different interleukin, like TSLP, IL-33 and IL-2 [141].

The skin microbiota plays a crucial role in AD developement. In individuals with AD, the microbial balance is often disrupted, with reduced diversity and an overgrowth of pathogenic bacteria, especially *S. aureus*. In fact, it is increasingly implicated as a possible trigger in the pathogenesis of atopic dermatitis; in fact, studies have shown that *S. aureus* colonizes the skin of up to 60-100% of individuals with moderate to severe atopic dermatitis, compared to just 5-30% of individuals without this condition [142]. In addition, it was been observed that in patients with AD, injured sites have a higher amount of *S. aureus* (70%)

than in sites with no lesions (39%) [143]. This strain can cause skin infections, leading to further inflammation and itching, and can also release toxins that exacerbate symptoms. In some cases, *S. aureus* may also trigger an immune response, leading to the release of cytokines that cause itching and redness.

Moreover, the AD-associated microbiome is characterized by a decrease in different commensals such as *C. acnes*, *Corynebacterium*, *Micrococcus* and coagulase-negative staphylococci, like *S. epidermidis* and *S. hominis*, associated with a higher amount of *Streptococcus* and various *Malassezia* species [144,145].

In contrast, *S. epidermidis* plays an antagonist role against *S. aureus* by antimicrobial mechanisms by the stimulation of the secretion of IL-1 α by dendritic cells and keratinocytes and regulate T-cell activity [146].

Due to its complexity, AD management involves different approaches aimed to restore the skin barrier, reducing inflammation and counteracting microbial imbalance. Regular use of emollients is essential to restore the skin barrier, to improve moisture and protect against irritants. Topical corticosteroids are commonly prescribed to reduce inflammation during flares, while calcineurin inhibitors are used for long-term management. Systemic immunomodulatory treatments such as cyclosporine, methotrexate, or biologics like dupilumab, which targets the Th2 inflammatory pathway, may be considered for moderate to severe AD [147].

Antimicrobial treatments, including topical or systemic antibiotics, may be necessary to counteract pathogens infections, but the misuse of them is enhancing the problem of antibiotic resistance. For this reason, emerging research suggests that probiotics and prebiotics may help restore microbial balance and improve AD symptoms [148]. This topic will be discussed in Chapter 1.2.

- Acne

Acne vulgaris is a multifactorial skin disorder that affect mainly the pilosebaceous units, characterized by comedones, papules and pustules (also cysts and nodules in severe grade disease). It is most prevalent during adolescence due to hormonal changes that stimulate sebaceous gland activity, leading to increased sebum production. The pathogenesis of acne involves four key factors: hyperkeratinization of the follicular epithelium, excessive sebum production, inflammation and alterations in the skin microbiota [149]. Among these, the role of microbial dysbiosis, particularly involving *C. acnes* (previously called *Propionibacterium acnes*), is well-documented as a pivotal contributor to the disease.

Cutibacterium acnes is a commensal gram-positive bacterium that resides in the anaerobic environment of hair follicles and contributes to maintain skin homeostasis by suppressing the growth of pathogenic strains, by producing virulence factors [54]. While generally harmless, specific strains of *C. acnes* associated with acne, exhibit increased virulence, producing lipases, proteases and porphyrins that degrade sebum and generate pro-inflammatory mediators, such as IL-1 β , IL-8, and TNF- α , attracting neutrophils and promoting hyperkeratosis [150,151]. Moreover, a virulence factor is represented by the ability of this bacterium to develop biofilms that enhance its resistance to antimicrobial therapies [152].

In normal conditions, *S. epidermidis*, a typical commensal, inhibits *C. acnes* proliferation by releasing succinic acid as a result of glycerol fermentation [153], limiting also inflammation thanks to lipoteichoic acid production that suppress proinflammatory cytokine release [154]. On the other hand, *C. acnes* is able to manage the proliferation of *S. epidermidis* by hydrolyzing sebum triglycerides and releasing propionic acid, that contribute to make the pilosebaceous follicle an acidic environment [155].

All the changes related to acne, generate a state of dysbiosis that involves not only to the increase in *C. acnes* colonization, but also to the shifts in the microbial populations. For example, overgrowth of the lipophilic yeast *Malassezia* species, has been implicated in triggering inflammatory responses in sebaceous areas, potentially aggravating acne in some individuals. In acne, *Malassezia* produce free fatty acids as the results of hydrolyzation of sebum triglycerides, causing the formation of comedons and hyper-keratinization of hair ducts [156].

Targeting the microbiota offers promising therapeutic avenues for managing *acne vulgaris*. Conventional treatments, such as topical antibiotics and benzoyl peroxide, aim to reduce *C. acnes* populations but often disrupt the overall skin microbiota, leading to resistance and other side effects [157]. Emerging approaches focus on microbiota modulation, such as using probiotics, prebiotics, and postbiotics to restore microbial balance. For instance, antimicrobial peptides, metabolites of *S. epidermidis* fermentation, the use of probiotics like *S. thermophiles*, *L. paracasei*, and *L. plantarum*, and natural compounds, could represent a novel strategy to inhibit *C. acnes* overgrowth, reduce inflammation, and enhance skin barrier function [156]. These innovations highlight the potential of microbiota-focused therapies to not only treat acne but also promote long-term skin health.

- Psoriasis

Psoriasis is a chronic autoimmune skin disorder characterized by the abnormal keratinocyte proliferation, immune dysregulation and the formation of erythematous, scaly plaques that can be itchy and painful. The condition involves a complex interplay between genetic predisposition, environmental triggers, and immune system responses, with the skin microbiota emerging as a critical player in its pathogenesis [158]. In healthy skin, a stable microbiota dominated by commensal bacteria like *S. epidermidis* and *C. acnes* supports

immune tolerance, skin barrier integrity and the suppression of pathogenic strains. However, in psoriatic lesions, microbial dysbiosis is frequently observed, with a notable increase in potentially pathogenic microorganisms, in particular *Staphylococcus* (*S. aureus*, *S. pettenkoferi* and *S. sciuri*), *Streptococcus pyogenes* and *Malassezia* species, as well as a reduction in protective commensals [159].

Staphylococcus aureus plays a significant role in exacerbating psoriatic inflammation by producing superantigens and toxins and stimulating T-cell activation and the release of pro-inflammatory cytokines, including IL-17, IL-23, and TNF- α [160]. It was noticed that 60% of patients with psoriatic lesion are colonized by *S. aureus* and 60% of isolates produce enterotoxins and toxic shock syndrome toxin-1 [161]. Similarly, *S. pyogenes*, related to upper respiratory tract infections, is implicated in triggering immune responses by T-cell activation and IFN- γ production, in particular in patients with chronic plaque lesions [162].

Fungal species like *Malassezia*, commonly found on the skin, can also contribute to the inflammatory environment and to an increased cell proliferation. In fact, it was found that *Malassezia* psoriatic lesion-associated, infiltrate in keratinocytes and upregulates several factors related with cell cycle like integrin chains, transforming growth factor-beta, heat shock protein 70, resulting in hyperproliferation of keratinocytes [163].

Interestingly, alterations in the skin microbiota may not only exacerbate the disease but also influence its chronicity and responsiveness to treatments. Restoring microbial balance through innovative approaches, such as the use of probiotics, aims to reduce pathogenic species while enhancing commensal populations, ameliorating the inflammation associated to lesions. For example, in a recent study, *L. sakei* showed to reduce the skin thickness, improved the condition of erythema and scaling scores and reduce the expression pro-inflammatory cytokines associated to psoriasis, such as IL-17A, IL-19, and IL-23 [164].

- Rosacea

Rosacea is a chronic inflammatory skin condition that typically affects the face, characterized by erythema, redness, pustules and papules. The National Rosacea Society (NRS), in 2002, defined rosacea as “a central face distribution of at least one of four primary features (flushing, persistent erythema, papules and pustules and telangiectasia)” and recognised four different types: erythematotelangiectatic rosacea, papulopustular rosacea, phymatous rosacea and ocular rosacea [165].

This condition has been shown to be associated with alteration of the skin microbiota, associated with the high colonization of *Demodex* species, that can represent a trigger of this condition. *Demodex* mites are commensal organisms that inhabit the pilosebaceous follicles of human skin, at low densities. In rosacea patients, there is often an increased density of these mites, such as *Demodex folliculorum* and *Demodex brevis* (from 0.7 mites/cm² to 10.8 mites/cm²), also promoting activation of the innate immune system by activating TLR [166].

The presence of these bacteria and the consequent immune response may contribute to the inflammation and vascular changes seen in rosacea. Additionally, microbial dysbiosis involving increased populations of certain bacteria and a decrease in microbial diversity may exacerbate the condition. In detail, patients with rosacea showed an increase in the colonization of the commensal beta-haemolytic *S. epidermidis* and decreased of *C. acnes* on a sebaceous skin site [167]. Moreover, *Bacillus oleronius* isolated from mites that colonize papulopustular lesion of rosacea-affected patients, is able to produce antigens that stimulate the mononuclear cells proliferation [168]. In this way, the inflammatory response is stimulated, leading to an increased local skin temperature that impact on the activity and on the colonization of some microbial strains, further exacerbating a situation of unbalance and dysbiosis [169].

1.2 Innovative strategies to counteract skin dysbiosis

As explained in the previous chapter, skin dysbiosis are characterized by an imbalance in the microbial community of the skin and it can be associated with different dermatological conditions, leading to damage of the skin barrier. This disruption involves an overgrowth of pathogenic microorganisms and a decline in beneficial commensals, leading to impaired skin barrier function and chronic inflammation. Conventional treatments, such as antibiotics and corticosteroids, often lead to the development of antimicrobial resistance, side effects and the recurrence of dysbiosis. For these reasons, especially in the last years, there has been a lot of research about innovative, natural and sustainable strategies to restore microbial balance and enhance skin health. Emerging approaches focus on the exploitation of the beneficial properties of microbial and natural compounds to restore eubiosis, promote skin homeostasis, and minimize environmental impact. They generated great attention in the cosmetic field, in particular for the daily-use care products, that can modify skin microbial composition, due to the ingredients contained inside as shown in recent studies [14,170]. For example, the lipidic component of cosmetics, can promote the growth of lipophilic bacteria like *C. acnes*, and preservatives or emulsifiers can inhibit the growth of commensals such as *S. epidermidis*, leading to an imbalanced microbiota [171,172]. For this reason, cosmetic industry has the main focus on the development of new strategies without altering the skin microbiota qualitative-quantitative composition, by using for example prebiotics, probiotics, postbiotics and natural extracts [173]. In this context, MyMicrobiome, the leader industry for microbiome-friendly cosmetics, pharmaceuticals, and tissues [174], introduced in 2019 the "Microbiome-friendly" certification, attesting that "*cosmetics products are free from*

contaminants, harmless to specific bacteria in the intended area, and non-disruptive to the skin's natural microbiome equilibrium” [175].

1.2.1 Probiotics

Probiotics, defined as live microorganisms that confer health benefits when administered in acceptable amounts, have emerged as a promising strategy for addressing skin dysbiosis [176]. While traditionally associated with gut health, probiotics are increasingly recognized for promoting skin health by restoring microbial balance, enhancing skin barrier function, and modulating immune responses, and for this reason they have generated interest in cosmetics applications [177].

Probiotic bacteria can modulate skin functions, both if they are directly applied topically or if they indirectly modulate them by acting on gut microbiome. This is justified by the presence of a bidirectional relationship between the gut and the skin microbiota, named “gut-skin axes”. In fact, several studies showed that gut microbiota modulates the immune system, influencing inflammatory and immune responses that can affect skin conditions, such as atopic dermatitis, psoriasis, acne and rosacea [178]. Moreover, it can produce metabolites that influence skin barrier function, hydration and inflammation.

For example, *Bifidobacterium adolescentis*, is able to inhibit the activity of the enzyme tyrosinase, leading to a decrease in melanin production [179]. In fact, although this pigment secreted by melanocytes, has a photoprotector activity against UV rays, if over expressed can lead to skin hyperpigmentation disorders. The antioxidant capacity of the culture filtrate of bifidobacterium adolescentis, showed skin whitening activity due to the ability to scavenge 2,2-diphenyl-1-picryl-hydrazyl (DPPH) and 2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) radicals in dose-dependent manners [179].

In addition, some probiotics show skin moisturizing action by decreasing TEWL. One study showed that oral supplementation of *Lactobacillus plantarum* increased levels of ceramides, lipids with important supporting and moisturizing functions in the skin barrier [180], while another one demonstrated that milk-fermented protein by *L. helveticus* led to a reduction in TEWL, confirming its moisturizing action [181]. In addition, Jung et al, showed that the probiotic *L. rhamnosus* was able to up regulate the gene expression of two proteins involved in the maintenance of the stratum corneum, Claudin-1 and Occludin [182].

The growing understanding of the skin microbiome has positioned probiotics as an innovative therapeutic option for conditions like acne, atopic dermatitis, and rosacea, where dysbiosis plays a key pathogenic role. Thanks to their multiple actions, probiotics can be used to improve inflammatory skin conditions related to an alteration of the superficial layers of the skin. Probiotics not only improve skin integrity and health, but also inhibit the growth of pathogenic bacteria by competing for nutrients and adhesion sites on the skin surface. For example, the lactic acid bacterium *Streptococcus thermophilus* promoted the synthesis of ceramides both *in vitro* and *in vivo*, and some of them showed antibacterial action against *C. acnes*, and to improve eczema symptoms related to atopic dermatitis [156,183].

Emollients containing *Lactobacillus*, like *Lactobacillus sakei* isolated from the Korean vegetable-based product kimchi, can improve inflammatory condition and suppress the proliferation of pathogens such as *S. aureus* [184]. Also the treatment with *Roseomonas mucosa* showed to improve inflammatory *S. aureus*-related conditions, decreasing the severity of AD symptoms without causing adverse effects [185]. Moreover, probiotic metabolites including SCFAs and bacteriocins, enhance the acidic pH of the skin, inhibiting the colonization of harmful bacteria while favouring commensals. They also modulate both innate and adaptive immune responses stimulating the production of anti-inflammatory

cytokines, such as IL-10, while suppressing pro-inflammatory mediators, helping to counteract chronic inflammation dysbiosis-associated [186].

Recently, thanks also to their safety, researchers have suggested that probiotics can be used as an efficient ingredient in cosmetics to address the above-mentioned skin issues to a better effect.

1.2.2 Prebiotics

Prebiotics are non-digestible complex carbohydrates, mainly derived from oligosaccharides, that act as substrates for commensal bacteria, indirectly promoting a favourable environment for maintaining or restoring skin eubiosis. They were defined from the International Scientific Association for Probiotics and Prebiotics (ISAPP) as “a substrate that is selectively utilized by host microorganisms conferring a health benefit” [187].

Some of them, such as fructo-oligosaccharides (FOS), galacto-oligosaccharides (GOS), isomalto-Oligosaccharides (IMO), arabinogalactan (LAG), inulin, dextran and konjac glucomannan hydrolysates (KGH), have been shown to support skin microbial modulation by selectively enhancing the growth and activity of beneficial strains like *S. epidermidis* while suppressing pathogenic species such as *S. aureus*. Several studies showed that prebiotics can modulate the severity of AD-like skin lesions, decreasing the expression of pro-inflammatory cytokine through toll-like receptors [188].

In particular, as showed by Akiyama et al, 5% GOS inhibited *S. aureus* growth and cell adhesion, without affecting *S. epidermidis* growth. Moreover, GOS act as inhibitors of *S. aureus* adhesion, while XYL affect the production of *S. aureus* biofilm matrix [189]. In addition, Di Lodovico et al, demonstrated that the combination of GOS, FOS, IMO and LAG with xylitol (XYL) at 1%, are able to modulate the *Staphylococcal* growth favouring the commensal microbial population, synergistically enhancing the XYL prebiotic activity.

These combinations expressed a selective species-specific effect against planktonic and sessile phases of *S. aureus* and *S. epidermidis*, suggesting their ability to restore the skin balance [190].

Also Bourgot et al, in another recent study, demonstrated that FOS selectively inhibit pathogenic growth while promoting at the same time the growth of the beneficial *S. epidermidis* [191].

β -glucans, the main active compound of oat bran, have a documented prebiotic efficacy on intestinal bacteria [192]. Colloidal oat, obtained from the very fine grinding of oat flakes, has always been used for its beneficial properties, especially to improve skin conditions such as atopic dermatitis [193]. In fact, 1% colloidal oat has been shown to improve skin barrier properties, including reducing TEWL and consequently increasing skin hydration, as well as increasing microbial diversity [194]. In a recent study of 2021 by Liu-Walsh et al, it was confirmed that 1% of colloidal oat acts on the lactic acid production, on the growth and on the metabolism of both *S. epidermidis* and *S. aureus* [195], probably due to the prebiotic β -glucans. In particular, the lactic acid levels increased with a consequently decreasing of pH, that favour the growth of *S. epidermidis* affecting the growth of *S. aureus* [195].

Moreover, some bacteria are able to produce SCFAs such as acetate, propionate, butyrate and lactic acid as fermentation metabolites with prebiotics applications; they can promote the growth of beneficial strains inhibiting the pathogenic ones both *in vitro* and *in vivo* [55,127]. In atopic dermatitis, these prebiotics help mitigate symptoms by increasing microbial diversity and reducing the prevalence of *S. aureus*, the main pathogen implicated in disease exacerbation.

Another example is konjac glucomannan hydrolysates (KGH), a plant-derived complex sugar extracted from the plant *Amorphophallus konjac*, that is a potential prebiotic due to its modulation of probiotic bacteria by promoting the production of SCFAs [196]. Patients affected by *acne vulgaris*, had a significant improvement of the skin health after 20 days of treatment with KGH, suggesting its applicability as novel topical therapeutic product [197].

It is possible to conclude that prebiotics, well-recognized ingredient for their positive action on gut microbiota, can modulate also the composition of the skin microbiota and for this reason it is possible to include them into cosmetic for topical application. These findings demonstrate the potential of prebiotic-based therapies as eco-friendly and effective strategies for maintaining skin health and managing dermatological conditions through microbiome modulation.

1.2.3 Postbiotics

In addition to probiotics and prebiotics, postbiotics are bioactive compounds that have gained attention for their role in modulating the skin microbiome and improving skin health. They are produced as a result of fermentation process by probiotic bacteria and they were recently defined by ISAPP as “preparation of inanimate microorganisms and/or their component that confers a health benefit on their host” [198].

Postbiotics have several advantages compared to probiotics, such as the ability to don't transfer antibiotic resistance make it useful also for immunosuppressed people, longer shelf life and as well as higher stability to temperature and pH variations. They include metabolites like enzymes, SCFAs, peptides, bacteriocins and bacterial cell wall components with antioxidant, antimicrobial, anti-inflammatory and immunomodulatory proprieties, by directly acting on human cells, or indirectly by the modulation of microorganisms [199].

Thanks to their stability and their proprieties, they were recently considered as ingredients to include in topical formulation for skin care also for the treatment of skin condition like atopic dermatitis, acne, alopecia and wound healing [200]. In particular, Golkar et al, by the direct addition of lyophilized supernatant of *L. fermentum*, *L. reuteri* and *B. subtilis*, tested the cold cream in wound healing [201]. Majeed et al evaluated the effect of postbiotic-based topical cream containing 2% of LactoSporin[®] (a protein metabolite produced by the probiotic *B. coagulans*), for the treatment of acne lesions, showing antimicrobial activity against *C. acnes* and also inhibiting biofilm formation [202]. The lysate of *S. thermophilus* contains sphingomyelinase, an enzyme that transform sphingomyelin to phosphocholine and ceramides; it was demonstrated that its use in topical application, can significantly improve the lipidic component in stratum corneum, reducing water loss and improving skin integrity [183]. Another recent study explored the effect of the *L. plantarum* K8 lysate on *in vitro* human keratinocyte cell line (HaCaT cells), observing an increase in the production of hyaluronan, one of the main polysaccharidic components of cell membrane, and an up-regulation of the moisturizing-related proteins HAS-2 and AQP-3 [203].

Postbiotics enhance the skin microbiome by suppressing the growth of pathogens, supporting beneficial species and strengthening the skin barrier integrity. For instance, SCFAs, such as acetate, propionate, and butyrate, produced by commensal bacteria, exhibit antimicrobial and anti-inflammatory properties. They help reduce overgrowth of pathogens like *S. aureus* and *C. acnes*, both implicated in atopic dermatitis and acne, respectively.

1.2.4 Eco-sustainable approaches

In 2019, Food and Agriculture Organization of the United Nations, declared that the waste coming from food production is about 33%, representing around 1.6 billion tons of

food waste annually threw away [204]. The food industry and associated consumption activities generate approximately 40-60% of all municipal solid waste globally [205]. This amounts to an estimated 5 billion tons annually from agricultural and forestry activities and another 500 million tons from food processing sectors alone [206]. A large portion of this waste includes by-products from food processing operations, such as fruit pulp, peel, pomace and seeds, as well as materials resulting from agricultural pruning, including leaves, branches, flowers and bark. The Food and Agriculture Organization (FAO) Food Loss Index tracks food losses at the national level along the whole supply chain, from agricultural production to retail. According to FAO estimates, this process results in a global loss of 13.8% [207].

These by-products, typically considered as waste, are rich sources of bioactive compounds, including proteins, lipids, carbohydrates and several secondary metabolites with potential applications in the biological fields. Among these compounds, polyphenols, are valued for their strong antioxidant properties, which contribute to cellular health and have been shown to counter oxidative stress effectively [208]. Consequently, the food and agricultural industries are increasingly prioritizing waste management and valorization strategies, transforming discarded material into valuable resources [209].

For this reason, one area of significant interest is represented by the use of natural antimicrobials derived from food by-products, such as pomegranate peel and almond hulls, to develop alternatives to traditional therapies. This choice can be crucial in the management of the rising problem of antimicrobial resistance, which presents a severe global health threat. Natural products, offer an eco-friendly and potentially safe alternative to synthetic antibiotics, aligning with global efforts to mitigate AMR [210].

To address the problem of AMR, it is important to adopt a One Health approach that integrates human health, animal health, and environmental health. This involves reducing the unnecessary use of antibiotics, improving hygiene and infection control measures, and promoting the development and use of alternative approaches to treating infections, like natural compounds [211]. Pomegranate peel, citrus peel, almond hulls, and other plant-derived materials represent key examples of agricultural by-products rich in bioactive compounds like polyphenols, antioxidants, and vitamins. These by-products are not just waste but potent reservoirs of functional compounds with broad applications, including antimicrobial, anti-inflammatory, and antioxidant activities [212].

In addition, the use of sustainable extraction methods increases its environmental friendliness, aligning with current trends in green chemistry and environmental responsibility. For example, the ultrasound-assisted extraction (UAE) is a technique widely used to obtain bioactive molecules from plant biomass, that provides the application of sonication, using high-power and low-frequency ultrasonic waves, to increase the yield and carry out the extraction of active molecules in a shorter time. In this way the vegetable membranes are disrupted and the solvent can easily penetrate inside the cells taking the active compounds [213].

For these reasons, using and combining food waste with green technologies of extraction can improve the yield, the percentage of bioactive compounds, obtaining a more effective extract and protecting the environment, at the same time.

2. AIMS OF THE PhD PROJECT

The skin microbiota community plays a critical role in maintaining skin health by providing a natural shield against harmful pathogens, regulating immune responses and preserving the integrity of the skin barrier. Eubiosis is characterized by a balanced skin microbiota, in which beneficial commensal species, such as *S. epidermidis*, prevent the overgrowth of pathogenic bacteria like *S. aureus*. When this balance is destroyed, the resulting dysbiosis can trigger infections, inflammation and various skin disorders, such as atopic dermatitis. Pharmacological therapies, including antibiotics and chemical-based topical treatments, often exhibit limited specificity, potentially causing collateral damage to the beneficial microorganisms essential for maintaining eubiosis. Additionally, the overuse of these treatments can contribute to the growing issue of antimicrobial resistance, further complicating skin health management.

The aim of my PhD thesis was to investigate eco-sustainable/eco-friendly strategies for restoring microbial eubiosis of the human skin microbiota. The focus was on the use of natural bioactive extracts derived from food waste, specifically pomegranate peel and almond hulls. The food by-products, often discarded as waste, represent a sustainable and cost-effective source of valuable compounds, including polyphenols, flavonoids, and tannins.

In addition, the plasma-activated water as potential, non-chemical intervention for managing microbial balance, was also studied.

Through an eco-friendly, low-impact extraction techniques, such as green chemistry approaches and plasma-assisted processes, the research aimed to optimize the yield and activity of these bioactive compounds while minimizing environmental degradation. This

approach aligns with the One Health concept, which advocates for the sustainable management of human, animal and environmental health as an interconnected system.

In details, the aims of my PhD project were to:

1. Analyze the specie-specific potential of pomegranate peel extract in terms of antimicrobial activity, focusing on its anti-adhesive effectiveness against *S. aureus* as one of the main skin pathogens, as well as its effect on promoting, or not affecting, *S. epidermidis* as one of the most representative among beneficial skin commensals.
2. Perform a preliminary study on the antimicrobial activity of almond hull extract. In fact, since in literature there are no previous researches regarding the action of this natural extract on microbial biofilm, the innovation of this work was to test for the first time antiadhesive and anti-biofilm action of almond hull extract against in formation and mature biofilms, respectively, of two reference strains (*S. aureus* ATCC 29213 and *E. coli* ATCC 9637).
3. Investigate the safety and biocompatibility of these natural extracts on *in vitro* human cells or on recognized *in vivo* model, ensuring they are not toxic and therefore suitable for being incorporated as part of topical formulations for re-balancing the skin microbiota.
4. Evaluate the efficacy of plasma activated water (PAW) as an additional or alternative treatment for restoring microbial balance, against strains isolated from the food chain. Future studies could evaluate the compatibility of PAW with natural extracts in synergistic formulations.

By integrating the antimicrobial power of food waste-derived bioactive compounds with innovative, non-chemical approaches like plasma-activated water, this thesis seeks to establish a novel, sustainable pathway for managing skin microbiota imbalances (preventing

or reversing dysbiosis). The long-term goal is to provide scientific evidence that supports the development of skincare products and therapeutic interventions that are not only effective but also aligned with global sustainability goals, particularly in the context of reducing waste, limiting chemical use and limiting the rise of antimicrobial resistance.

In conclusion, by developing natural and eco-friendly interventions to restore eubiosis, the thesis will contribute to increasing knowledge on sustainable resources to support human health and well-being.

3. POMEGRANATE PEEL EXTRACT (PPE): Effect on skin microbiota

3.1 Introduction

Pomegranate (*Punica granatum L.*) is a fruit widely used in the food industry, in particular to produce juices and extracts; it is known for its beneficial properties and so it is widespread in many countries such as India, Iran, Turkey, China, United States of America, Israel, Egypt, Spain, Afghanistan, Tunisia, Azerbaijan, Morocco, Argentina, Brazil, Chile, Peru, South Africa, Australia and Italy [214].

Pomegranate is composed by three parts: arils seeds and (inner and outer) peel (**Figure 4**). A recent study highlights how the composition of pomegranate significantly changes depending on the variety; some cultivars showed thicker and differently coloured peels than others, or bigger arils, or different juice yield due to different arils/peel ratio [215].

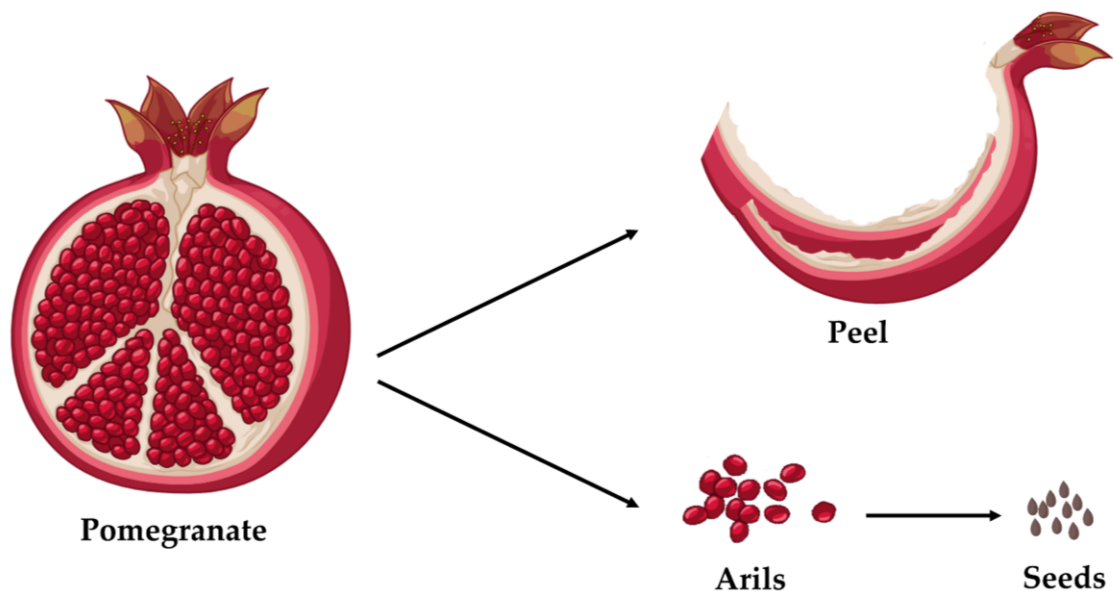


Figure 4. Pomegranate fruit.

Anyway, the peel (by-product) constitutes on average approximately 40-50% of the weight of pomegranate, the arils account for about 30-50% of the fruit and the seeds, located inside the arils, make up about 10-20% of the total weight and they represent an important source of dietary fibers and oils rich in punicic acid [216].

In the past the pomegranate was rarely cultivated, due to the difficulty of extracting the arils from the peel, while today it is widely used thanks to the discovery of its properties. Moreover, this fruit has a very pleasant taste, and then the consumption of pomegranate around the world is growing every year, reaching 3.8 tons in 2017, and consequently about 1.9 tons/year of waste are generated from this fruit [214].

In addition to the juice, the peel has also attracted increasing attention for its high concentration of bioactive compounds, which offer significant potential in nutraceutical [217], pharmaceutical [218] and cosmeceutical applications [219,220].

Interestingly, different studies, highlights that the peel shows the highest concentration of polyphenols, flavonoids and tannins, compared to arils and seed [221,222], which are the primary contribute to its therapeutic properties and they give the typical colour to the peel [223]. Among polyphenols, ellagitannins, including punicalagin, are recognized for their remarkable antioxidant activity. Punicalagin, a hydrolyzable tannin, is able to neutralizes free radicals and also exerts anti-inflammatory and antimicrobial effects. Ellagic acid, a derivative of ellagitannins, has been widely studied for its ability to reduce oxidative stress, inhibit tumour growth and enhance skin healing [217]. Flavonoids such as quercetin and catechins, exhibited strong free radical scavenging abilities, modulating immune responses and contributing to cardiovascular and neuroprotective health [224].

Thanks to these bioactive compounds, pomegranate peel extract shows different biological activities, which make it a natural product with possible applications in different fields [225,226]. The antioxidant property of PPE is due to its polyphenolic content, which protects cells and tissues from oxidative damage [227,228]. Since oxidative stress is a key contributor to chronic conditions such as cardiovascular diseases, neurodegenerative disorders, diabetes, the ability of PPE to mitigate these effects highlights its preventive and therapeutic potential.

Furthermore, regarding its anti-inflammatory activity, PPE shown to modulate the expression of pro-inflammatory cytokines and mediators, such as interleukin-6 (IL-6), interleukin-1 beta (IL-1 β) and tumour necrosis factor α (TNF- α). Moreover, PPE showed to down-regulate inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) expression, probably due to the inhibition of MAPKs signalling pathway, making it a promising candidate for managing inflammatory conditions, including dermatological disorders like psoriasis and atopic dermatitis and improve the outcomes [217,229].

Moreover, the bioactive molecules inside the peel of pomegranate showed a remarkable antimicrobial activity, which has generated increasing attention due to the global problem of antimicrobial resistance. PPE has demonstrated broad-spectrum antimicrobial effects against a wide range of pathogens, including both Gram-positive bacteria and Gram-negative bacteria, as well as fungi such as *Candida albicans* [230].

Pomegranate peel extract acts with different antibacterial mechanism, including the disruption of bacterial membranes, due to the phenolic compounds that can modify the bacterial cell membrane permeability and integrity [231,232], interacting for example with the sulfhydryl groups in the cell membrane selectively suppressing membranous enzymes [233]. Moreover, different studies demonstrated the efficacy of PPE against bacterial and

fungal biofilm formation and removal [234,235]. Since biofilms represent one of the major obstacles in treating chronic infections due to their resistance to antibiotics, PPE ability to disrupt biofilms and prevent their formation indicates the possibility of its usefulness in the treatment of persistent infections.

The role of PPE in terms of microbial modulation, extends beyond its direct antimicrobial effects, as it also contributes to maintaining microbial homeostasis. By selectively targeting pathogenic species while promoting, or without affecting, beneficial microorganisms, PPE supports a balanced microbiome which is essential for preventing infections. In fact, recent studies shown that ellagitannins, acts on bacterial growth, not only as pathogens inhibitors, but it also showed capability to improve the viability and the metabolism of intestinal bacteria, suggesting that pomegranate peel may work as a potential prebiotic [236,237]. In this PhD thesis the potential activity of this extract to support skin commensals growth was investigated, showing that PPE supports the growth of the beneficial *S. epidermidis*, collected from healthy skin, and its potential prebiotic effect was confirmed.

For all these reasons, the peel of the pomegranate, previously considered as waste of the food industry, is now recognized as a rich reservoir of bioactive molecules. Its multiple proprieties have significant implications for applications in wound care, where PPE can favour the healing process while preventing microbial colonization.

This chapter explored the antimicrobial and antiadhesive ability of PPE against previously collected skin microbiota samples, and its eventual ability to selectively inhibit pathogenic bacteria like *S. aureus* while preserving and supporting beneficial commensals, such as *S. epidermidis*, with the purpose to eventually include it in topical formulations, to support an healthy and balanced microbiota.

3.2 Materials and Methods

3.2.1 Study design

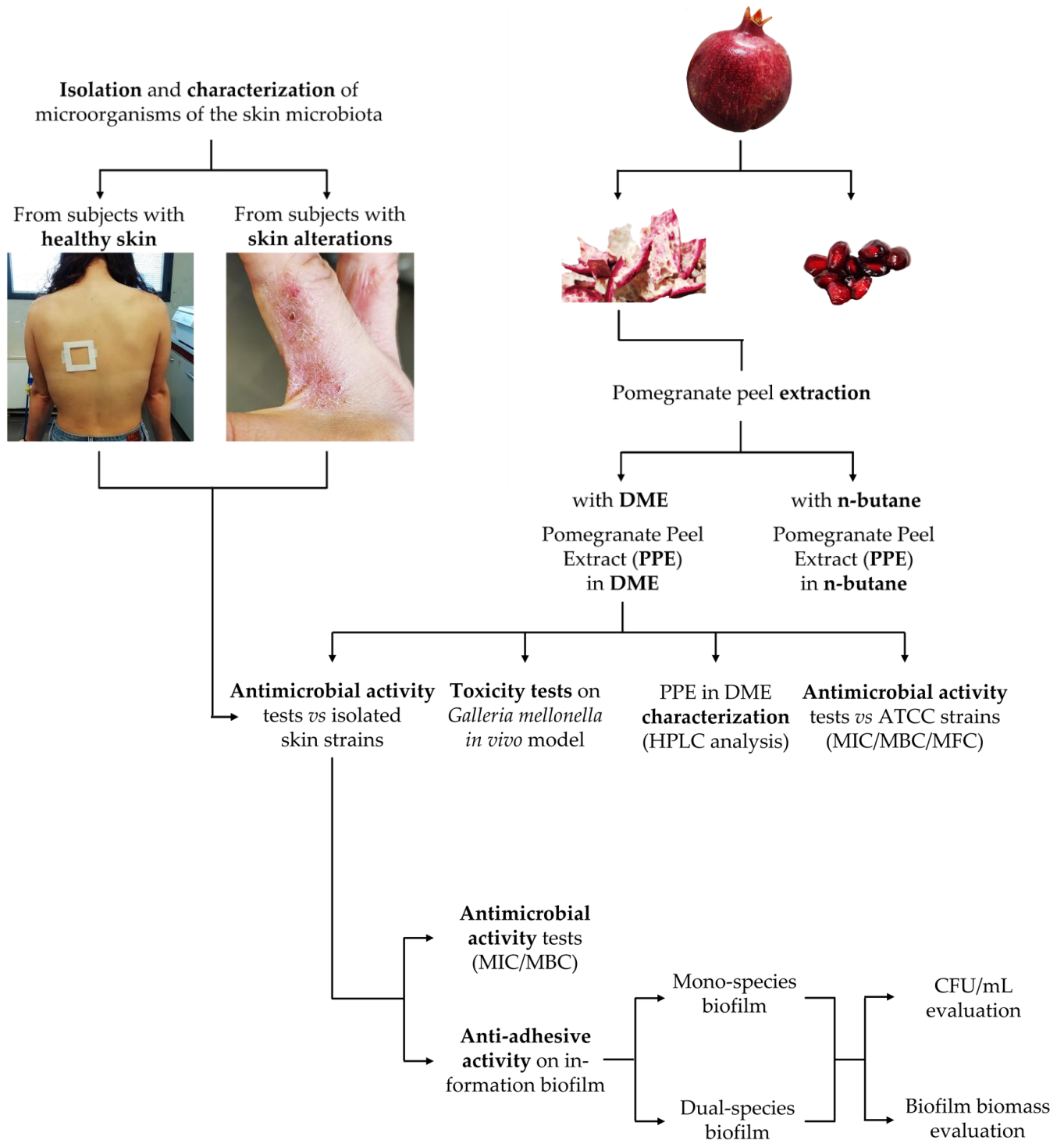


Figure 5. Experimental design.

3.2.2 Skin microbiota sampling and bacterial culture

The collection of skin microbiota samples took place between April and June 2022. The samples were obtained from six healthy volunteers (three men and three women, aged 20-32 years) and from three volunteers with skin alterations (atopic dermatitis, one man and two women, aged 25-32 years); all participants provided written informed consent for the study, that was approved by the Inter Institutional Ethics Committee of University "G. d'Annunzio" Chieti-Pescara, Chieti, Italy (ID: richycnvw). Participants declare that, in the four months preceding the study, they did not receive systemic or topical antibiotic therapies. They also confirmed that they had not used any cream or lotions on the affected area in the four days before the sampling and they only used neutral soap and water to clean the swabbing area. Microorganisms were collected from the back skin for healthy subjects, following the method of Ogai et al [238], and from the local area of skin alteration for subjects with atopic dermatitis.

To obtain skin samples, a sterile cotton swab soaked in sterile saline solution was used to swab a 4.5 x 4.5 cm². The sampling procedure is illustrated in **Figure 6**. The swab was gently rubbed ten times horizontally and vertically on the chosen skin area. After that, 1 mL of sterile saline solution was used to soak the swab; than in was vortexed for 2 minutes and 100 µL was spread on blood agar plates (Tryptic Soy agar-TSA, Oxoid, Milan, Italy, with 5% sheep blood). The plates were incubated at 37°C both in aerobic and anaerobic conditions for 3 and 5 days, respectively. Vitek 2 system (BioMérieux, France) was used to identify the isolated colonies that were stored at -80°C untile the use.

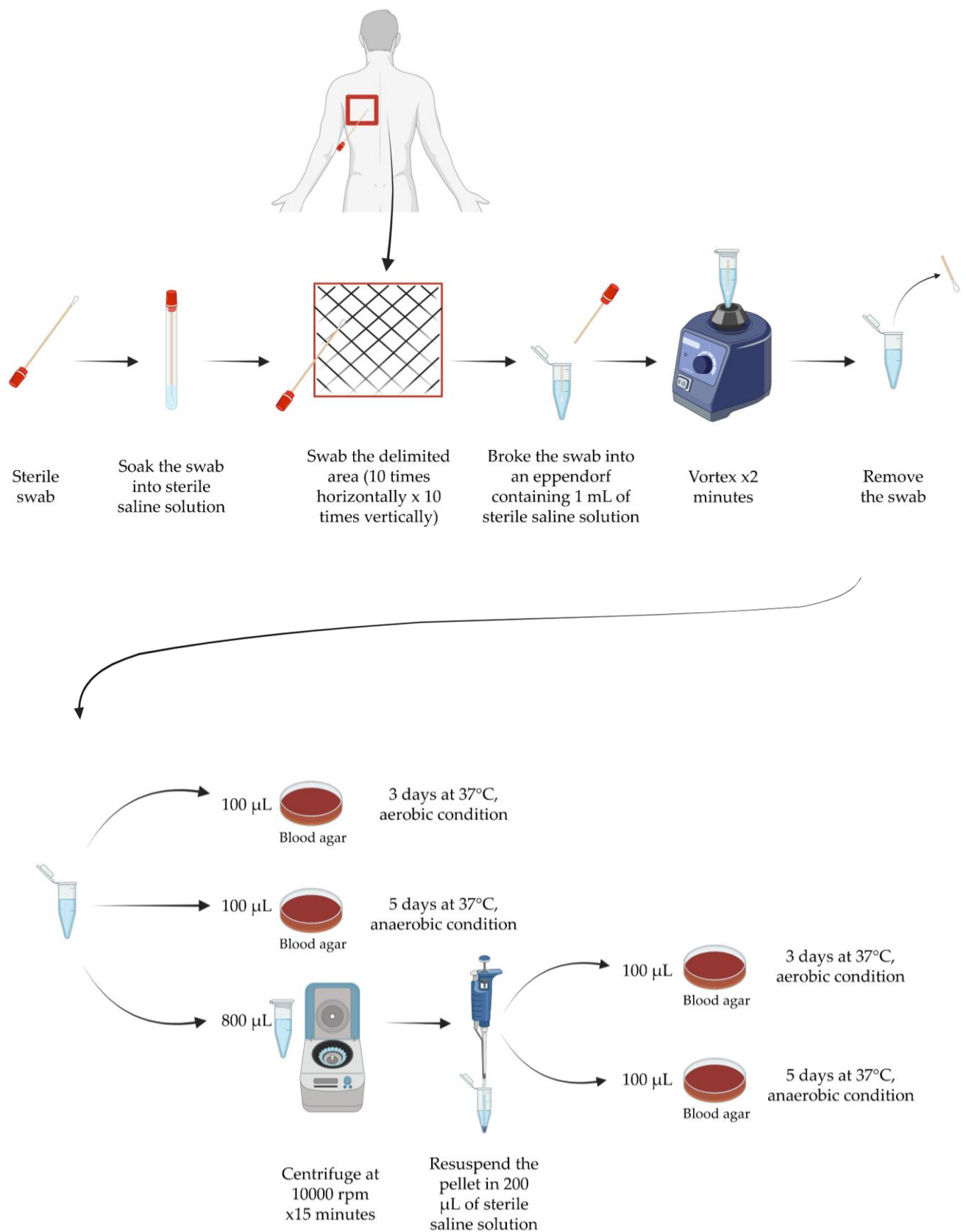


Figure 6. Skin microbiota sampling and collection.

3.2.3 PPE extraction and characterization

The pomegranates used in the research were harvested from a private farm in Abruzzo, Italy, and the peels were obtained by manually removing the arils.

According to Ciulla et al the extract, coming from both the internal and external pomegranate peel, was obtained using an eco-sustainable extraction method with low environmental impact [239].

This apparatus comprises a 250 mL evaporation chamber, a 500 mL condenser column, tubing, and a 30 mL extraction chamber, all constructed from AISI 316L steel. It is versatile, capable of processing both liquids and solids. The entire system is under the control of an Advanced Process Controller/Programmer (Eurotherm 2604, Schneider Electric, Italy), providing precise control over pressure and temperature (with an accuracy of >0.002 MPa and >0.01 K, respectively).

Operating conditions are monitored using temperature probes (RTD-PT100 class 1/3 DIN) acquired from OMEGA Engineering, Inc. (USA), positioned in both the evaporator chamber and the condenser column. Additionally, a 4–20 mA pressure transducer (Gems Sensors and Controls, UK) with a measurement range of 0–10 MPa is employed. Temperature regulation for all components is managed by two chiller/heater units with a cooling capacity of 1000 W. The evaporation chamber operates within a pressure range of 0.101–10 MPa. The solvent flow rate is controlled by a flowmeter (Bronkhorst EL-Flow, NL) and is visually monitored through a quartz window situated between the condenser and the extraction chamber. The tubing is insulated with a 5 cm layer of foam rubber. The influx of liquefied gas is measured both by the weight of the input gas and by flowmeter

measurements. The extraction apparatus is illustrated in **Figure 7**.

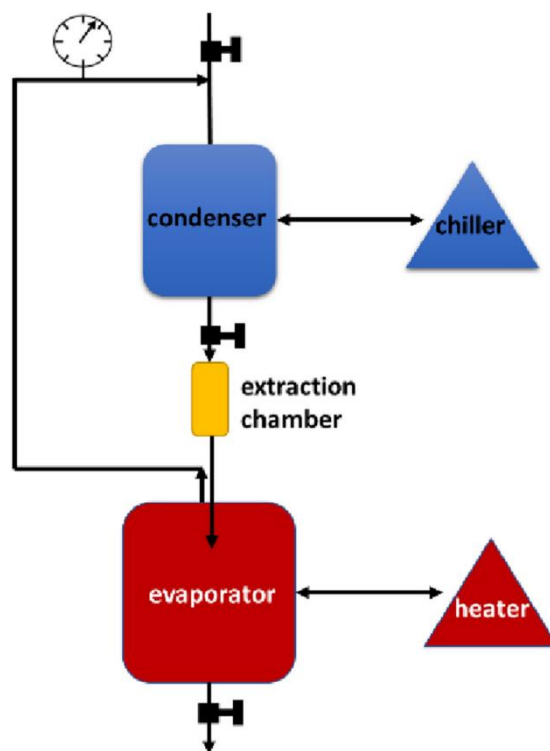


Figure 7. Extraction apparatus used to obtain Pomegranate Peel Extract using two different solvents: dymetil-ether and n-butane [239].

Two different extraction conditions were utilized: Dimethyl Ether (DME) and n-butane. The extraction process involved specific temperatures and flow rates for each solvent. The extraction of pomegranate peel was performed under two different conditions. The setup for extraction with DME is as follows: a condenser column temperature of 287 K, an evaporation chamber temperature of 318 K, and a liquefied DME flow rate of 9–10 mL/min. For extraction with nB, the setup is as follows: a condensation column temperature of 283 K, an evaporation chamber temperature of 318 K, and a liquefied nB flow rate of 8–9 mL/min. The sample, consisting of approximately 3.5–3.7 g of peels, was placed into the extraction chamber using a cellulose thimble for physical support and filtration. Subsequently, a vacuum (approximately 5×10^{-3} MPa) was applied to remove air from the

tubing. The liquefied gas eluent was injected once the set temperatures were reached. In each run, 40.0 g of eluent (nB or DME) was introduced, and the experiments were conducted for durations of 30 minutes, 1 hour, and 2.5 hours, with fresh samples loaded each time. At the end of the extraction, the liquefied gas was extracted from the sample by vaporizing it into a recovery chamber. The extract was stored under a nitrogen atmosphere at 275 K. After each experiment, the tubing was disassembled and thoroughly cleaned using polar and nonpolar solvents before being reassembled for the next run.

High-performance liquid chromatography (HPLC) was used to chemically characterize the pomegranate peel extract. The system was used composed by a Waters 600 controller, a quaternary pump, a column heater, an Agilent Infinity 1260 High Performance Degasser, and a Waters 2996 photodiode array detector. A Kinetex EVO column (250 x 4.6 mm I.D., 5 μ m particle size) was used. Separation was achieved using gradient elution of a mobile phase composed by a solution of water and 0.1 % acetic acid (Line A) and a mixture of acetonitrile and 2-propanol (75-25 %, v/v, Line B). Gradient elution starts with 98% of Line A, then in 25 minutes become 80%, and after 15 minutes become 35%, then remain constant for 10 minutes, after that become 5% in 2 minutes and remain constant for 5 minutes, finally, come back to the initial composition in 2 minutes and remain constant for 12 minutes for the re-equilibration step. The analytes were detected at their maximum wavelength, and the total run time was 70 minutes. Standard solutions of the investigated polyphenols were obtained by weighing an exact amount of each reference powder into an analytical balance and solubilizing them into a volumetric flask with methanol. Working standard solutions containing the analytes were obtained diluting stock solution with a mixture of water and methanol (50-50 %, v/v). The calibrators were obtained in the range 0.5-50 μ g/mL by further dilution of the standard solutions. the samples to be analyzed were prepared using 50 μ L of

extract and diluting it to 500µL with methanol. 20 µL was injected into the HPLC system. Calibration curve was obtained by linear regression analysis of concentrations (X-axis) to response (area, Y-axis) using Graph-Pad software. Before conducting microbiological tests, the characterized PPE was treated at 70°C for 30 minutes three times to ensure sterility, which was confirmed by spreading on nutrient agar plates.

3.2.4 Antimicrobial activity of PPE

- Minimum Inhibitory Concentration Minimum Bactericidal/ Fungicidal Concentration assay

In the study, the effectiveness of PPE was evaluated in terms of antimicrobial activity, by the comparison of two extract obtained with two different solvents (DME or n-butane). The antimicrobial activity of PPE was tested against reference strains: *S. aureus* ATCC 6538, *E. coli* ATCC 10536 and *C. albicans* ATCC 10231, and it was evaluated in terms of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal/Fungicidal Concentration (MBC/MFC) using the microdilution method, following the guidelines provided by the Clinical & Laboratory Standards Institute (CLSI) [240]. PPE in DME and n-butane extracts, were tested at different concentrations ranging from 0.06 mg/mL to 32 mg/mL. Bacterial strains were cultivated in Tryptic Soy Broth (TSB, Oxoid, Milan, Italy) at 37°C for 24 h. Then, a 1:10 dilution and a refresh in a thermostatic bath at 37°C in agitation (120 rpm) for 2 h, were performed. Bacterial suspensions were standardized to obtain an optical density (OD₆₀₀) of 0.12 (corresponding approximately 10⁷ CFU/mL). The yeast reference strain (*C. albicans* ATCC 10231) was cultured without overnight growth, in RPMI supplemented with 2% of glucose, following the refresh conditions of bacterial strains. Then it was standardized at OD₆₀₀ 0.15 (corresponding approximately to 10⁶ CFU/mL).

After standardization, the bacterial cultures followed a 1:100 dilution in Muller Hinton

Broth (MHB, Oxoid, Milan, Italy), while the yeast was diluted 1:10 in RPMI + 2% glucose and used for the experiments. Each standardized broth culture (100 µL) was inoculated in a 96-well microtiter plate and mixed with different concentrations of PPE in DME and PPE in n-butane. Plates were then incubated for 24-48 h.

Positive and negative controls were included for comparison. In detail, positive controls were represented by free-extract broth culture of each strain, free-broth culture diluted-extract represents the negative control. Only medium without PPE was included to verify the sterility of the medium.

The MBC and MFC were determined by sub-culturing suspensions, from the non-turbid wells, on Muller Hinton Agar (MHA, Oxoid, Milan, Italy) and Sabouraud Dextrose Agar (SDA, Oxoid, Milan, Italy) plates respectively, and incubated at 37°C for 24 h.

Once it was verified that PPE in DME was the most effective extract on bacteria, the MIC/MBC values of PPE in DME were determined on the following selected skin bacterial strains: *S. aureus* DAS 68, *S. aureus* DLS 69, *S. aureus* SP 70, *S. epidermidis* DLS 29 and *S. epidermidis* DAS 31.

- PPE activity against in formation biofilm

The effectiveness of PPE in DME to prevent biofilm formation was assessed using *S. epidermidis* DAS 31 and *S. aureus* DLS 69, both in mono and dual-species biofilms.

Figure 8 shows the setting of the 96-well microtiter plates used for the experiment and the dilution scheme. Sub-MIC concentrations of PPE (1/2 MIC, 1/4 MIC, 1/8 MIC; MIC *S. epidermidis* DAS 31 = 32 mg/mL; MIC *S. aureus* DLS 69 = 16 mg/mL; for the dual-species biofilm, *S. epidermidis* DAS 31 = 16 mg/mL + MIC *S. aureus* DLS 69 = 16 mg/mL) were inoculated with standardized bacterial cultures in 96-well flat-bottomed microtiter plates and incubated at 37°C in aerobic conditions for 3 and 24 h. Then, the supernatant was removed

and each well was rinsed with sterile water. Biofilm CFU/mL determination and quantification of biofilm biomass were then performed. In detail, the bottom of each well was scraped for the CFU/mL determination, then it was diluted and spread on Mannitol Salt Agar (MSA, Mannitol Salt Agar, Oxoid, Milan, Italy). For the biofilm biomass analysis, the washed and dried biofilm was stained with 0.1% Crystal violet solution, and the biomass was measured by absorbance (OD₅₇₀) using a microplate ELISA reader (BioRad, Milan, Italy) [190].

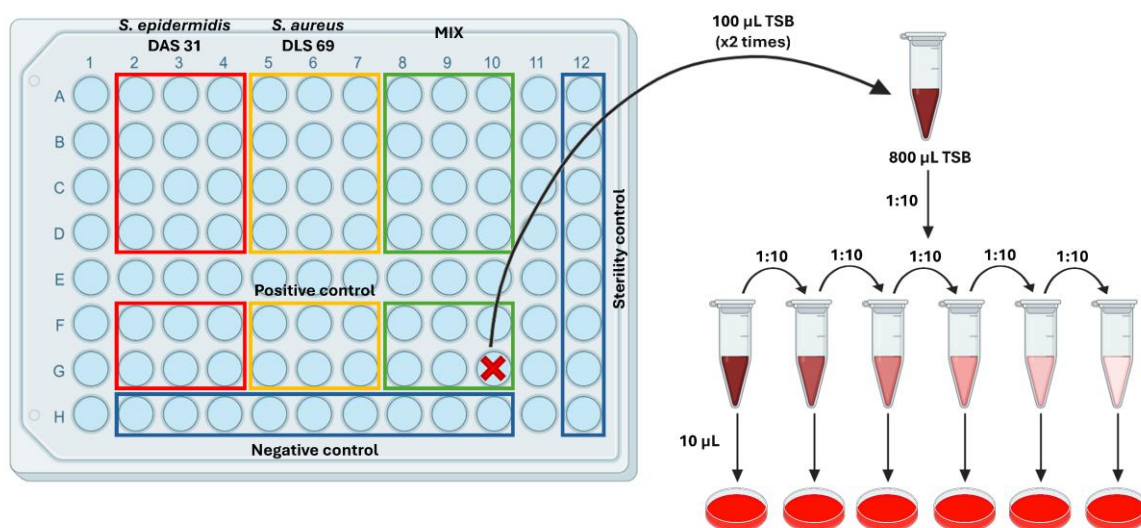


Figure 8. Antiadhesive experiment setting. 96-well plate setting for the antiadhesive activity evaluation of PPE against mono and dual-species biofilm. Positive control represents the non-treated sample. Negative control is represented by free-strain PPE. Sterility control is represented by free-strains and free-PPE culture medium.

3.2.5 PPE Toxicity Test

The toxicity of PPE in DME was assessed using *Galleria mellonella* larvae *in vivo* model, following the same methodology of Di Lodovico et al [241]. For the toxicity test, seven groups of 10 *G. mellonella* larvae, weighing between 0.2 and 0.3 g, were used. As shown in **Figure 9**, each larva received an injection of 10 µL of PPE, Phosphate Buffered Saline (PBS), or no injection at all (as control), into the last left proleg using 0.3 mL micro-

fine needle insulin syringes. In detail, five groups received different doses of PPE (8000 mg/kg, 1000 mg/kg, 500 mg/kg, 250 mg/kg, and 125 mg/kg), while two control groups were included - one treated with PBS and the other was left untreated. The larvae were then incubated at 37°C in the dark for seven days in Petri dishes, and their survival rate was monitored daily. Larvae were considered dead when they turned black and were unresponsive to touch. Throughout the assays, the larvae did not receive any nutrition. The survival rate was calculated using a Kaplan-Meier curve [242].

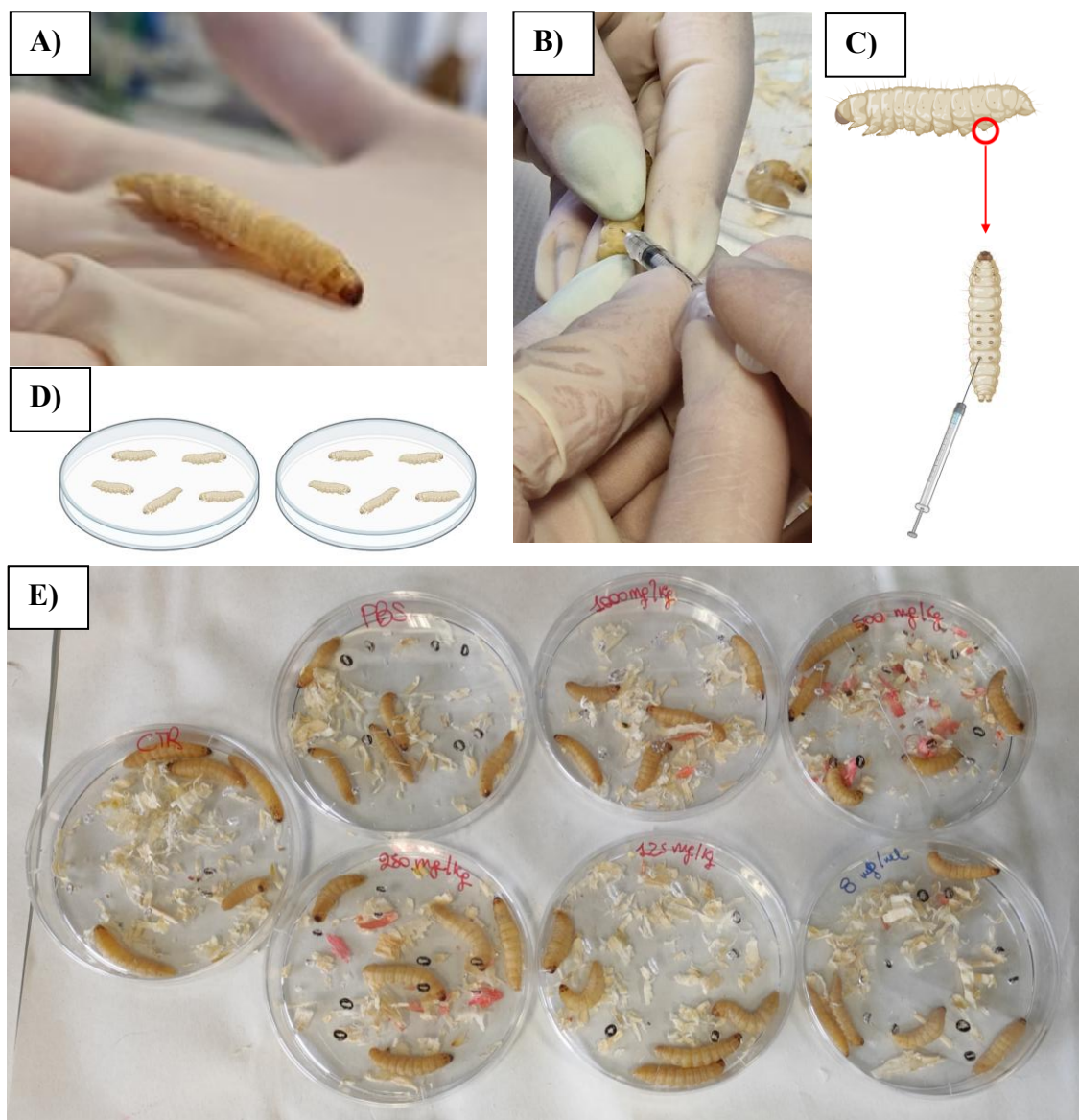


Figure 9. *Galleria mellonella* larvae used in the study. **A:** *G. mellonella* larvae. **B** and **C:** *G. mellonella* modality of injection. **D** and **E:** larvae setting for the experiments.

3.2.6 Statistical analysis

The results were obtained from at least three separate experiments, each performed twice, and are expressed as average $\pm$ standard deviation. $p \leq 0.05$ were regarded as statistically significant. Group variances were assessed using the ANOVA test.

3.3 Results

3.3.1 Skin microbiota sampling

A total of 67 microorganisms were identified from the skin of healthy volunteers, as documented in **Figure 10**. The most prevalent bacteria on the skin were *S. epidermidis* (13%) was the most abundant, followed by *Micrococcus luteus* (12%), *C. acnes* (12%), and *S. hominis* (11%).

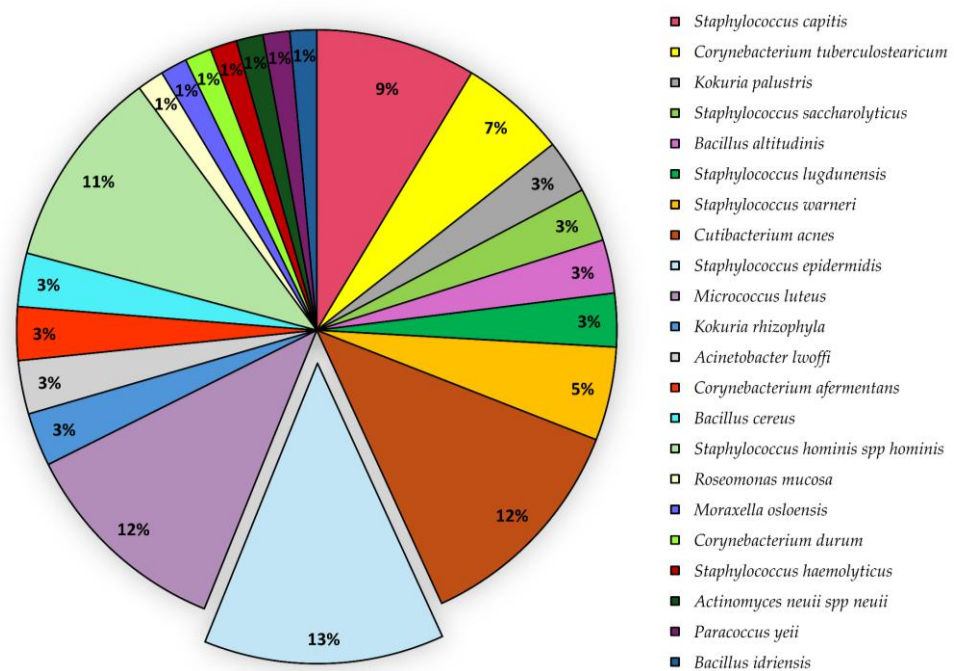


Figure 10. Collection of skin microbiota samples. Percentage of skin microbiota species identified from healthy volunteers enrolled in the study.

Figure 11 illustrates the main relative abundance of bacterial species isolated from male and female volunteers. The predominant bacterial genus isolated from both sexes was *Staphylococcus* (35% in males and 51% in females). In male skin, the dominant taxonomic groups were *M. luteus* (16%), *C. acnes* (11%), *S. hominis* (11%), *S. epidermidis* (8%), and *S. capitis* (8%). In female skin, they were *S. epidermidis* (17%), *C. acnes* (14%), *S. hominis* (10%), *M. luteus* (10%), and *S. capitis* (7%). *Staphylococcus saccharolyticus*, *Kokuria*

rhizophyla, *Corynebacterium afermentans*, and *Bacillus cereus* were exclusively detected in male subjects, while *S. lugdunensis* was only found in female subjects.

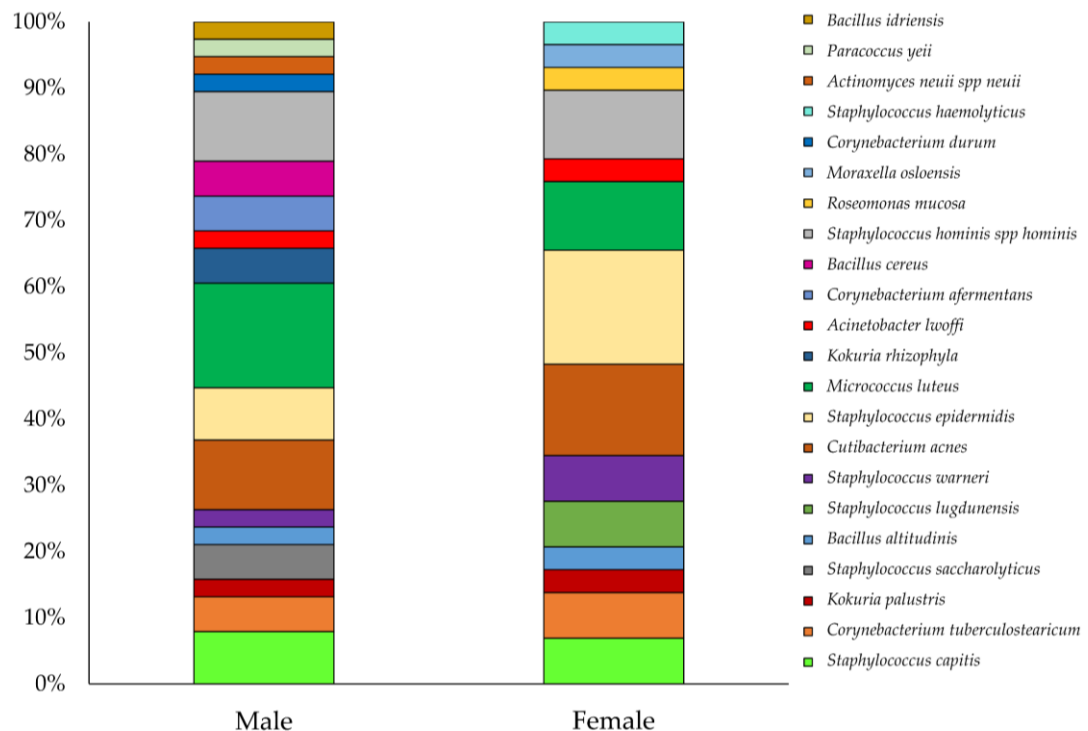


Figure 11. Male and female distribution of isolated skin bacteria from the back of volunteers enrolled in the study.

Corynebacterium durum and *Paracoccus yeii* strains were only identified in a volunteer from the Middle East region. Furthermore, a subject that declared who had previously experienced skin irritation hosted *Actinomyces neuui spp. neuui* and *B. idriensis*, which were not found in the other volunteers. This data highlights the variability in skin microbiota among subjects from different geographic areas or with skin conditions. *Staphylococcus aureus* was the only strain isolated from subjects with AD.

Identifies bacterial species and their antimicrobial profile are listed in **Table 2**. *Staphylococcus aureus* DAS 68, *S. aureus* DLS 69, *S. aureus* SP 70, *S. epidermidis* DLS 29, and *S. epidermidis* DAS 31 were selected for the experiments.

Table 2. Antimicrobial profile of collected skin strains.

Collected and identified skin strains	Antibiotic resistance profile		
	Resistant	Intermediate	Sensitive
<i>Staphylococcus capitis</i> DLS 1			D-G-L-O-Te-T-V
<i>Staphylococcus capitis</i> DF 2	G		D-L-O-Te-T-V
<i>Staphylococcus capitis</i> RRA 3			D-G-L-O-Te-T-V
<i>Staphylococcus capitis</i> CG 4			D-G-L-O-Te-T-V
<i>Staphylococcus capitis</i> DMA 5			D-G-L-O-Te-T-V
<i>Corynebacterium tuberculostearicum</i> DLS 6	B-Cl	C	L-R-V
<i>Corynebacterium tuberculostearicum</i> DAS 7	Cl	C	B-L-R-V
<i>Corynebacterium tuberculostearicum</i> DF 8	B-Cl	C	L-R-V
<i>Corynebacterium tuberculostearicum</i> RRA 9	Cl	C	B-L-R-V
<i>Kokuria palustris</i> DAS 10	\	\	\
<i>Kokuria palustris</i> RRA 11	\	\	\
<i>Staphylococcus saccharolyticus</i> RRA 12	\	\	\
<i>Staphylococcus saccharolyticus</i> DLFM 13	\	\	\
<i>Bacillus altitudinis</i> DLS 14	E	C-Le	Cl-V-L-M
<i>Bacillus altitudinis</i> RRA 15		C-Le	Cl-L-E-V-M
<i>Staphylococcus lugdunensis</i> DLS 16		Le	AF-B-Cl-D-E-G-O-R- Te-Tet-T-TS-V
<i>Staphylococcus lugdunensis</i> DAS 17		Le	AF-B-Cl-D-E-G-O-R- Te-Tet-T-TS-V
<i>Staphylococcus warneri</i> DAS 18			D-G-L-O-Te-T-V
<i>Staphylococcus warneri</i> CG 19			D-G-L-O-T-V
<i>Staphylococcus warneri</i> DMA 20			D-G-L-O-Te-T-V
<i>Cutibacterium acnes</i> DLS 21	\	\	\
<i>Cutibacterium acnes</i> DAS 22	\	\	\
<i>Cutibacterium acnes</i> DF 23	\	\	\
<i>Cutibacterium acnes</i> DLFM 24	\	\	\
<i>Cutibacterium acnes</i> DLFM 25	\	\	\
<i>Cutibacterium acnes</i> CG 26	\	\	\
<i>Cutibacterium acnes</i> CG 27	\	\	\
<i>Cutibacterium acnes</i> DMA 28	\	\	\
<i>Staphylococcus epidermidis</i> DLS 29			D-G-L-O-T-V

<i>Staphylococcus epidermidis</i> DLS 30			D-G-L-O-T-V
<i>Staphylococcus epidermidis</i> DAS 31			D-G-L-O-T-V
<i>Staphylococcus epidermidis</i> RRA 32			D-G-L-O-T-V
<i>Staphylococcus epidermidis</i> DLFM 33			D-G-L-O-T-V
<i>Staphylococcus epidermidis</i> DLFM 34			D-G-L-O-T-V
<i>Staphylococcus epidermidis</i> CG 35			D-G-L-O-T-V
<i>Staphylococcus epidermidis</i> CG 36			D-G-L-O-T-V
<i>Micrococcus luteus</i> DLS 37	\	\	\
<i>Micrococcus luteus</i> DLS 38	\	\	\
<i>Micrococcus luteus</i> DAS 39	\	\	\
<i>Micrococcus luteus</i> DF 40	\	\	\
<i>Micrococcus luteus</i> RRA 41	\	\	\
<i>Micrococcus luteus</i> RRA 42	\	\	\
<i>Micrococcus luteus</i> DLFM 43	\	\	\
<i>Micrococcus luteus</i> DLFM 44	\	\	\
<i>Micrococcus luteus</i> DMA 45	\	\	\
<i>Kokuria rhizophyla</i> RRA 46	\	\	\
<i>Kokuria rhizophyla</i> DLFM 47	\	\	\
<i>Acinetobacter lwoffii</i> DLS 48	\	\	\
<i>Acinetobacter lwoffii</i> DMA 49	\	\	\
<i>Corynebacterium afermentans</i> DF 50	Cl	C	B-L-R-V
<i>Corynebacterium afermentans</i> DLFM 51	Cl		B-C-L-R-V
<i>Bacillus cereus</i> RRA 52		C-Le	C-E-L-M-V
<i>Bacillus cereus</i> DLFM 53		C-Le	Cl-E-L-M-V
<i>Staphylococcus hominis spp hominis</i> DLS 54			D-G-L-O-T-V
<i>Staphylococcus hominis spp hominis</i> DAS 55			D-G-L-O-T-V
<i>Staphylococcus hominis spp hominis</i> DF 56	O		D-G-L-T-V
<i>Staphylococcus hominis spp hominis</i> RRA 57			D-G-L-O-T-V
<i>Staphylococcus hominis spp hominis</i> RRA 58			D-G-L-O-T-V
<i>Staphylococcus hominis spp hominis</i> DLFM 59			D-G-L-O-T-V
<i>Staphylococcus hominis spp hominis</i> CG 60			D-G-L-O-T-V
<i>Roseomonas mucosa</i> DLS 61	\	\	\

<i>Moraxella osloensis</i> DLS 62	\	\	\
<i>Corynebacterium durum</i> DF 63	Cl-C		B-R-L-V
<i>Staphylococcus haemolyticus</i> CG 64	\	\	\
<i>Actinomyces neuuii spp neuuii</i> DLFM 65	\	\	\
<i>Paracoccus yeii</i> DF 66	\	\	\
<i>Bacillus idriensis</i> DLFM 67		C-Le	E-Cl-L-M-V
<i>Staphylococcus aureus</i> DAS 68	Cl-E	Le	AF-B-Ce-D-G-L-O-R- Te-Tet-T-TS-V
<i>Staphylococcus aureus</i> DLS 69	L-P-Fox	E	Le-Te-V-Tet-R-Net-G
<i>Staphylococcus aureus</i> SP 70	E-L-P-Fox	Te-Tet	Le-V-R-Net-G

D: Daptomycin; G: Gentamicin; L: Linezolid; O: Oxacillin; T: Tigecycline; V: Vancomycin; Te: Teicoplanin; C: Ciprofloxacin; Cl: Clindamycin; E: Erythromycin; Le: Levofloxacin; M: Meropenem; B: Benzylpenicillin; R: Rifampicin; AF: Fusidic Acid; Ce: Ceftriaxone; Tet: Tetracycline; TS: Trimethoprim/Sulfamethoxazole; P: Penicillin; Net: Netilmicin; Fox: Cefoxitin.

In **Figure 12**, it's highlighted the differences between healthy skin and altered skin; while healthy skin is associated with a high variability of microbial species and a low microbial load, skin with dysbiosis is characterized by the predominance of one or few harmful species and by an increase in terms of bacterial load.

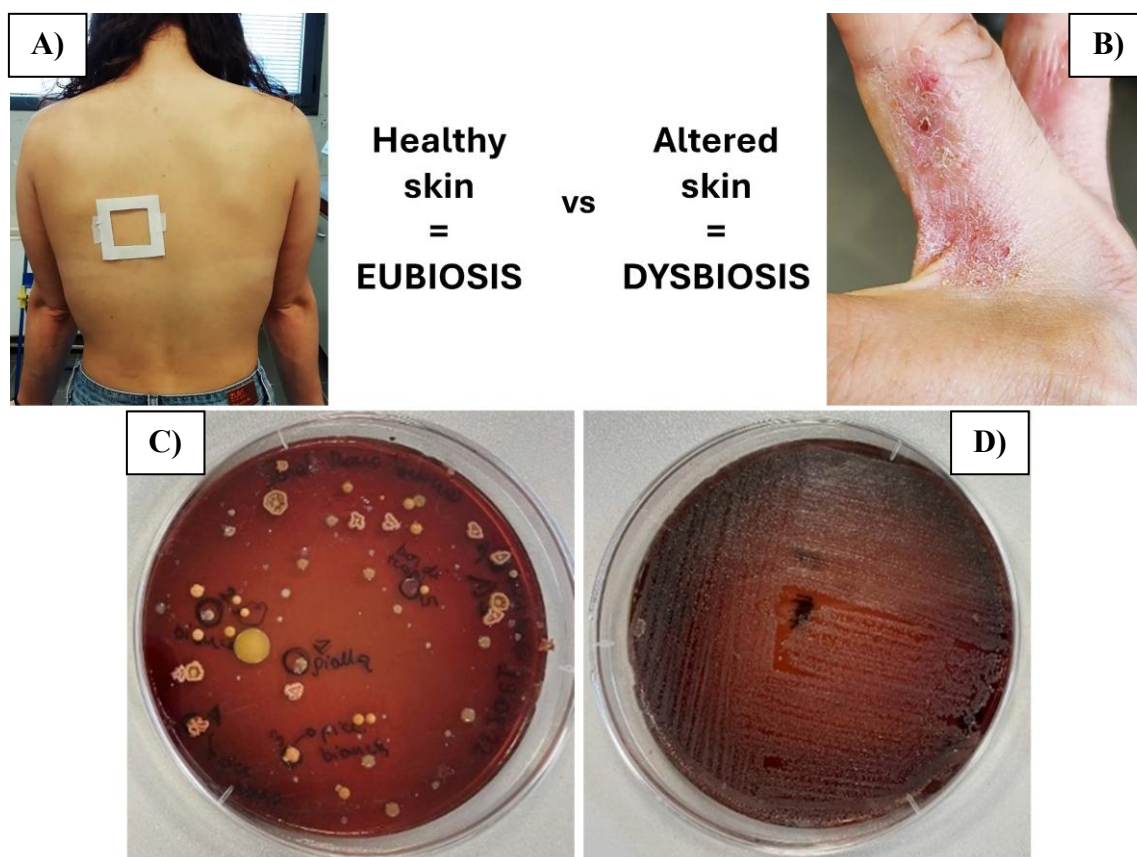


Figure 12. Healthy skin volunteers (A) and skin of a person affected by dysbiosis (B). Representative plates of skin microbiota samples coming from skin eubiosis (C) and skin dysbiosis (D), from volunteers enrolled in the study.

3.3.2 PPE characterization

Following the tyndallization process, no differences in antimicrobial activity were observed between tyndallized and non-tyndallized extracts of PPE in DME (data not shown). The phytochemical analysis outlined in **Table 3** denotes that the predominant components included Catechin, Quercetin, Vanillic acid, and Gallic acid.

Table 3. Phytochemicals characterization of PPE in DME (Concentrations are expressed in $\mu\text{g/mL}$)

Phytochemical compounds							
Gallic acid	Catechin	Vanillic acid	Chlorogenic acid	Caffeic acid	Rutin	Quercetin	Kaempferol
7.49	24.32	8.35	4.52	3.52	3.27	9.69	7.40

3.3.3 Antimicrobial activity of PPE

- Minimum Inhibitory Concentration Minimum Bactericidal/ Fungicidal Concentration assay

In this research the antimicrobial efficacy of PPE extracted using DME and n-butane was evaluated against reference strains. **Table 4** outlines the MIC and MBC/MFC of PPE in DME and n-butane against the reference strains, with MIC values ranging from 1 to 32 mg/mL. PPE in DME demonstrated the highest activity against bacteria, with MIC values of 1 mg/mL for *S. aureus* ATCC 6538 and 2 mg/mL for *E. coli* ATCC 10536. Meanwhile, PPE in n-butane was most effective against *C. albicans* ATCC 10231. All MBC/MFC values were higher than 32 mg/mL. Since only bacterial species were isolated from the skin microbiota of the volunteers enrolled in the study, subsequent tests were carried out using PPE in DME.

Table 4. MIC and MBC/MFC values of PPE in DME and n-butane against reference strains
(Concentrations are expressed in mg/mL)

Strains	PPE in DME		PPE in n-butane	
	MIC	MBC/MFC	MIC	MBC/MFC
<i>S. aureus</i> ATCC 6538	1	>32	32	>32
<i>E. coli</i> ATCC 10536	2	>32	16	>32
<i>C. albicans</i> ATCC 10231	32	>32	4	>32

MIC: Minimum Inhibitory Concentration; MBC/MFC: Minimum Bactericidal/ Fungicidal Concentration; PPE: Pomegranate Peel Extract; DME: dimethyl ether.

Table 5 shows MIC and MBC values of PPE in DME against skin bacterial isolates. The MIC values for clinical strains ranged from 16 mg/mL to 128 mg/mL, while all MBC values were higher than 128 mg/mL. Specifically, MIC values of the clinical strains *S. aureus* DAS 68, *S. aureus* DLS 69 and *S. aureus* SP 70 were 128, 16 and 128 mg/mL respectively, while MIC values for *S. epidermidis* DLS 29 and *S. epidermidis* DAS 31 were

128 and 16 mg/mL, respectively. Among these strains, *S. aureus* DLS 69 and *S. epidermidis* DAS 31 were selected to perform experiments about anti-adhesive activity.

Table 5. MIC and MBC values of PPE in DME of isolated skin bacteria (Concentrations are expressed in mg/mL)

PPE in DME		
Strains	MIC value	MBC value
<i>S. aureus</i> DAS 68	128	>128
<i>S. aureus</i> DLS 69	16	>128
<i>S. aureus</i> SP 70	128	>128
<i>S. epidermidis</i> DLS 29	128	>128
<i>S. epidermidis</i> DAS 31	32	>128

MIC: Minimum Inhibitory Concentration; MBC: Minimum Bactericidal Concentration; PPE: Pomegranate Peel Extract; DME: dimethyl ether.

- PPE anti-adhesive activity

In **Figure 13**, the anti-adhesive effect of PPE in DME is shown against mono-species biofilm. The biofilm of *S. epidermidis* DAS 31 exhibited a significant increase in CFU/mL at 1/8 MIC (4 mg/mL) after 3h (**Figure 13A**) and at all tested concentrations after 24 hours (**Figure 13C**), compared to the control. Conversely, there was a notable reduction in *S. aureus* DLS 69 CFU/mL at 1/2 MIC and 1/4 MIC (8 mg/mL and 4 mg/mL, respectively) both after 3 and 24h, compared to the control (**Figure 13B, D**). At these tested concentrations, the PPE in DME selectively affected *S. aureus* DLS 69, promoting the growth of *S. epidermidis* DAS 31, showing a specie-specific action.

Figure 14 shows that after 24h, PPE in DME displayed a remarkable anti-adhesive effect on *S. aureus* DLS 69 mono-species biofilms at 8 mg/mL and 4 mg/mL, resulting in a 12.2% and 16.2% reduction in biomass, respectively, without interfering with the growth of *S. epidermidis* DAS 31.

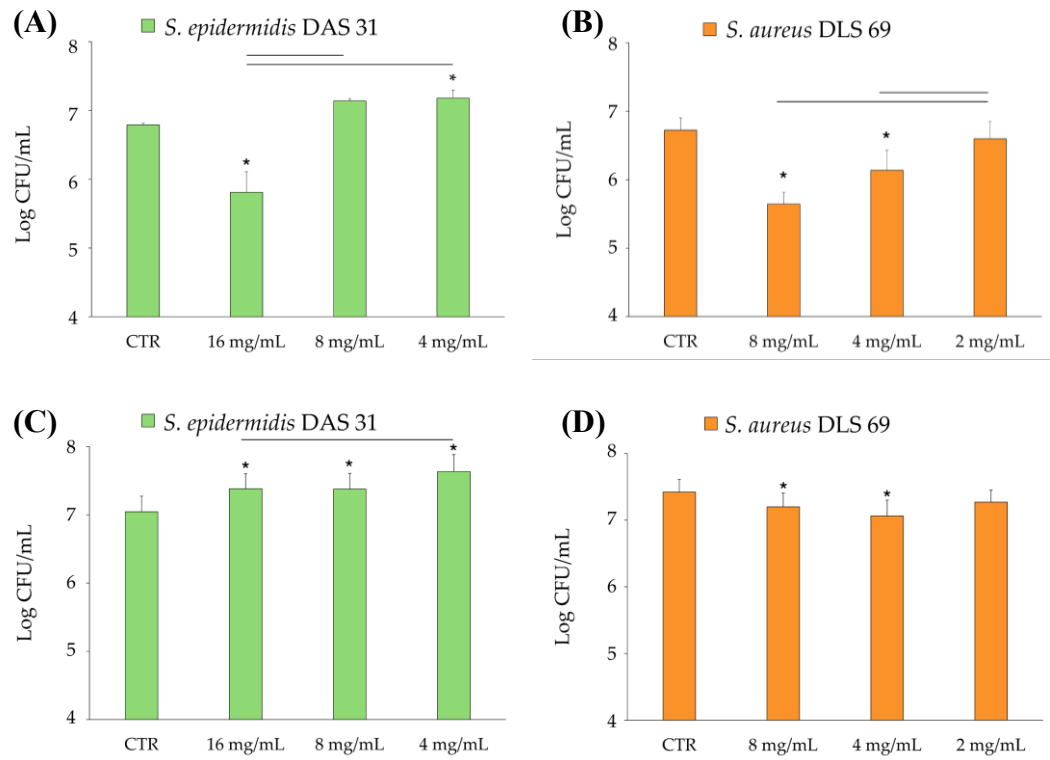


Figure 13. PPE in DME effect on mono-species biofilm formation after 3h and 24h. Log CFU/mL of *S. epidermidis* DAS 31 (A, C) and *S. aureus* DLS 69 (B, D) in mono-species biofilms in presence of PPE in DME at 16, 8, 4 mg/mL (1/2, 1/4 and 1/8 MIC *S. epidermidis* DAS 31) and 8, 4, 2 mg/mL (1/2, 1/4 and 1/8 MIC of *S. aureus* DLS 69) at 3 h (A, B) and 24 h (C, D). *Statistically significant in respect to the control. The lines indicate the intragroup significance. (CTR: control)

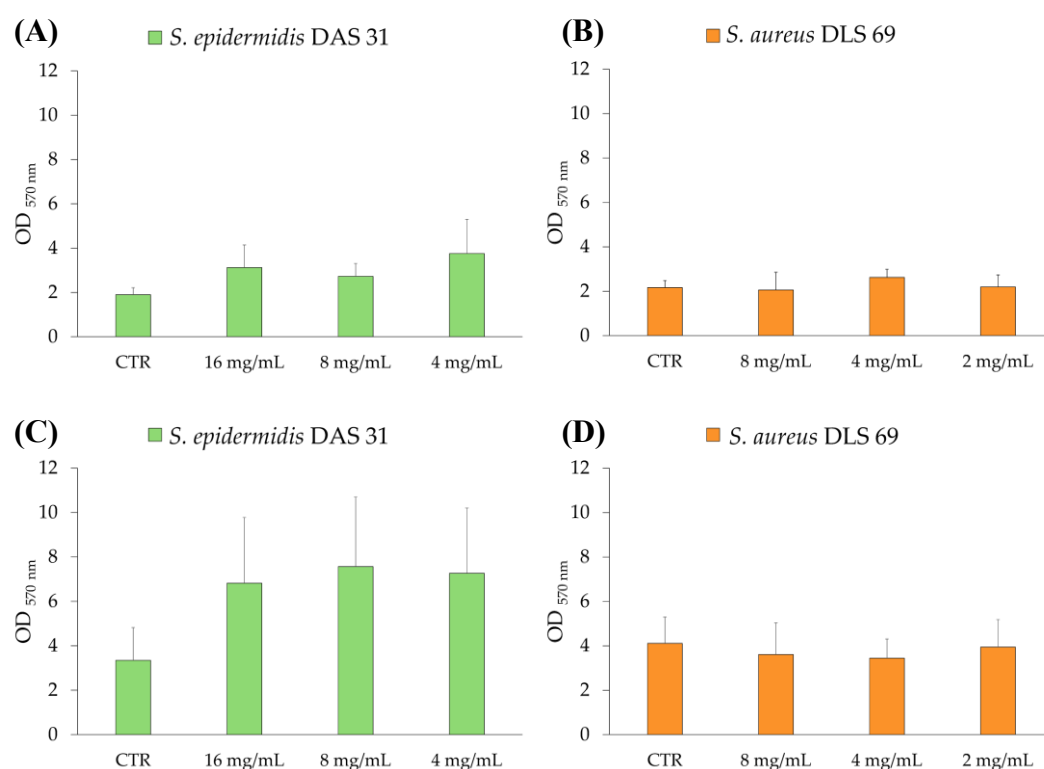


Figure 14. PPE in DME effect on mono-species biofilm formation after 3h and 24h. *S. epidermidis* DAS 31 (A, C) and *S. aureus* DLS 69 (B, D) biofilm biomass after treatment with 16, 8, 4 mg/mL (1/2, 1/4 and 1/8 MIC *S. epidermidis* DAS 31) and 8, 4, 2 mg/mL (1/2, 1/4 and 1/8 MIC of *S. aureus* DLS 69) of PPE in DME at 3 h (A, B) and 24 h (C, D). (CTR: control)

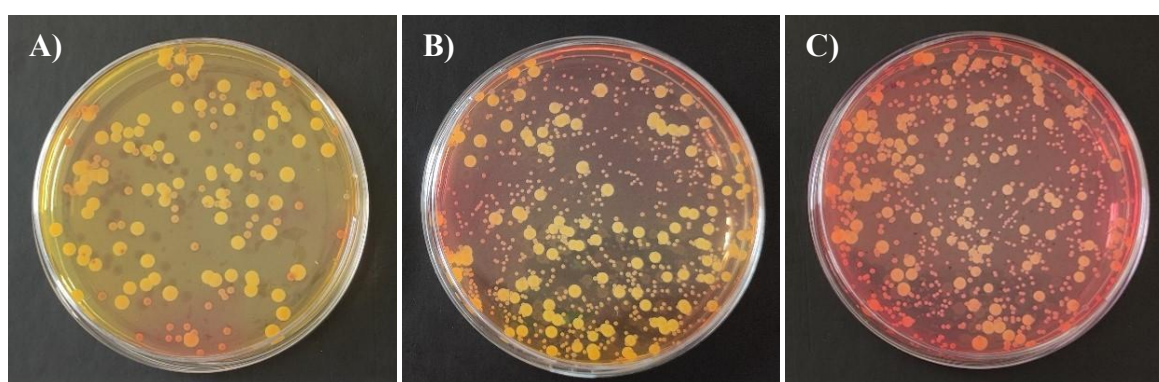


Figure 15. Representative plates of dual-species biofilm of *S. epidermidis* DAS 31 and *S. aureus* DLS 29 non-treated (A) treated with 4 mg/mL and 8 mg/mL (B and C, respectively) of PPE in DME after 24 h of incubation.

In **Figure 15**, they are reported representative pictures of dual-species bacterial growth of *S. epidermidis* DAS 31 and *S. aureus* DLS 69 on MSA plates; the first one (**Figure 15A**), is the non-treated sample that represents the control while the **Figures 15B** and **15C** represent samples treated with 4 and 8 mg/mL of PPE in DME, respectively.

In **Figure 16**, the Log CFU/mL of dual-species biofilm is shown after treatment with 1/2 MIC (8 mg/mL), 1/4 MIC (4 mg/mL), and 1/8 MIC (2 mg/mL) of PPE in DME at 3 h and 24 h, in respect to the control. A significant increase in CFU/mL of *S. epidermidis* DAS 31 was observed at 4 mg/mL and 2 mg/mL after 3 and 24 h, and at 8 mg/mL after 24 h.

Staphylococcus aureus DLS 69 growth was not significantly affected at any concentration, both after 3 and 24 h of treatment.

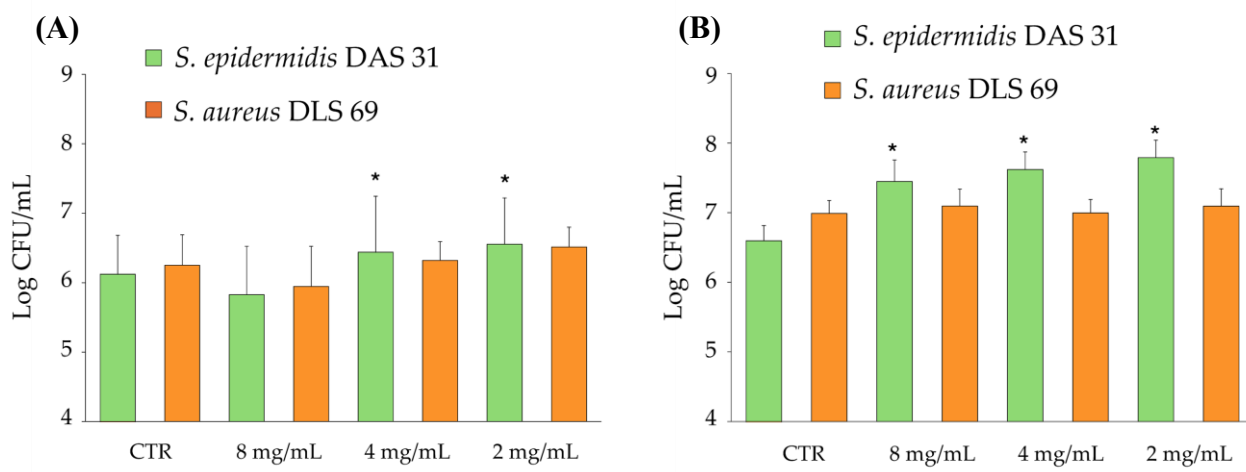


Figure 16. PPE in DME effect on dual-species biofilm formation after 3h and 24h. Log CFU/mL of *S. epidermidis* DAS 31 and *S. aureus* DLS 69 in dual-species biofilm, in presence of 8, 4, 2 mg/mL (1/2, 1/4 and 1/8 MIC) PPE in DME after 3 h (**A**) and 24 h (**B**). *Statistically significant in respect to the control. (CTR: control)

3.3.4 Toxicity test

The toxicity of PPE in DME was examined at several extract concentrations using the *G. mellonella* larvae *in vivo* model. As displayed in **Figure 17**, at 8000 mg/kg, that represents the highest tested concentration, the survival rate of larvae was 90% after 1 day and 80%

after 7 days. Similarly to the injection control (treated with PBS only), there is the same survival rate in larvae treated with PPE, indicating the safety of the extract. At lower concentrations (125 mg/mL, 250 mg/mL, 500 mg/mL and 100 mg/kg), the survival rates ranged from 80% to 90%. The control group displayed a survival rate of 80% after 7 days. As shown in **Figure 18**, larvae were considered dead when they turned into black, and they were unresponsive to touch.

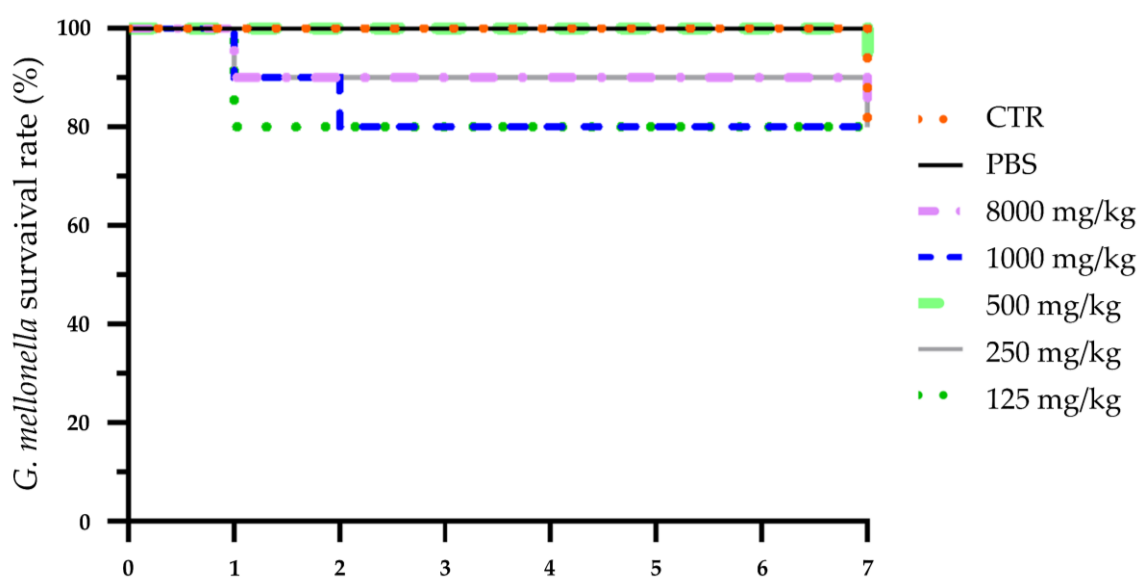


Figure 17. In vivo toxicity assay. Survival rate of *Galleria mellonella* larvae after treatment with PPE in DME, shown through Kaplan-Meier curve.

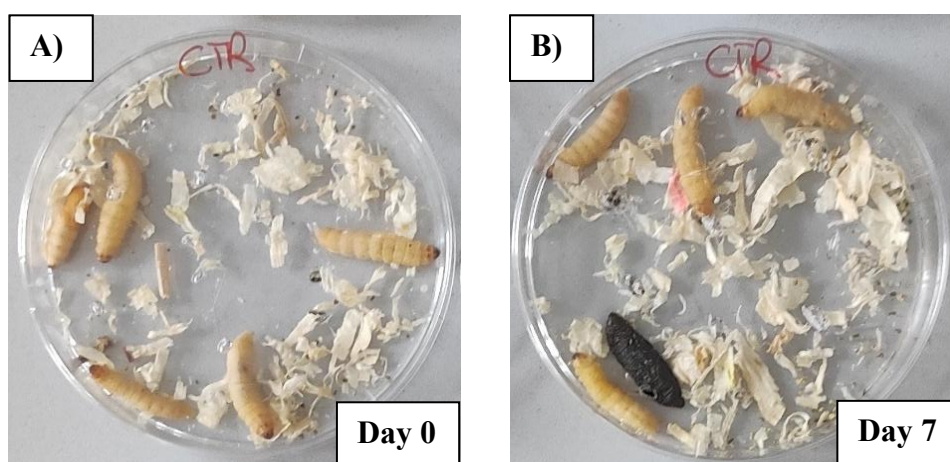


Figure 18. Comparison of the control sample (CTR) of *G. mellonella* larvae at day 0 (A) and after 7 days (B). Dead larvae turn black and they are unresponsive to touch.

3.4 Discussion

Human skin is a complex ecosystem where the microbiota plays an essential role in maintaining skin health and resilience. This PhD thesis chapter highlight the findings about pomegranate peel extract, particularly when extracted using dimethyl ether, representing a promising, innovative and eco-sustainable strategy for restoring skin microbial balance. The selective antimicrobial and anti-biofilm activities of PPE observed in this study align with and extend the growing body of evidence supporting the therapeutic potential of natural plant extracts rich in bioactive compounds [243].

The bacterial composition of the skin strains isolated from healthy subject, corresponds to the eubiosis-associated microbiota described in previous literature [13]. However, unlike the literature, the absence of significant differences in microbiota composition between males and females, is not in accordance with other findings, probably due to the limited number of enrolled subjects and the homogeneity of the studied population in terms of age, lifestyle and environmental exposure [244]. These results emphasize the influence of external and geographical factors on microbial diversity, as supported by prior studies showing shifts in microbiota related to regional differences [245]. Dysbiosis, characterized by the overgrowth of pathogens like *S. aureus* and the loss of beneficial commensals such as *S. epidermidis*, is an indicator of skin disorders. Rebalancing this microbial ecosystem through selective stimulation of commensals and inhibition of pathogens represents a promising therapeutic strategy.

The antimicrobial activity of PPE in DME against *S. aureus* and *E. coli* demonstrated in this study confirms its broad-spectrum efficacy against both Gram positive and Gram negative, which is attributed to its high content of bioactive polyphenols, including catechin, quercetin, vanillic acid and gallic acid. These phenolic compounds act through various

mechanisms, such as disrupting bacterial membranes, impairing energy metabolism, and inducing oxidative stress [246]. The higher susceptibility of *S. aureus* compared to *E. coli* probably reflects the structural differences between Gram-positive and Gram-negative bacteria, as the outer membrane of Gram-negative provides an additional protection against the penetration phenolic compounds [247].

The anti-biofilm activity of PPE, and in particular, its ability to disrupt *S. aureus* biofilms and simultaneously promote *S. epidermidis* biofilms, underscores its species-specific effects. This finding aligns with previous studies indicating that PPE polyphenols like quercetin and gallic acid, interfere with quorum sensing, downregulating biofilm-related genes such as *icaA*, *icaD*, and *agrA*, which are critical for biofilm formation in *S. aureus* [248,249]. In particular, quercetin is capable to interfere with the initial bacterial attachment to surface, by extracellular pili deletion [250]. Conversely, the ability of PPE to enhance *S. epidermidis* biofilm formation suggests a prebiotic-like effect, potentially supporting the role of commensals in maintaining skin barrier integrity and inhibiting pathogen colonization. In fact, as demonstrated also in recent researches, PPE is known to have a prebiotic activity against intestinal bacteria, improving the viability and metabolism of probiotic bacteria of the gut, representing a safe and eco-sustainable alternative to restore the balance in this site [226,236,237]. Likewise, probably the PPE acts on *S. epidermidis*, which represents one of the most representative commensal strains of the skin that contribute to maintaining skin health by production of ceramides, AMP and other metabolites [92].

This effect is probably due to the ability of phenolic compounds in pomegranate peel to influence the expression of genes critical for *S. aureus* biofilm formation, disrupting the production of the extracellular matrix, as documented in previous studies [190].

The use of liquefied gases, specifically DME and n-butane, as extraction solvents

represents a significant step toward eco-friendly processing of agricultural waste [251,252]. Using dimethyl ether (DME) as a solvent in green extraction can be advantageous as it is a non-toxic and biodegradable solvent with low boiling point, which reduces the energy required for evaporation and reduces the risk of environmental contamination. Additionally, DME has a low critical temperature, which allows for efficient extraction at low temperatures, reducing the thermal degradation of heat-sensitive compounds [253]. This makes DME a suitable choice for green extraction of valuable compounds from plant materials, such as essential oils, phytochemicals, and natural pigments. For this reason, it was defined by Takeishi as “a clean fuel/energy for the 21st century” [254]. The green extraction method employed in this study not only ensures the preservation of bioactive compounds but also aligns with eco-sustainability by minimizing energy consumption and solvent waste. This approach takes advantage from the natural richness of pomegranate peels, an important by-product of juice production, turning waste into a valuable resource in line with the circular economy model.

The *in vivo* toxicity results demonstrated the no-toxicity of PPE. The high survival rate of *G. mellonella* larvae further validates the potential for PPE to be used in topical formulations for managing skin infections and dysbiosis.

The species-specific antimicrobial and anti-biofilm activity of PPE underscores its potential as a targeted solution for managing skin dysbiosis and related conditions. Its non-cytotoxic nature further supports its use in topical formulations for dermatological applications. Future research should focus on exploring synergistic combinations of PPE with other natural or synthetic agents to enhance its therapeutic efficacy and broad-spectrum application. Additionally, *in vitro* studies on human skin models and *in vivo* studies, would provide deeper insights into its practical use in clinical and cosmetic formulations.

4. ALMOND HULL EXTRACT (AHE): Effect on microbial biofilm

4.1 Introduction

The almond (*Prunus dulcis* L.) is one member of the tree nuts family, that includes also cashews, Brazil nuts, walnuts, hazelnuts, macadamia nuts, pistachios, pecans and pine nuts [255] and it represent one of the earliest domesticated trees [256].

Since the optimum temperature for sprouting and flowering of the tree is 24°C, almonds originated in the Mediterranean basin, and their ability to thrive in arid and semi-arid climates established them as a stable crop in regions such as Portugal, Spain and Italy [257]. In particular, in Italy, almond cultivation is concentrated in the southern regions, specifically in Sicily and Apulia. In Avola, a city in the south of Sicily, the “Pizzuta” variety is cultivated and it is internationally recognized for its exceptional quality [258].

As shown in **Figure 19**, almond consists of three distinct layers: the hull, shell and kernel [259,260].

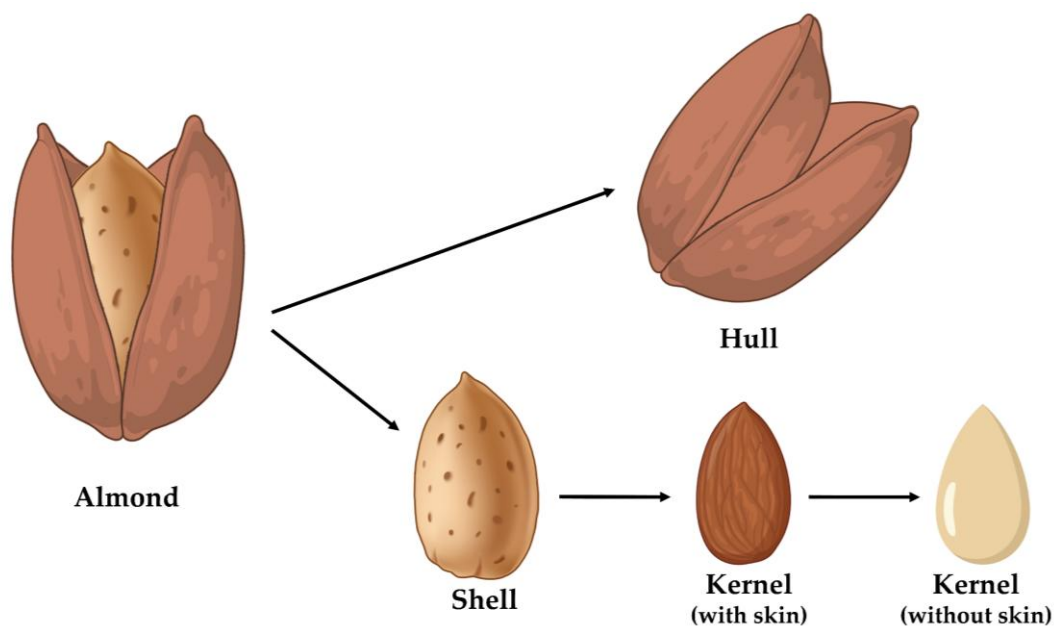


Figure 19. Almond fruit.

The outermost layer, known as the hull, is a fibrous, green structure that surrounds and protects the inner layers during the early stages of fruit development. When the fruit matures, the hull dries out and opens, exposing the shell inside. The hull represents the largest portion of the fruit by weight, accounting for approximately 35-62%, depending on the almond variety [261]. Although it is traditionally considered as an agricultural waste, the hull contains a lot of bioactive compounds, including polyphenols, flavonoids and dietary fibers, making it a valuable by-product with emerging uses in pharmaceuticals, cosmetics and sustainable agriculture [262].

Below the hull there is the shell, a woody layer that encloses the kernel of the almond. It is mainly composed by lignin-carbohydrate complexes with antioxidant antidiabetic properties due to its capacity to inhibit α -glucosidase [263]. The shell, contributing around 25-35% of the weight of the fruit, plays a crucial role in protecting the kernel from mechanical damage and environmental factors.

The kernel, commonly referred to as the almond nut, is the edible seed of the fruit and is prized for its high nutritional value. Covered by a thin and brown skin, the kernel represents 15-25% of the total fruit weight. It is rich in proteins, monounsaturated fatty acid (such as oleic acid), vitamins and minerals, as well as secondary metabolites so much so that their consumption has been associated with several therapeutic and protective health benefits [264].

The relatively small proportion of the kernel compared to the hull and shell underscores the importance of utilizing the non-edible components of the almond fruit to minimize waste and promote sustainability. In particular, the significant weight and bioactive composition of the almond hull highlight its potential for valorization, through the extraction of bioactive compounds.

Almond hulls, are rich source of bioactive compounds, including polyphenols, flavonoids, dietary fibers, and secondary metabolites, but their composition significantly change among different varieties and it also depends from the extraction method [262,264]. These compounds contribute significantly to the antioxidant, antimicrobial and anti-inflammatory properties of almond hull extracts, making them a valuable by-product with numerous applications in health, agriculture and industry.

Almond hulls are a rich source of bioactive compounds, such as polyphenols, flavonoids, and dietary fibers, providing significant antioxidant, anticancer, antimicrobial and anti-inflammatory benefits [260].

In particular polyphenols are among the most abundant bioactive compounds in almond hulls. The main polyphenols include protocatechuic acid, chlorogenic acid, and p-coumaric acid, which are known for their strong antioxidant properties, by scavenging free radicals and reducing oxidative stress, playing an important role in preventing cellular damage [262]. Flavonoids, another important group of bioactive compounds in almond, include catechin, epicatechin, quercetin, isorhamnetin, morin, quercetin 3-O-rhamnoside, kaempferol 3-O-glucoside, kaempferol 3-O-rutinoside, and isorhamnetin 3-O-glucoside as the major flavonoids in almond kernels, skins and hulls, which display too antioxidant activity [265].

Recently, the antimicrobial properties of almond hull extract have gained attention due to the growing widespread phenomenon of antimicrobial resistance, which led to an increasing necessity of natural alternatives to traditional antimicrobial therapies. AHE demonstrates significant activity against different pathogens, including gram-positive, like *S. aureus* and gram-negative bacteria, such as *E. coli* and *P. aeruginosa* [258,266,267]. This

activity is largely attributed to the high presence of the bioactive compounds, mentioned above.

In particular, polyphenols such as protocatechuic acid, chlorogenic acid and p-coumaric acid, alter bacterial membranes, increasing permeability and leading to cell lysis [268]. These compounds also interfere with DNA and RNA synthesis, thanks to B-ring of flavonoids which bind nucleic acid bases of bacteria, by DNA gyrase and nucleic acid synthesis [269]. Flavonoids such as quercetin, catechin, and epicatechin can also enhance antibacterial effects through their ability to form bonds to bacterial proteins and disrupt their structural integrity [270].

These activities make it particularly attractive for applications in skin care, wound healing and food preservation.

However, there is a lack of studies regarding the ability to prevent or inhibit biofilm formation or facilitate removal. For this reason, in this chapter the activity of AHE against bacterial biofilm, was assessed, both in terms of anti-adhesive and antibiofilm activity.

4.2 Materials and Methods

4.2.1 Study design

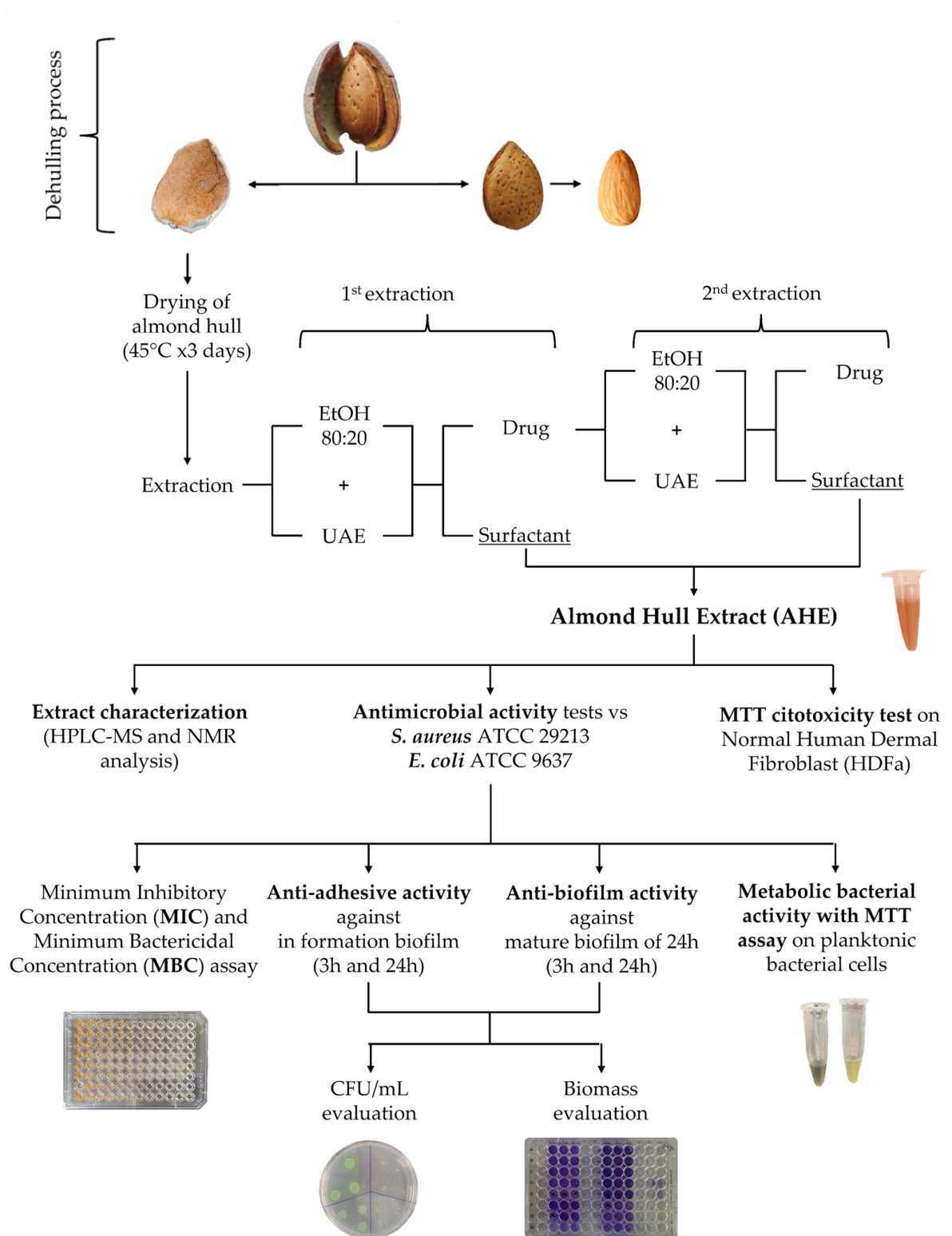


Figure 20. Experimental design.

4.2.2 Almond Hulls Extraction and characterization

- Chemicals

Ethanol (CAS 64-17-5, >99.8%), Acetonitrile (CAS 75-05-8, $\geq 99.9\%$, gradient grade, suitable for HPLC), Formic acid (CAS 64-18-6, MS grade), Water (MS grade) and Methanol-d₄ (99.80 atom %D, CAS No. 811-98-3) were purchased from Sigma-Aldrich (Steinheim, Baden-Württemberg, Germany).

- Extraction

The almond fruits (*Prunus Dulcis Mill.*) of the Pizzuta cultivar, were harvested in the eastern part of Sicily (Avola, SR, Italy) in August-September. The hulls were carefully separated by hand and then dried at 45°C for 48 hours. Each 24h, the weight loss was measured. Subsequently, they were crushed in a mechanical blender. The resulting hull powder underwent an extraction process using an 80:20 ethanol/water solution in an ultrasonic device (UP 400 S, Dr. Hielscher GmbH, Stuttgart, Germany) for 22.5 minutes while maintaining a temperature of approximately 50°C in an ice bath. Following this, the extract was stirred for 3h at room temperature and left to decant overnight. Then, the precipitate undergone again to the extraction process using the same solvent mixture for 6h under stirring and decanted again. Both supernatants were filtered through a paper filter and dried under vacuum and then mixed together [271]. The resulting extract was concentrated and dried using a rotavapor (Buchi R-300, Milan, Italy) and stored at -20°C until use.

- NMR analysis

Nuclear Magnetic Resonance (NMR) was used to analyze the crude reaction extract. 600 μ L of Deuterated Methanol (MeOD) were used to dissolve the dried crude extract, that was subsequently filtered with a nylon syringe filter with 0.45 μ m pore size and 13 mm

diameter. To obtain homogeneous solutions, the prepared sample was placed in a 5 mm NMR tube and thoroughly mixed.

Proton NMR (^1H -NMR) spectra were recorded using a Varian Unity Inova 500 MHz spectrometer. The experiments were conducted in MeOD at a temperature of 25 °C, with chemical shifts reported as δ (ppm) relative to the resonance of the Trimethyl Silane (TMS) signal. The utilized parameters were the following: 16 scans, a 90° pulse (5 μs), and a relaxation delay (D1) of 2 seconds. Coupling constants (J) are expressed in Hertz (Hz).

- HPLC-MS analysis

LTQ XL was used to perform all mass spectrometry (MS) experiments; it was equipped with an H-ESI II source (Thermo Fisher, San Jose, CA, USA) in full scan mode, covering an m/z range of 150–2000. Measurements were performed in positive ion mode with a spray voltage of 2–3 kV. The capillary temperature was set at 250 °C, the capillary voltage at 20 V, and the tube lens voltage at 120 V. Collision-induced dissociation (CID) experiments were carried out in the ion trap with a precursor ion selection window of 1–2 m/z , using an activation time of 30 ms and helium as the collision gas. External calibration was performed using Pierce LTQ ESI Positive Ion Calibration Solution. Data processing was completed with FreeStyle Software version 1.6 SP1 (Thermo Fisher Scientific).

Chromatographic analyses were conducted in positive ion mode, utilizing a Thermo Scientific Vanquish high-performance liquid chromatography (HPLC) system (Thermo Fisher Scientific, Bremen, Germany) equipped with a Phenomenex Kinetex C-18 column (100 mm $\times$ 2.10 mm, 2.6 μm) from Torrance, CA, USA. The mobile phases consisted of water with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B). The gradient conditions for LC separation were as follows: 10% B at 0 minutes, ramping to 100% B over

60 minutes, held for 10 minutes. The flow rate was set at 0.25 mL/min, with an injection volume of 5 μ L, and the column temperature was maintained at 40 °C.

4.2.3 Antibacterial activity of AHE

- Minimum Inhibitory Concentration and Minimum Bactericidal Concentration assay

The effectiveness of AHE in terms of antimicrobial activity was tested against bacteria using the broth microdilution method to evaluate the MIC and the MBC, following the CLSI guidelines [240].

Reference strains *S. aureus* ATCC 29213 and *E. coli* ATCC 9637 from the American Type Culture Collection (ATCC) were used for the tests. AHE was placed into 96-well plates (Nunc, Euroclone SpA, Life Science Division, Milan, Italy) and diluted in MHB ranging from 0.2 mg/mL to 100 mg/mL. The strains were recovered from -80°C and plated on MHA. Broth cultures were prepared by inoculating individual colonies pre-cultured overnight in MHB, in aerobic conditions at 37°C. A 1:10 culture refresh was then performed in MHB with shaking at 120 rpm for 2 h. After the incubation period, the bacterial cultures were diluted to obtain an optical density (OD₆₀₀) of 0.125 (approximately 5×10^7 CFU/mL). After, the standardized broth cultures were diluted 1:100 in MHB to obtain a final concentration of 5×10^5 CFU/mL. 100 μ L of each broth culture was added to each well and the plates were then incubated at 37 °C for 24 h.

As positive control, to compare bacterial growth levels, strains were added to the free-extract medium. AHE diluted at tested concentrations was added to strain-free culture media and was used as negative control. The MBC values were determined by sub-culturing 10 μ L of each bacterial suspension, from the non-turbid wells, on Muller Hinton Agar MHA plates and incubated at 37°C for 24 h.

- Effect on bacterial growth curves

The impact of AHE on the growth patterns of *S. aureus* ATCC 29213 and *E. coli* ATCC 9637 was investigated by using the microdilution method in 96-well plates. The plates were set up following the same procedure as the MIC determination, with the same bacterial and extract concentrations. The setting of the microtiter plates is shown in **Figure 21**.

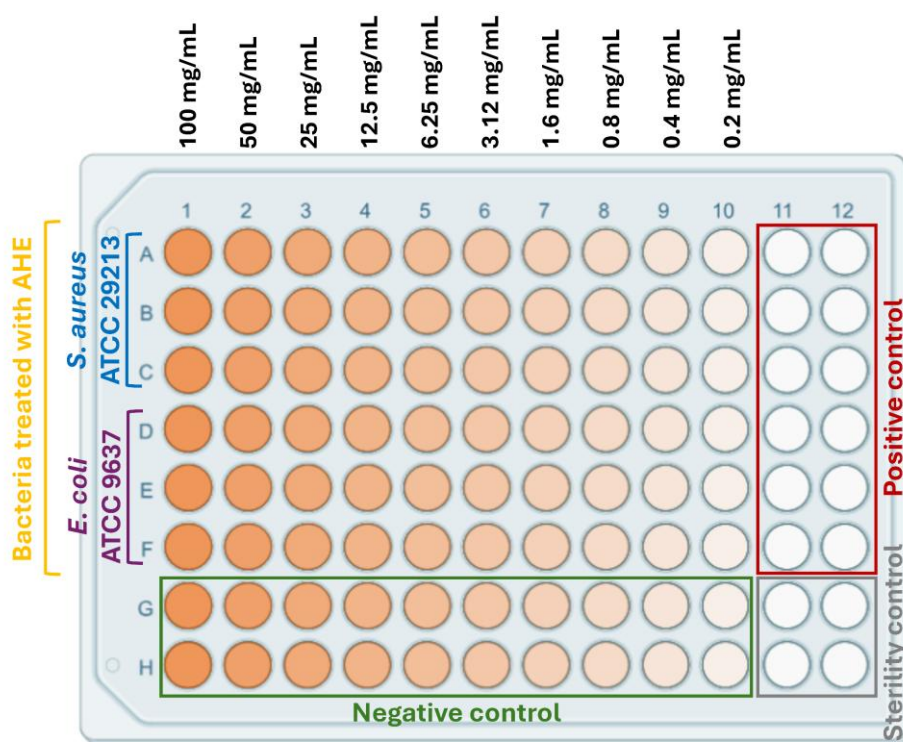


Figure 21. Setting of the 96-well plates containing *S. aureus* ATCC 29213 and *E. coli* ATCC 9637 treated with different concentration of AHE (ranging from 100 mg/mL to 0.2 mg/mL). Plate setting was used both for MIC assay and bacterial growth curves evaluation. As positive control, bacteria were added to free-extract medium. Negative control is represented by AHE diluted in free-strain medium. Sterility control is represented by free-extract and free-strain medium.

Incubation took place, following standardized conditions, using the ELx808 Incubating Absorbance Plate Reader (Lonza, Basel, Switzerland). The optical density was measured at 630 nm after regular shaking at 30-minute intervals. Bacterial growth curves were generated

by plotting the mean of each OD₆₃₀ values over time using Gen5 software (v.3.14 Agilent BioTek). After 6 h, the percentage of inhibition was determined using the following formula:

$$(CTR - \text{treated sample}) / CTR \times 100\%$$

where "CTR" and "treated sample" represent the positive control and bacteria treated with extract at different concentrations, respectively.

- Effect on bacterial biofilm

The effectiveness of AHE in influencing bacterial adhesion and in the removal of mature mono-species biofilm, was examined at different concentrations and time intervals (3 h and 24 h) against biofilm-producing strains of *S. aureus* ATCC 29213 and *E. coli* ATCC 9637. The biofilm experiments were conducted using an abiotic surface represented by 96-well flat-bottomed microtiter polystyrene plates, and the schematic procedures are displayed in **Figure 22**.

For the inoculation, bacterial suspensions were prepared following the same method used for determining the MIC, but TSB was used instead of MHB. After preparation, the bacterial suspensions were diluted in TSB to achieve an optical density of 0.250 at a wavelength of 600 nm, which corresponds to 1.0 McFarland.

The impact on bacterial adhesion was assessed using three different sub-MIC concentrations of AHE (1/2 MIC, 1/4 MIC, 1/8 MIC) diluted in TSB [148].

Furthermore, the anti-biofilm activity of AHE against mature biofilm was analyzed using 2-fold, 4-fold, and 8-fold AHE MIC concentrations [272].

~Anti-adhesive effect

100 µL of AHE (at the concentrations described above) and 100 µL of bacterial suspension, were combined in each well for the anti-adhesive assay. The plates were then

incubated in aerobic conditions at 37 °C for 3 and 24 h. After incubation, the wells were rinsed with sterile water, and the adherent cells were removed by scraping, resuspended in TSB, and plated on TSA to calculate the bacterial viable load by the evaluation of CFU/mL.

~ Anti-biofilm effect

To test the effectiveness of AHE against biofilm formation, 200 µL of bacterial suspensions were placed in each well and then incubate at 37 °C in aerobic conditions for 24 h, in order to obtain a mature biofilm. After that, the planktonic bacteria (supernatant) were removed and 200 µL of AHE were added to each well. The samples were then incubated for 3 and 24 h at 37 °C in aerobic conditions. The supernatant was removed, and the wells were rinsed with sterile water. After that, the contents of each well were scraped and suspended in TSB and then spread on TSA to determine the bacterial viable load by evaluating CFU/mL.

~ Biofilm biomass evaluation

The bacterial growth was assessed by measuring the biomass using a spectrophotometer ELx808 (Lonza, Basel, Switzerland). For both the antiadhesive and anti-biofilm tests, three replicates for each treatment condition were washed and then used for biomass measurement. To do this, 200 µL of 0.1% Crystal violet stain (Sigma Aldrich, Italy) was added to each well after washing and drying at 25 °C. After 1 minute, the stain solution was removed, and the wells were washed with sterile water to remove excess stain. The stained biomass in each well was then eluted in ethanol, and the optical density was measured at a wavelength of 570 nm.

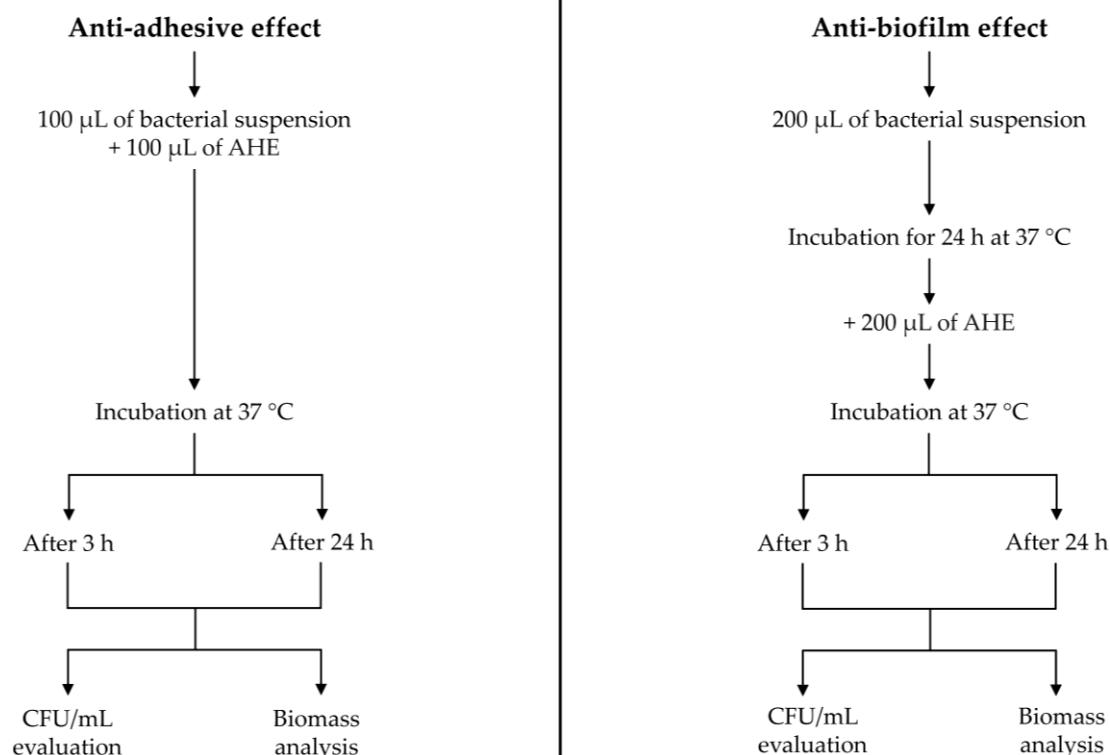


Figure 22. Test scheme of anti-adhesive and anti-biofilm evaluation against in formation and mature biofilm, respectively. Tests were performed on both *S. aureus* ATCC 29213 and *E. coli* ATCC 9637.

- Metabolic bacterial activity

The effect of AHE on planktonic bacterial metabolic activity was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay [273]. Bacterial strains were cultured in MHB at 37 °C for 24 h. Overnight bacterial suspensions grown in MHB at 37 °C in aerobic conditions, were adjusted to an optical density of 0.18 at a wavelength of 600 nm. The working broth cultures were prepared by diluting this suspension at a ratio of 1:50. The bacterial cultures were then treated with on-scale MIC concentrations of AHE, ranging from 0.2 mg/mL to 12.5 mg/mL and from 1.6 mg/mL to 100 mg/mL for *S. aureus* ATCC 29213 and for *E. coli* ATCC 9637, respectively.

Non-treated bacterial samples represented positive control. Following a 24 h incubation at 37 °C, the bacterial suspensions were adjusted to an optical density of 0.1 at a wavelength of 600 nm. Subsequently, the samples were centrifuged at 10000 rpm for 60 seconds, and the supernatants were removed. The pellets were resuspended in MHB, and the optical density of each sample was recorded. Then, 200 µL of each sample were transferred to a 1.5 mL tube, and 20 µL of MTT solution (5 mg/mL) was added. The tubes were incubated at 37°C for 15 minutes, after which the supernatants were removed, and the pellets were resuspended in 2500 µL of Dimethylsulfoxide (DMSO). The optical density was measured at a wavelength of 550 nm (OD₅₅₀). The MTT reduction unit (MRU) was calculated using the formula:

$$\text{MRU} = \text{OD}_{550} \times (2500 \text{ } \mu\text{L} / 220 \text{ } \mu\text{L}) \times (1000 \text{ } \mu\text{L} / 220 \text{ } \mu\text{L}) \times \text{DF} / \text{OD}_{600}$$

where OD₅₅₀ is the absorbance read after the suspension of the MTT-treated pellet in DMSO, DF is the dilution factor used to standardize the bacterial cultures, and OD₆₀₀ is the absorbance after dilution of the pellet in MHB. The samples were analyzed in duplicate.

4.2.4 In vitro viability assay on human dermal fibroblast

The cytotoxicity of AHE was assessed by using the MTT assay on *in vitro* adult human dermal fibroblast primary cells (HDFa) obtained from ATCC (ATCC® PCS-201-012).

The HDFa cells were cultured in fibroblast basal medium (ATCC® PCS-201-030) supplemented with fibroblast growth kit–serum free (ATCC® PCS-201-040), Phenol Red (ATCC® PCS-999-001), and Penicillin-Streptomycin-Amphotericin B solution (ATCC® PCS-999-002). The cells were then incubated at 37°C with 5% CO₂ for 48 h (using CO-150, pbi New Brunswick Scientific, Enfield, CT, USA). Subsequently, the cells were harvested using Trypsin-EDTA (ATCC® PCS-999-003) and Trypsin Neutralizing Solution (ATCC® PCS-999-004).

Fresh medium was carefully added to the cells to obtain a final concentration of approximately 1.0×10^4 cells/well. Then, 50 μL of the cell suspension was gently added to each well of a flat-bottomed 96-well plate and incubated at 37°C with 5% CO_2 for 24 h until reaching 80% confluence.

Once the confluence was reached, the cells were treated with 50 μL of AHE at different concentrations (ranging from 0.2 mg/mL to 100 mg/mL) in a 1.0% RPMI/DMSO (1:1) solution. The plates were then incubated for 72 h using the conditions previously described.

The positive control consisted of cells in a free-extract suspension, while AHE diluted at the tested concentrations was added to cells-free culture media as a negative control.

Following the incubation period, 10 μL of MTT solution (5 mg/mL) were added to each well and incubated for 3 h with shaking at 500 rpm. The absorbance at a wavelength of 570 nm was measured after adding 100 μL of DMSO and incubating for 30 minutes with shaking at 500 rpm [274].

4.2.5 Statistical Analysis

The results were obtained from three separate experiments, each performed three times. They are displayed as average $\pm$ standard deviation. Any $p \leq 0.05$ were considered statistically significant. Group variations were assessed using an ANOVA test.

4.3 Results

4.3.1. Almond Hulls Extraction and characterization

- Extraction and weight reduction

Extraction modalities were chosen to obtain a high yield and preservation of bioactive compounds. An ethanol/water solution (80:20, v/v) and a 1:40 powder/solvent solution ratio (g/mL) were used for the extraction that was carried out at 50°C [266,275,276]. To maximize the extraction yield, the obtained extract was subjected to two steps of stirring at 3 and 6 h, respectively [271].

After one day of drying, the almonds lost 6.6% of their weight, and this percentage increased to 12.6% after three days. The weight losses after two and three days were 3.9% and 2.1%, respectively. Monitoring continued until day 5, and no significant differences were observed compared to day 3 (**Table 6**). The initial weight of 1000 g trays decreased to a final weight of 874 g.

Table 6. Weight and reduction rate of almond hull after drying at 45 °C for 3 days. Reductions are expressed as percentage.

Days	Weight (g)	Reduction in respect to "Day 0" (%)	Reduction than the previous day (%)
0	1000		
1	934	6.6	
2	895	10.5	3.9
3	874	12.6	2.1

- NMR analyses

The ¹H-NMR spectrum (**Figure 2**) shows a characteristic profile with different signals corresponding to carbohydrate structures, in agreement with existing literature on both simple and complex carbohydrates [277,278]. Proton signals are notably observed at position

1 for the two anomeric forms of glucose (α : 5.07 ppm, β : 4.44 ppm). Comparable signals for fructose and sucrose appear at 4.13 ppm and 4.99 ppm, respectively. In the chemical shift range of 3.0 to 4.5 ppm, signals related to the carbohydrate backbone are clearly visible, along with methyl signals from the methoxy groups found in polyphenols. In the aliphatic region, which range from 0 to 2 ppm, only a few signals appear, indicating that there is a minimal lipid component extracted by this method. The presence of chlorogenic acid can be identified by distinctive signals, specifically two doublet signals located at 2.52 and 2.75 ppm. These signals correspond to the diastereotopic protons at the sixth position of the chlorogenic acid backbone. Additionally, a correlation can be observed in the COSY spectrum (**Figure 23**) with the signal at 4.25 ppm, which originates from the proton at the fifth position.

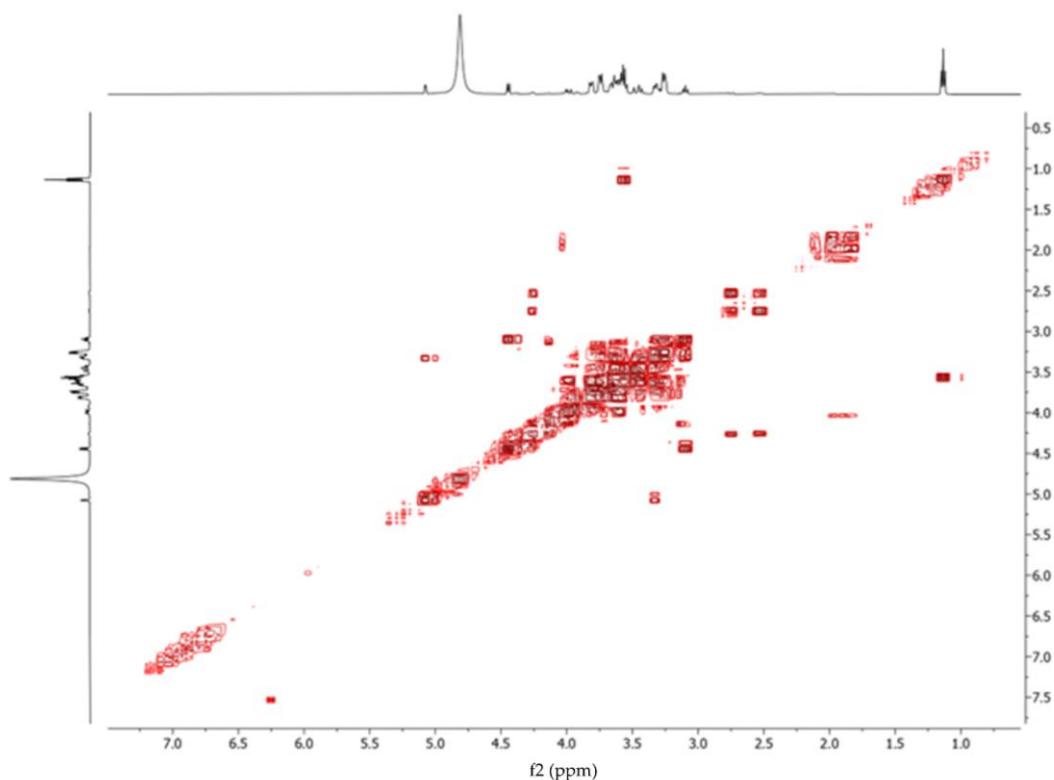


Figure 23. gCOSY spectrum of crude extract in MeOD.

Furthermore, the ^1H -NMR spectrum displays (**Figure 24**) signals associated with the aromatic rings of polyphenols (8-6 ppm). These signals exhibit relatively low intensity, which can be attributed to inefficient vibrational relaxation in an aqueous solvent, where stacking interactions of aromatic rings are favored. The ^1H -NMR spectrum also shows signals corresponding to the aromatic rings of polyphenols within the range of 8 to 6 ppm. The observed signals display a low intensity, likely attributable to inadequate vibrational relaxation within an aqueous environment that promotes stacking interactions among the aromatic rings.

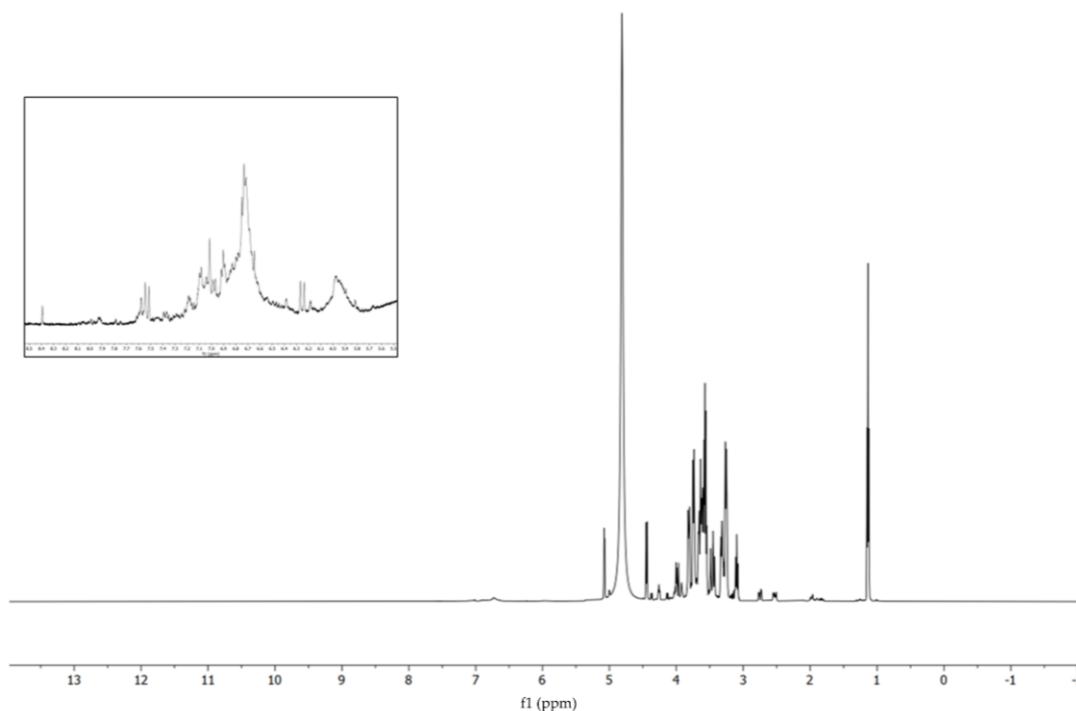


Figure 24. ^1H -NMR spectrum and magnification of aromatic field (insert).

- HPLC-MS analyses

The use of mass spectrometry in negative electrospray ionization mode allowed the identification of all ions at m/z values that have been documented in previous studies [262,279–281]. This encompassed the detection of phenolic acids from both the benzoic and

hydroxycinnamic classes, including protocatechuic acid, p-coumaric acid, caffeic acid and chlorogenic acid. Additionally, the key aglycones within the flavonoid group, such as catechin, epicatechin, and quercetin, were also successfully identified, as shown in **Figure 25**. The analysis was carried out in positive mode, which facilitated the identification of glycosylated flavonoids. This result corroborates the findings from the NMR spectroscopy experiment, highlighting the substantial presence of the sugar component (see **Figure 26**),

The main compounds detected, along with their corresponding MRM transitions, are listed below: Chlorogenic acid (black line, m/z 355.3 and MS/MS: 192.7; 180.8); Delphinidin-3-O-galactoside (red line, m/z , 465.1 and MS/MS 303.1; 257.0; 229.2); Delphinidin-3-O-glucoside (red line m/z , 465.1 and MS/MS 303.1; 257.0; 229.2); Cyanidin-3-O-glucoside (green line, m/z 449.1 and MS/MS 287.1; 241.0); Petunidin-3-O-glucoside (blue line, m/z 479.1 and MS/MS 317.1; 302.1; 274.1; 257.1); Peonidin-3-O-(p-coumaroyl-glucoside) (yellow line, m/z 609.2 and MS/MS 301.1; 286.3; 258.2); Peonidin-3-O-glucoside (purple line, m/z 463.1 and MS/MS 301.1; 286.0; 258.2).

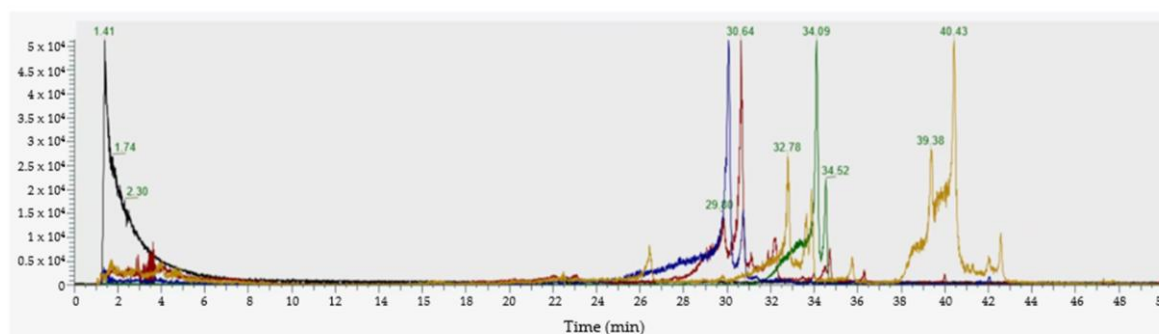


Figure 25. Chromatogram of crude extracted in ESI(-)MS mode.

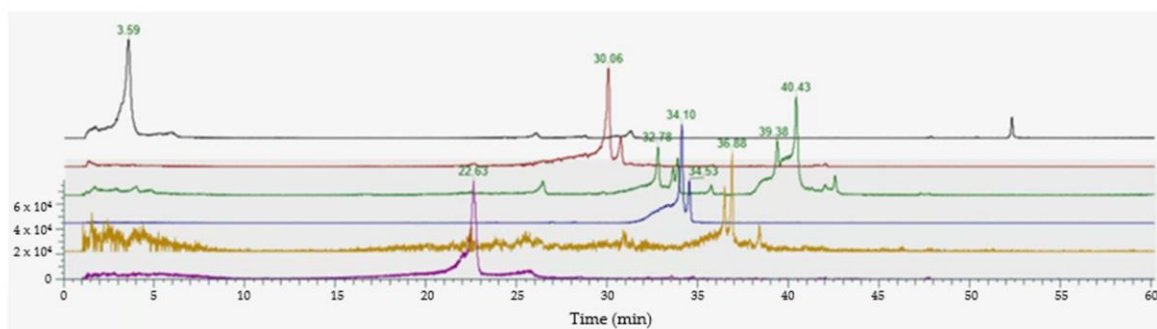


Figure 26. MRM profile of Chlorogenic acid (black line); Delphinidin-3-O-galactoside and Delphinidin-3-O-glucoside (red line), Cyanidin-3-O-glucoside (green line), Petunidin-3-O-glucoside (blue line), Peonidin-3-O-(p-coumaroyl-glucoside) (yellow line), Peonidin-3-O-glucoside (purple line).

4.3.2 Antibacterial activity of AHE

- Minimum Inhibitory Concentration and Minimum Bactericidal Concentration assay

Table 7 displays the results of the antibacterial activity of AHE against *S. aureus* ATCC 29213 and *E. coli* ATCC 9637. Both strains were tested for their minimum inhibitory and bactericidal concentrations. The MIC for *S. aureus* ATCC 29213 was found to be 7.8 times lower than that of *E. coli* ATCC 9637. Additionally, the MBC was determined to be 3.2 mg/mL for *S. aureus* ATCC 29213, while for *E. coli* ATCC 9637, the MBC was higher than 100 mg/mL, which was the highest concentration tested.

Table 7. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) values of AHE against reference strains. Concentrations are expressed in mg/mL.

Strains	MIC	MBC
<i>S. aureus</i> ATCC 29213	1.6	3.2
<i>E. coli</i> ATCC 9637	12.5	>100

- Effect on bacterial growth curves

The AHE had a dose-dependent effect on bacterial growth, as shown in the growth curves (**Figure 27** and **Figure 28**, growth curves of *S. aureus* ATCC 29213 and *E. coli* ATCC

9637, respectively). The MIC concentration values were confirmed, and AHE, at a concentration of 1.6 mg/mL, inhibited *S. aureus* ATCC 29213 growth by 76% after 6 h of incubation compared to the untreated control. Notably, at sub-MIC concentrations (1/2, 1/4, and 1/8 of MIC value), the growth of *S. aureus* ATCC 29213 was inhibited by 56%, 24%, and 11%, respectively, compared to the control. For *E. coli* ATCC 9637, the inhibition at MIC concentration (12.5 mg/mL), was about 85% compared to the untreated control after 6 hours of incubation, with reductions of 68%, 52%, and 46% observed at 1/2, 1/4, and 1/8 of the MIC concentration value, respectively.

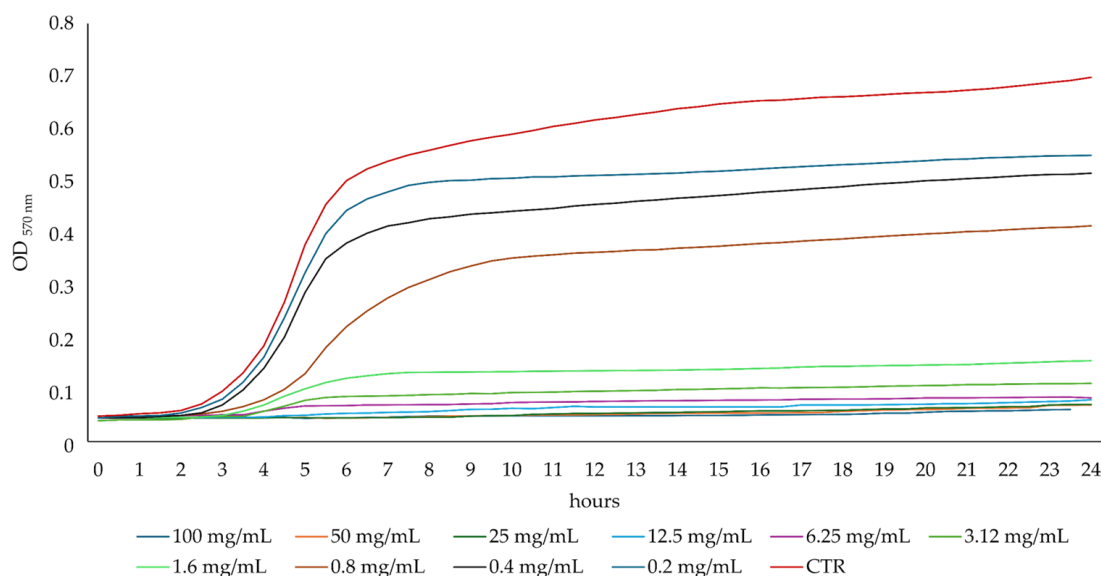


Figure 27. Growth curves of *S. aureus* ATCC 29213 treated with different concentrations of AHE (ranging from 0.2 to 100 mg/mL). CTR (control) represents the non-treated sample.

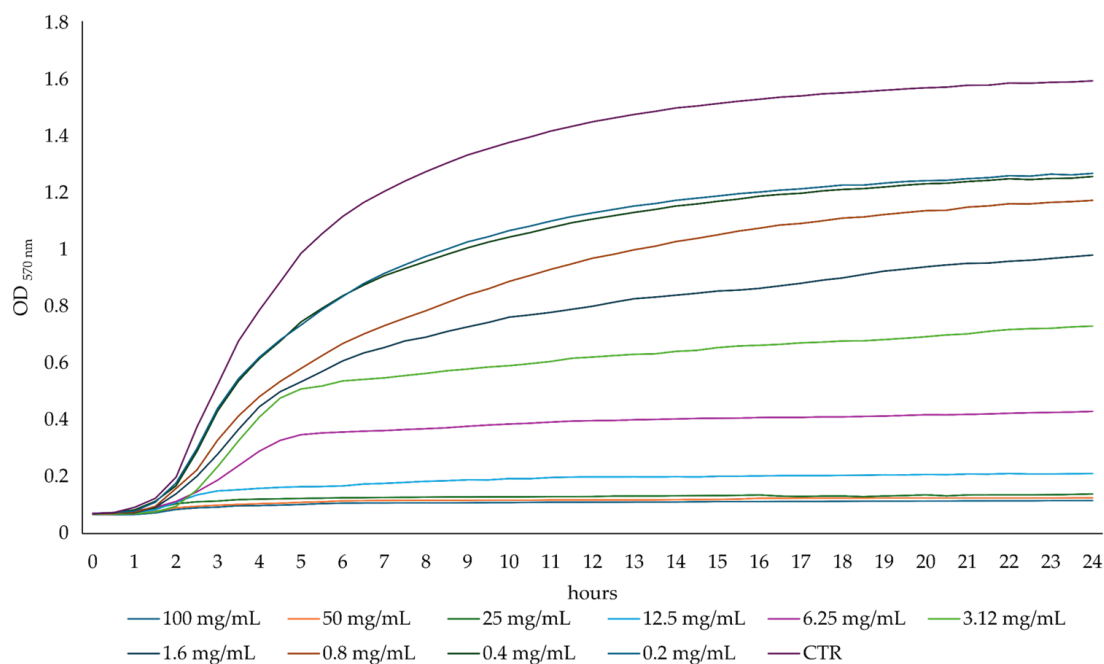


Figure 28. Growth curves of *E. coli* ATCC 9637 treated with different concentrations of AHE (ranging from 0.2 to 100 mg/mL). CTR (control) represents the non-treated sample.

- Effect on bacterial biofilm

The effect of AHE on *S. aureus* ATCC 29213 and *E. coli* ATCC 9637 biofilms, was evaluated both against in formation and mature biofilm to evaluate the antiadhesive and anti-biofilm capabilities, respectively. Both experiments were performed at two different treatment times (3 h and 24 h) using microtiter 96-well flat-bottomed plates.

~ Anti-adhesive effect

Figure 29 illustrates the anti-adhesive activity of AHE against the biofilm formation of *S. aureus* ATCC 29213 and *E. coli* ATCC 9637 after 3 and 24 h of incubation. A significant reduction in CFU/mL was observed in comparison to the control group when using 1/2 MIC for each strain (MIC of *S. aureus* and *E. coli* ATCC 9637ATCC 29213 = 1.6 mg/mL and 12.5 mg/mL, respectively). After 3 h of incubation (as shown in **Figures 29A and B**), a decrease of 39% for *S. aureus* ATCC 29213 and 46% for *E. coli* ATCC 9637 was recorded compared

to the untreated control group. This corresponds to reductions of 0.27 and 0.58 logarithmic values, respectively, indicating a significant impact on the viability of these bacteria.

After 24 h (**Figure 29C and D**), the growth of *S. aureus* ATCC 29213 was affected by 0.8 mg/mL of AHE, resulting in a 27% reduction in terms of CFU/mL, which corresponds to a decrease of 0.13 logarithms. In contrast, the growth of *E. coli* ATCC 9637 was inhibited to a much greater extent at all tested concentrations. The reductions in CFU/mL compared to the untreated control were significant, with decreases of 58%, 71%, and 67% at concentrations of 1/8, 1/4, and 1/2 of the MIC value, respectively. These correspond to sub-MIC concentrations of 1.6, 3.12, and 6.25 mg/mL, which reflect decreases of 0.33, 0.56, and 0.41 logarithms, respectively.

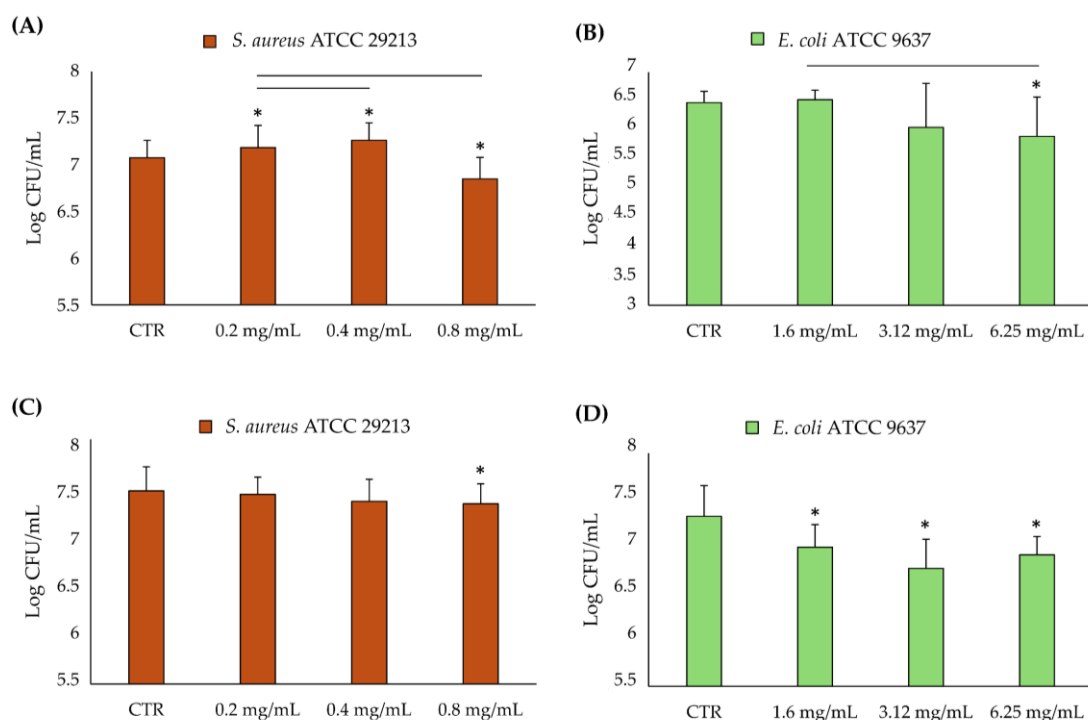


Figure 29. Log CFU/mL of *S. aureus* ATCC 29213 (**A,C**) and *E. coli* ATCC 9637 (**B,D**) in formation biofilms treated with sub-MIC concentrations of AHE after 3 h (**A,B**) and 24 h (**C,D**). CTR represents the non-treated sample (control). *Statistically significant in respect to the control. The lines indicate the intragroup significance.

~ Anti-biofilm effect

The anti-biofilm activity of AHE against mature biofilm was assessed using *S. aureus* ATCC 29213 and *E. coli* ATCC 9637 following treatments for both 3 and 24 h.

The AHE demonstrated a significant reduction in the mature biofilm of *S. aureus* ATCC 29213 compared to the untreated control, across all tested concentrations and treatment time. Specifically, after 3 h, the observed reduction rates were 37%, 57%, and 39%, which correspond to logarithmic reductions of 0.41, 0.57, and 0.49, respectively, at 2-, 4- and 8-fold MIC concentration values (**Figure 30A**). After 24 h, the observed reduction rates were 44%, 40%, and 70%, which correspond to logarithmic reductions of 0.29, 0.26, and 0.65, respectively, at 2-fold, 4-fold and 8-fold MIC values (**Figure 30C**).

Moreover, the application of AHE resulted in a significant reduction of the biofilm of *E. coli* ATCC 9637 only at the highest concentration, represented by 100 mg/mL (8-fold MIC concentration), after an exposure duration of 3 h (**Figure 30B**). After 24 h, AHE was found to elevate the CFU/mL of *E. coli* ATCC 9637 across all tested concentration levels (**Figure 30D**).

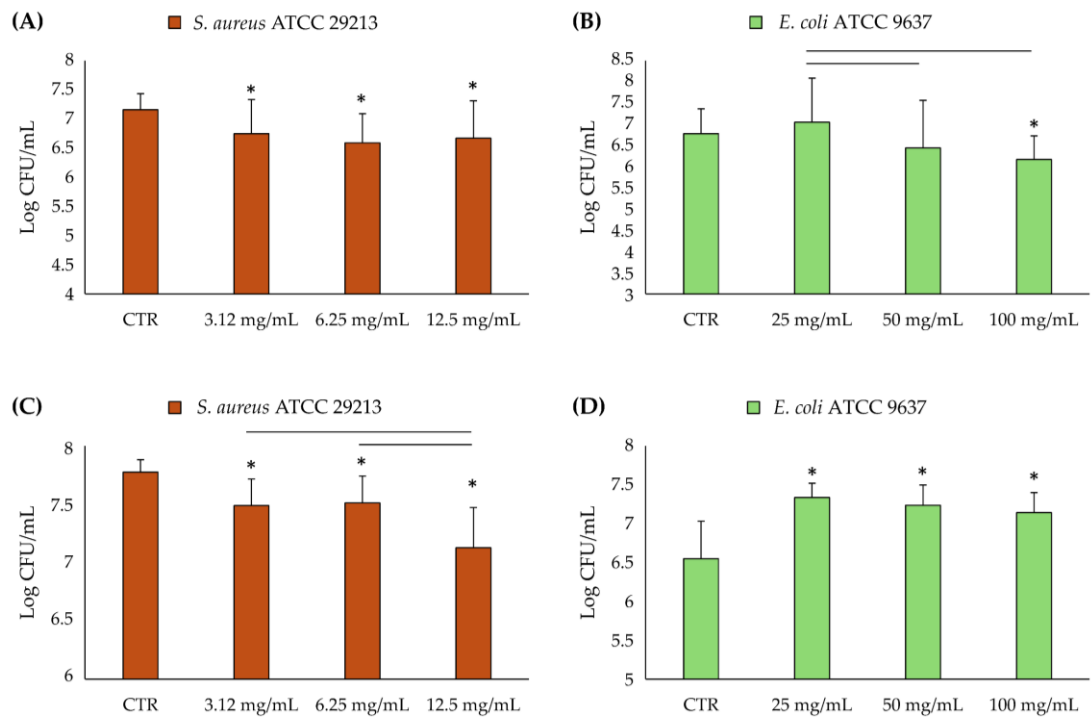


Figure 30. Log CFU/mL of *S. aureus* ATCC 29213 (A,C) and *E. coli* ATCC 9637 (B,D) mature biofilms treated with over-MIC concentrations of AHE after 3 h and 24 h. CTR represents the non-treated sample (control). *Statistically significant in respect to the control. The lines indicate the intragroup significance.

~ Biofilm biomass evaluation

The results concerning biofilm biomass formation are displayed in **Figure 31**. A significant reduction in biofilm formation was observed in *E. coli* ATCC 9637 across all concentrations, both at 3 and 24 h, compared to the control (**Figures 31B** and **D**). Specifically, after 3 h, there was a decrease in biomass formation of 26%, 35%, and 60% at concentrations of 1/8, 1/4, and 1/2 of the MIC value, respectively. After 24 h, the decreases were 21%, 19%, and 31% at the same respective concentrations. In addition, the analysis of mature biofilm biomass revealed a significant increase in *S. aureus* ATCC 29213 at a concentration of 6.25 mg/mL (4-fold MIC) and for *E. coli* ATCC 9637 at 25 mg/mL (2-fold MIC) after 3 h (**Figure 32**).

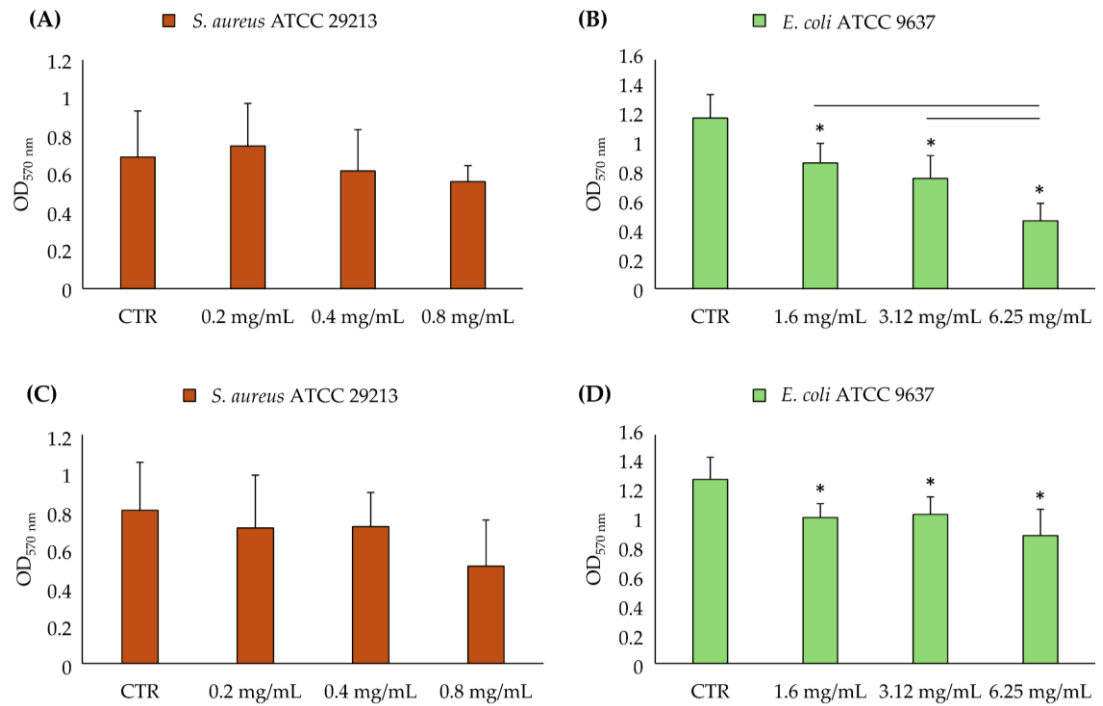


Figure 31. Biomass analysis of in formation biofilm of *S. aureus* ATCC 29213 (A,C) and *E. coli* ATCC 9637 (B,D) in biofilms formation treated with sub-MIC concentrations of AHE after 3 h and 24 h. CTR represents the non-treated sample (control). *Statistically significant in respect to the control. The lines indicate the intragroup significance.

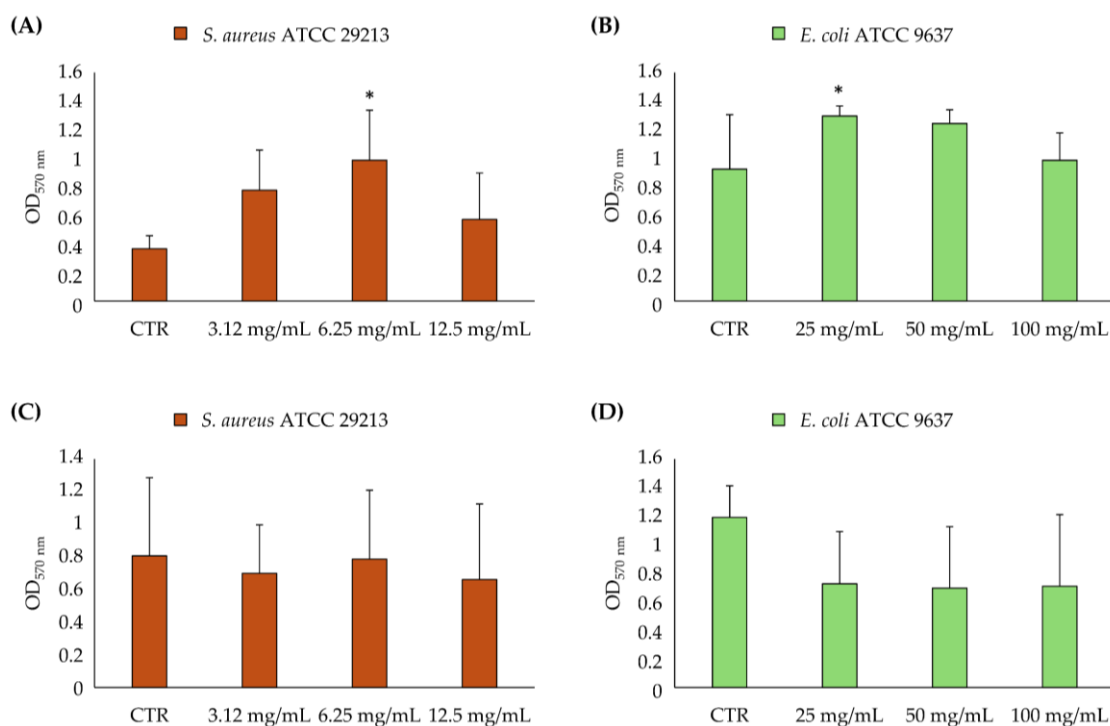


Figure 3. Biomass analysis of mature biofilm of *S. aureus* ATCC 29213 (A,C) and *E. coli* ATCC 9637 (B,D) treated with over-MIC concentrations of AHE after 3 h and 24 h. CTR represent the non-treated sample (control). *Statistically significant in respect to the control. The lines indicate the intragroup significance.

- Metabolic bacterial activity

The MTT cytotoxicity assay conducted on bacterial planktonic cells revealed a dose-dependent inhibition of growth for both bacterial strains examined, as depicted in **Figure 33** (*S. aureus* ATCC 29213) and **Figure 34** (*E. coli* ATCC 9637).

Notably, for *S. aureus* ATCC 29213, there was a reduction greater than 90% in cell metabolic activity at the MIC concentration of 1.6 mg/mL. In comparison, *E. coli* ATCC 9637 exhibited a 70% decrease in metabolic activity at its MIC value of 12.5 mg/mL. The most significant reductions were observed at 8-folds MIC for both bacterial strains, where it was displayed a decrease of 95% and 94% of the growth of *S. aureus* and *E. coli*, respectively. The outcomes of the MTT cytotoxicity assay are reported in terms of MRU.

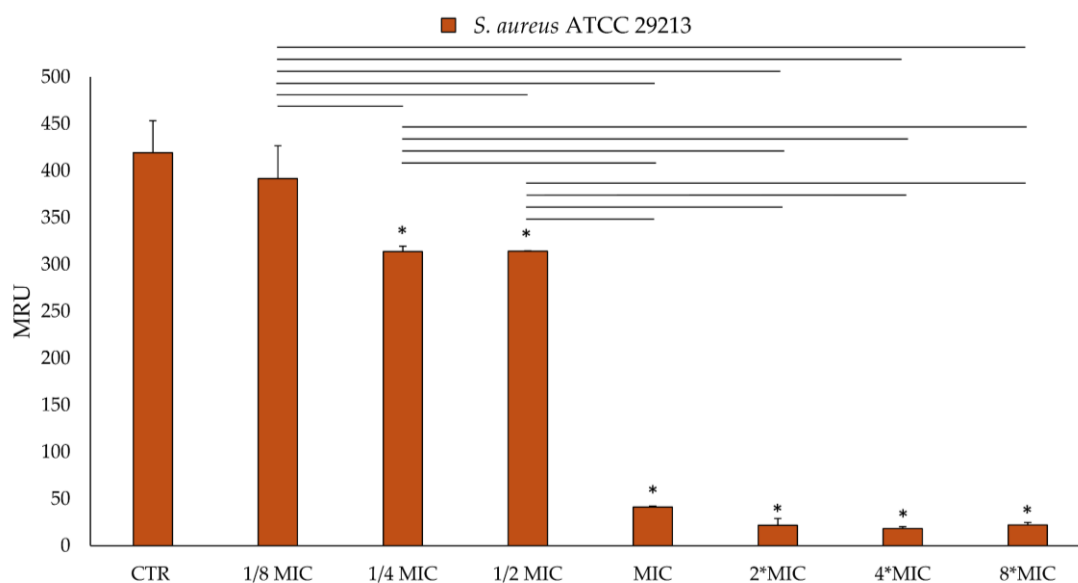


Figure 33. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) cytotoxicity on planktonic cells of *S.aureus* ATCC 29213 treated with different concentrations of AHE. CTR represents the non-treated sample (CTR). *Statistically significant in respect to the control. The lines indicate the intragroup significance.

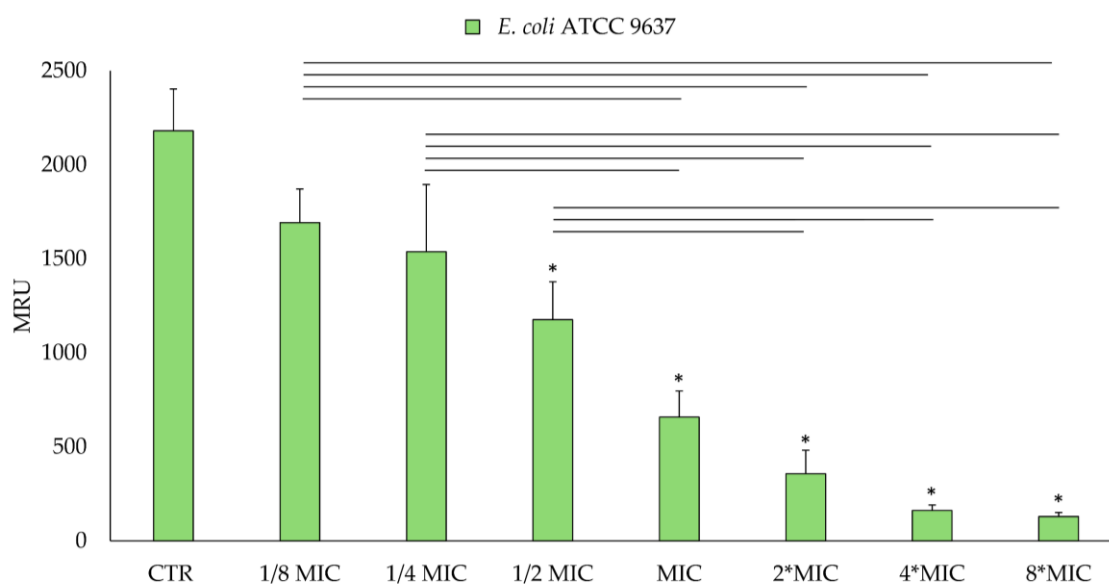


Figure 34. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) cytotoxicity assay on planktonic cells of *E. coli* ATCC 9637 treated with different concentrations of AHE. CTR represents the non-treated sample (CTR). *Statistically significant in respect to the control. The lines indicate the intragroup significance.

4.3.3 In vitro viability assay on human dermal fibroblast

The viability of HDFa was assessed through the analysis of metabolic activity. The MTT assay demonstrated no significant differences between the non-treated fibroblasts (representing the control group) and the treated samples at all the tested concentrations. However, a significant increase in metabolic activity was observed at the lowest concentration of 0.2 mg/mL (**Figure 35**).

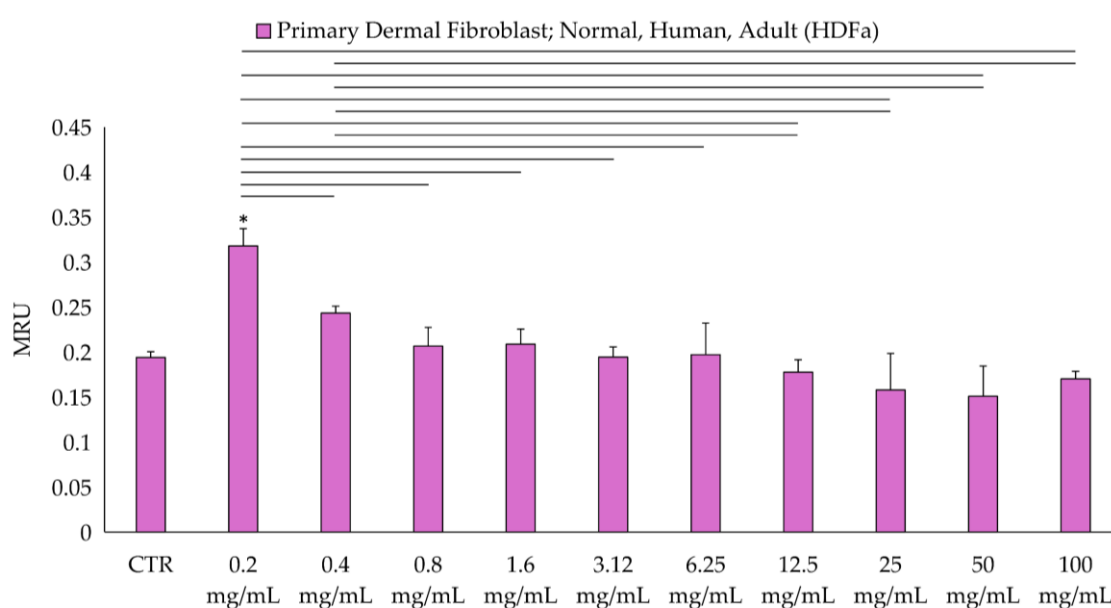


Figure 34. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) cytotoxicity assay on Primary Dermal Fibroblast; Normal, Human, Adult (HDFa) treated with different concentrations of AHE. CTR represents the non-treated sample. *Statistically significant in respect to the control. The lines indicate the intragroup significance.

4.4 Discussion

Almond hulls, the primary by-product of almond production, constitute around 50% of the almond's total weight. These hulls are rich in bioactive compounds such as protocatechuic acid, caffeic acid, chlorogenic acid, and catechins, making them a promising candidate for applications in health and industry. Ultrasound-assisted extraction was employed in this study as an eco-friendly method to maximize the yield and quality of these bioactive components. UAE disrupts plant cell membranes through sound waves, enabling efficient solvent penetration while preserving the integrity of bioactive compounds [271]. In particular, several studies evaluated the different combinations of solvent extraction and mechanical extraction (such as UAE), optimizing the method to extract the highest amount of bioactive compounds, focusing on total phenolic content (TPC) [266,275,276]. This aligns with sustainability principles by reducing energy use and using an optimized powder/solvent ratio (1:40) and ethanol/water mixture (80:20 v/v), which enhances polyphenol extraction efficiency from almond hulls, as previously demonstrated [266].

The growth inhibition of *S. aureus* and *E. coli* at sub-MIC concentrations is in line with studies indicating that flavonoids and polyphenols, such as those found in AHE, interfere with bacterial enzymes and DNA synthesis, inhibiting bacterial proliferation.

The HPLC analysis identified major phytochemicals such as catechins, quercetin, and chlorogenic acid, which are well-documented for their antimicrobial properties [282,283]. The antibacterial action of polyphenolic compounds is primarily linked to their capacity to form hydrogen bonds with proteins in the bacterial cell membrane, leading to the disruption of electron transport chains and compromising membrane integrity [270]. Additionally, chlorogenic acid enhances the permeability of both the outer and plasma membranes, further destabilizing bacterial cells and facilitating antimicrobial effects [268]. Catechins, as

reported in the literature, exhibit potent antibacterial activity against *E. coli*, with a MIC ranging from 1 to 2 mg/mL and an MBC of 2 to 4 mg/mL. Additionally, catechins have been shown to significantly downregulate the *acrA* gene, which plays a critical role in biofilm formation, achieving reductions more than 60% [284]. Similarly, protocatechuic acid is recognized as a promising natural compound with broad-spectrum antimicrobial properties effective against both Gram-positive and Gram-negative bacteria, with synergistic effects when combined with antibiotics, enhancing their efficacy [285,286]. Recent studies further confirm that AHE possesses antimicrobial activity, effectively inhibiting the growth of bacteria such as *S. aureus* and *E. coli*. These findings support AHE's potential as a natural antimicrobial agent targeting both planktonic and biofilm-forming bacteria.

While the antioxidant property of AHE is well-documented in the literature, research on its antimicrobial capabilities is limited, and there is currently a lack of studies specifically addressing its antibiofilm activity against microorganisms. This study demonstrated the remarkable antimicrobial potential of almond hull extract against both *S. aureus* and *E. coli* (MIC values of 1.6 and 12.5 mg/mL, respectively), confirming its broad-spectrum activity as demonstrated in previous study [264,287] and its activity against bacterial biofilm was also explored.

This observation aligns with studies by Jincanet al, which documented the role of flavonoids like catechin and quercetin, as the main secondary metabolites of vegetables and plants, in bacterial growth inhibition and in biofilm reduction by interfering with different mechanisms like quorum sensing, ATP synthase, DNA or protein synthesis, biofilm formation, the production of EPS and more [288].

The anti-biofilm activity of AHE revealed a differential impact on Gram-positive and Gram-negative bacteria. Against *S. aureus*, a significant reduction in biofilm CFU/mL was

observed at all tested concentrations (2,4,8-fold MIC), both at 3 and 24 h. In contrast, *E. coli* biofilms showed significant reductions only at highest concentration (8-fold MIC) after 3 h, suggesting that AHE is able to impact not only on the bacterial adhesion to surfaces, but also in the biofilm removing, probably due to the modification of EPS.

The anti-adhesive activity of AHE against *S. aureus* and *E. coli* highlights the different impact of sub-MIC concentrations of the extract on biofilm formation over time. Interestingly, after 3 h the extract showed a slight reduction in biofilm formation only at the highest concentration for both bacteria, suggests that AHE effectively affects the early stage of biofilm development, particularly by preventing bacterial adhesion, a key step in biofilm formation. After 24 h, AHE displayed a stronger inhibitory effect on *E. coli* biofilm formation than on *S. aureus* biofilm, may suggesting differences in biofilm-formation process between Gram-positive and Gram-negative, probably interfering with biofilm matrix development, a critical factor for biofilm structural integrity. These differences in efficacy against gram-positive and gram-negative biofilms may be attributed to the distinct structural and functional characteristics of these bacterial groups. For instance, Gram-negative bacteria have an outer membrane rich in lipopolysaccharides, which can act as a barrier to bioactive compounds, reducing their penetration and efficacy [289]. Additionally, the QS mechanisms of both Gram-positive and Gram-negative bacteria shows a lot of differences, leading the bacteria to evade the immune system and increase cell density [290].

The MTT metabolic assay revealed a dose-dependent decline in planktonic cell viability for both *S. aureus* and *E. coli*, with bacterial growth reduced by over 94% at the highest tested concentrations. This indicates that the antimicrobial efficacy of AHE intensifies at higher doses, likely due to enhanced disruption of bacterial membranes or

interference with essential metabolic pathways, further affirming its potent antibacterial properties.

The non-cytotoxic nature of AHE at tested concentrations on human dermal fibroblasts highlights its safety profile, an essential consideration for potential applications in wound healing and dermatological formulations. This finding aligns with previous studies on almond-derived extracts that emphasize their biocompatibility and potential for use in skin care products.

The variability in biofilm inhibition between Gram-positive and Gram-negative bacteria underscores the need to explore synergistic combinations of AHE with other natural or synthetic antimicrobials to enhance efficacy.

In conclusion, this study highlights the potential of almond hull extract as a natural, eco-sustainable antimicrobial agent coming from the re-use of food waste with significant activity against biofilm-forming bacteria. Its broad-spectrum activity, safety profile, and alignment with green chemistry principles position AHE as a valuable alternative in addressing the challenges of antimicrobial resistance and biofilm-associated infections. Future research aimed at evaluate its activity against clinical strains coming from healthy skin and from altered skin sites, optimizing its formulation and understanding its mechanisms will further expand its applications in healthcare.

5. PLASMA ACTIVATED WATER (PAW): Effect on microbial biofilm

5.1 Introduction

Plasma activated water (PAW) is the result of the direct exposure of the water to Non-Thermal Atmospheric Plasma (NTAP). NTAP is a partially ionized gas obtained at room temperature and atmospheric pressure, as the result of the application of an electrical discharge to water, which causes ionization, dissociation and excitation of the atoms inside it [291]. NTAP systems utilize a variety of electrode configurations to generate plasma, where dielectric barrier discharge (DBD), plasma jets and spark or glow discharges are commonly used. These configurations differ in their mechanisms of plasma generation and application versatility, making them widely applicable in both research and industrial settings [292].

Plasma-treated samples show different physico-chemical characteristics in respect to the untreated controls in terms of reactive species, both reactive oxygen species (ROS) and reactive nitrogen species (RNS). In fact, during plasma treatment, these reactive species are generated, and they dissolve into the water altering its chemical properties. Moreover, they show an increased conductivity and the pH value significantly decrease, becoming more acidic [293].

Due to its effectiveness, its safety and its eco-sustainability, PAW antimicrobial efficacy was largely evaluated during the last few years as an efficacious alternative to antibiotic traditional therapies. In addition to microbial decontamination, PAW is also involved in other activities related to the food industry, like pesticide degradation, it works as a curing agent of meat products limiting the use of synthetic nitrates, it favours sprouts production and others [291].

It showed also antimicrobial action against different microorganisms, such as *E. coli*, *S. aureus*, *Salmonella enteritidis*, *Listeria monocytogenes*, [294–297]. The ability of NTAP, and consequently of PAW, to inhibit bacterial growth is due to different mechanisms of action [298]. In particular, ROS, such as superoxide radical anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hydroxyl radicals ($OH^{\cdot}$), ozone (O_3) and singlet oxygen ($^1O_2$), and RNS are the main ones responsible of this activity, causing mechanical or oxidative damage to cell envelopes, due to both the impact of electrons and excited atoms and the phenomenon of electroporation in which it occurs the accumulation of charges on the cell surface, leading to its permeabilization as the result of the formation of pores. These conditions lead to the release of intracellular components and to severe damage to cellular structures, causing microbial death. Singlet oxygen, in particular, has a high ability to affect the polyunsaturated fatty acid of the cytoplasmatic membrane, leading to microbial inactivation due to lipid peroxidation [298].

However, the action of these reactive species is not limited to damage to the outer portion of the cell, also attacking other structures in addition to lipids, like proteins, nucleic acids and carbohydrates. In normal conditions, bacterial cells develop systems to neutralize the effects of radicals, such as catalases, superoxide dismutase and glutathione peroxidase, limiting and repairing cell damage. However, when these species accumulate, the antioxidant capacity of the cell is not able to counteract the action of free radicals leading to oxidative stress. This stress can lead to DNA damage due to oxidation of the bases [291,298].

Recent studies showed that plasma activated water exhibits significant potential in disrupting bacterial biofilms, which represent a microbial virulence factor [297,299–303]. In fact, reactives can degrade the polysaccharides, proteins, and lipids in the EPS matrix, compromising the structural integrity of the biofilm. Consequently, the degradation of the

EPS matrix exposes bacterial cells within the biofilm to antimicrobial agents, enhancing the efficacy of treatments. In detail, Fernández-Gómez et al, demonstrated a direct relationship between PAW generation parameters, regarding plasma power and activation time, and PAW antimicrobial activity against planktonic and sessile phase of *L. monocytogenes* [297]; in particular, while 15 minutes-treated PAW showed a 1.9 and 1.8 log reductions for biofilms formed on polystyrene and stainless steel plates, respectively (*versus* 4.6 log reduction for planktonic cells), an increased treatment time of 60 min led to a reduction of more than 5.4 log. Northage et al, highlights how the disinfectant treatment with PAW can increase log reduction (from 4.39 to 7.30 log) of multi-species biofilm of *S. aureus*, *S. epidermidis*, *P. aeruginosa* and *E. coli*, on reusable flexible endoscopic equipment [299]. Interestingly, Basiri et al, analyzed the effect of PAW treatment on the different stages of biofilm formation of *S. enteritidis*. Data indicated an inhibitory effect of PAW on the biofilm formation process, highlighting that this exposure resulted in a decreased expression of the genes involved in the early stages of biofilm formation (*csgD*, *agfA*, *fimA*, *lpfE* and *rpoS*). Moreover, findings showed also an alteration in the metabolic activity related to biofilm structure [300]. In addition to its biological effects, the cytotoxicity of PAW was assessed. For example, Xu et al, during the evaluation of PAW activity on wound healing with a mice model infected with *P. aeruginosa*, found that there was a faster healing in samples treated with PAW in respect to the controls treated with tap water, indicated the biosafety of PAW. In particular, plasma treatment both enhanced the migration of keratinocytes and fibroblasts and decrease the bacterial load in wound site [304].

For these reasons, PAW represents a possible strategy used in to prevent and to treat skin conditions and chronic wounds infections, in order to increase the efficacy of topical antiseptics [301].

5.2 Material and methods

5.2.1 Study design

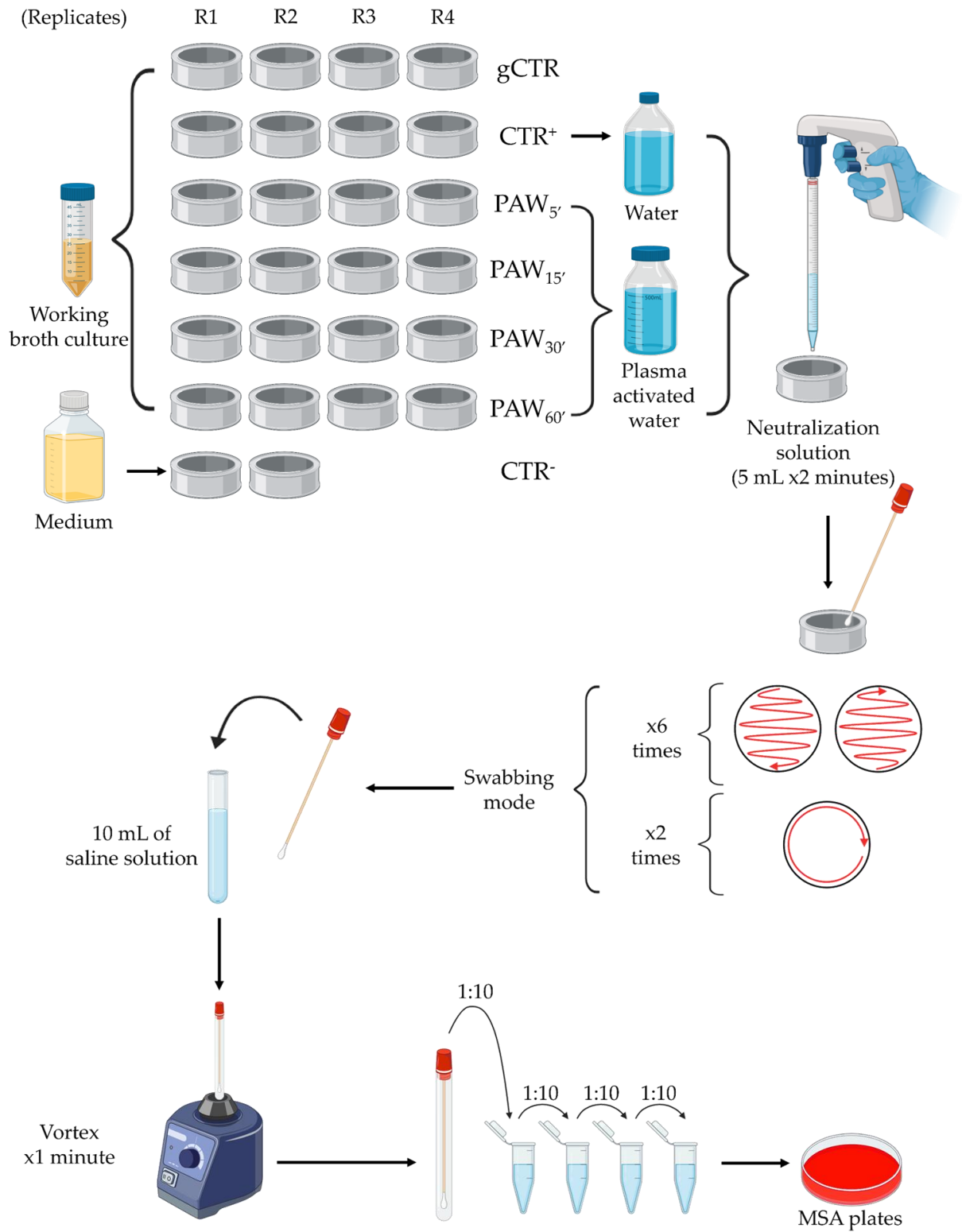


Figure 36. Experimental plan.

5.2.2 Plasma system

Plasma activated water generation was carried out using a plasma system, allowing the formation of plasma that after its production, is introduced into the water in the form of bubbles. The system consisted of a power source (Leap100, PlasmaLeap Technologies, Australia), a gas source (synthetic air), a flowmeter to regulate the flow of gas introduced into the water, two electrodes (one for high voltage and one ground electrode), and a glass tube to protect the high voltage electrode, as shown in **Figure 37**. This glass tube had eight radially placed microholes (400 μm in diameter) located 5 mm above the end sealed to allow gas flow and bubble generation.

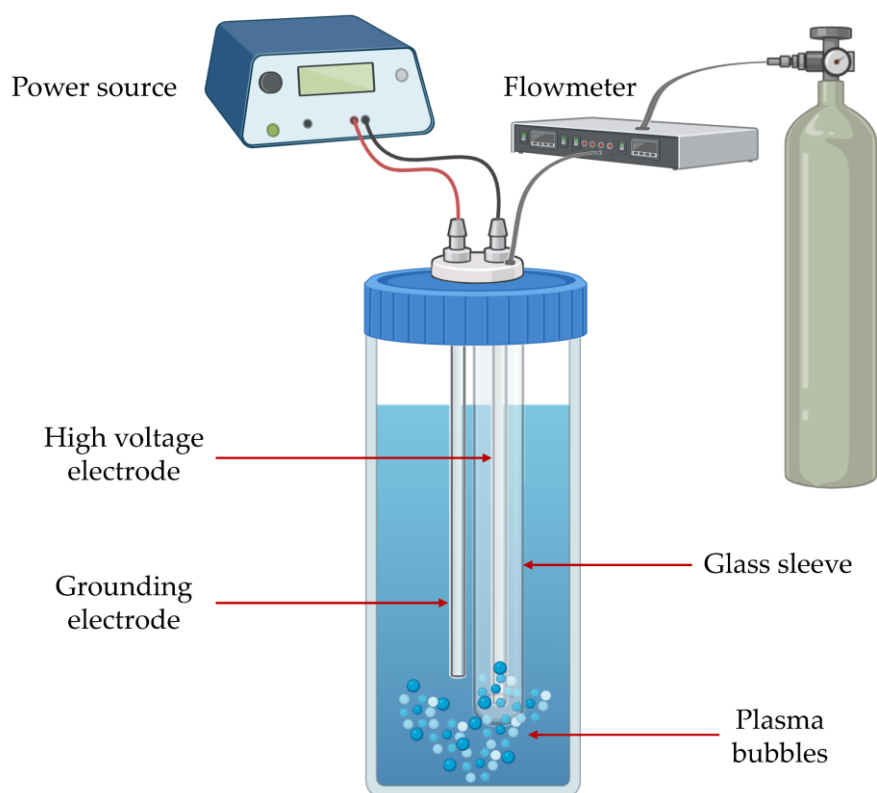


Figure 37. Plasma-producing device.

5.2.3 PAW generation

Plasma-activated water was generated using a voltage of 175 V, setting a discharge frequency of 1600 Hz, for 240 seconds (resonance frequency was kept at 60 kHz and duty cycles at 50 μ s). PAW was generated inside glass bottles, previously sterilized, containing 500 mL of deionized water. Synthetic air was used as gas to generate the plasma, and the flow rate was kept constant at 0.8 L/min for the entire treatment time. The physicochemical characteristics of PAW in terms of pH, oxidation-reduction potential (ORP) and electrical conductivity (EC) were evaluated before treatment and immediately after. In addition, nitrate concentrations were determined using the Spectroquant[®] test kit for Nitrates (#114773, Merck, Darmstadt, Germany), with absorbance measured at 516 nm using a SpectraMax[®] iD3 spectrophotometer (San José, CA, USA). Calibration curve was prepared with a standard solution. The range used was 0.5–100.0 mg/L for nitrate with distilled water serving as the blank. Ozone levels were quantified using a Hanna Instruments multiparameter photometer (HI83399-02) equipped with a colorimetric kit. Moreover, the antibacterial activity of PAW was evaluated against mature biofilms (formed in 24 h), as subsequently described. The plasma-activated water generation conditions are listed in **Table 8** and expressed in terms of voltage, frequency and treatment time.

Table 8. PAW generation conditions.

Voltage (V)	Frequency (Hz)	Time (s)
175	1600	240

V= Volt; Hz= Hertz; s= seconds

5.2.4 Bacterial culture

Staphylococcus aureus 32, *S. epidermidis* M19C, *S. epidermidis* NM128 and *S. epidermidis* MI282, from the private collection of the Food and Hygiene Department of the University of León (Spain), were used in the study. The strains, previously characterized

using the MALDI Biotyper® identification System (Bruker, Billerica, Massachusetts, US), were stored at -80°C until the use. For recovering, the strains were recovered on Brain Heart Infusion (BHI, Condalab, Madrid, Spain) agar plates (1.5% of agar) and then incubated at 37°C overnight in aerobic conditions.

5.2.5 Anti-biofilm activity of PAW

Isolated colonies of each strain were cultured in BHI and incubated again at 37°C overnight in aerobic conditions. After incubation, the broth cultures were diluted 1:10 in the same medium and refreshed for 2 h at 37°C with shaking (120 rpm). Finally, the broth cultures were adjusted using a spectrophotometer Elisa (BioTek 800 TS, Agilent, Santa Clara, California, US) to obtain an optical density of 0.25 at a wavelength of 600 nm. Standardized cultures represent the working broth cultures. PAW anti-biofilm activity evaluation is displayed in **Figure 36** (experimental plan). Briefly, stainless steel plates with a diameter of 3.5 cm were used. For mono-species biofilm, each plate was inoculated with 4 mL of the working broth culture of *S. aureus* 32, or *S. epidermidis* MIX (*S. epidermidis* M19C + *S. epidermidis* NM128 + *S. epidermidis* MI282), while for dual species-biofilm plates were inoculated with 4 mL of all strains together (2 mL of *S. aureus* 32 + 2 mL of *S. epidermidis* MIX). Plates were incubated at 37°C for 24 h. As illustrated in **Figure 36**, 24 plates were inoculated with bacterial suspension. Each condition, that were bacterial growth control (gCTR) non-treated, positive control (CTR⁺) treated with sterile water only and samples treated with PAW for 5 minutes (T_{5'}), 15 minutes (T_{15'}), 30 minutes (T_{30'}) and 60 minutes (T_{60'}), had 4 replicates (R1, R2, R3, R4) each one. Two plates containing only medium, were included to check the sterility of the medium itself, representing the negative control (CTR⁻).

After incubation time, non-adherent bacterial suspensions were removed from each plate, and then they were washed 3 times with 5 mL of sterile saline solution. Then, plates were left to dry at 25°C. For the control of bacterial growth (gCTR), once plates were dried, a sterile swab previously soaked in a sterile saline solution was swabbed into the plates. The swab was inserted in a sterile tube containing saline solution and it was subsequently diluted and spread on MSA plates. In positive control (CTR⁺) and treated samples (T_{5'}, T_{15'}, T_{30'}, T_{60'}), after washing and drying, there were added 5 mL of sterile water and 5 mL of PAW, respectively. After 60 minutes for CTR⁺ and after 15', 30' and 60' for treated samples (for T_{5'}, T_{15'}, T_{30'}, T_{60'}, respectively), the plates were emptied and then 5 mL of sterile neutralization solution (Tween 80 10 g/L, L-histidine 0.5 g/L, lecithin of soya 1 g/L, Na₂S₂O₃ 2.5 g/L in PBS, pH 7.0) were added to each plate and left to act for 2 minutes [297]. Plates were left to dry again, and then they were swabbed, followed by dilution and spread, as previously described for gCTR. MSA plates were incubated at 37°C for 24-48 h and CFU/mL reduction was evaluated using the following formula:

$$\% \text{ reduction}_{\text{CFU/mL}} = 100 \times (\text{CTR}^+_{\text{CFU/mL}} - \text{T}_{5'/15'/30'/60'}_{\text{CFU/mL}}) / \text{CTR}^+_{\text{CFU/mL}}$$

5.2.6 Statistical analysis

The results were obtained from at least two independent experiments, each performed in quadruplicate, and they are expressed as average $\pm$ standard deviation. $p \leq 0.05$ were considered as statistically significant. Group variances were assessed using the ANOVA test.

5.3 Results

5.3.1 Characteristics of PAW

The chemical and physical properties of water were measured before and after plasma treatment; the differences between sterile water and PAW in terms of pH, oxidation-reduction potential and conductivity, are highlighted in **Table 9**.

Table 9. Changes of parameters in PAW than sterile water.

	pH	ORP (mV)	EC ($\mu\text{S}/\text{cm}$)
Deionized water	6.4 ± 0.2	378.7 ± 61.4	1.8 ± 0.8
PAW	4.2 ± 0.02	765.0 ± 41.61	51.2 ± 7.6

mV= millivolt; $\mu\text{S}/\text{cm}$ = Microsiemens Per Centimeter

The content of nitrate and ozone generated in the water after treatment with plasma system, were measured and they are listed in **Table 10**.

Table 10. Content of Nitrate and Ozone generated in PAW.

	NO_3^-	O_3
Water	0.00	0.00
PAW	8.25 ± 0.35	0.19 ± 0.09

NO_3^- = Nitrate; O_3 = Ozone

5.3.2 Anti-biofilm activity of PAW

As displayed in **Figure 38**, the anti-biofilm activity of PAW showed a specie-specific activity against mono-specie biofilm of *S. epidermidis* MIX and *S. aureus* 32. In detail, there was no reduction in *S. epidermidis* MIX biofilm CFU/mL at all treatment times, while there was recorded a significant CFU/mL reduction of *S. aureus* 32 biofilm, after 15 minutes, 30 minutes and 60 minutes, where the percentage of reduction where respectively 11%, 13%

and 16%, corresponding to 0.7, 0.8 and 1 log reduction, respectively. It is possible to conclude that PAW showed a time-dependent reduction of *S. aureus* biofilm.

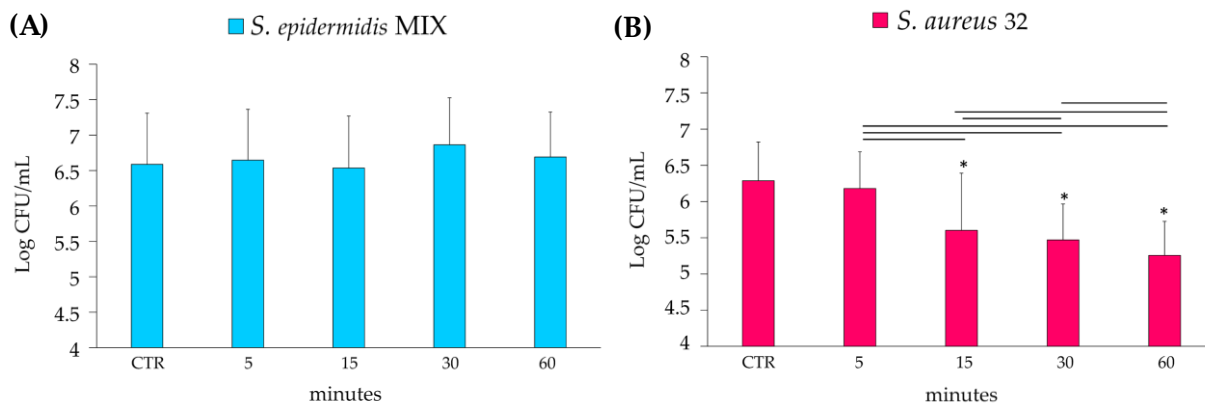


Figure 38. Anti-biofilm activity of PAW against mono-species biofilm of *S. epidermidis* MIX (A) and *S. aureus* 32 (B). The antibacterial effect was evaluated during the time (5, 15, 30 and 60 minutes). CTR represent the non-treated sample (control). *Statistically significant in respect to the control. The lines indicate the intragroup significance.

In dual-species biofilms, the strain-selectivity showed in mono-species biofilm was lost. Both bacteria showed a significant reduction after 60 minutes, corresponding to 0.5 log reduction for *S. epidermidis* MIX and 0.3 log reduction for *S. aureus* 32 (see **Figure 39**).

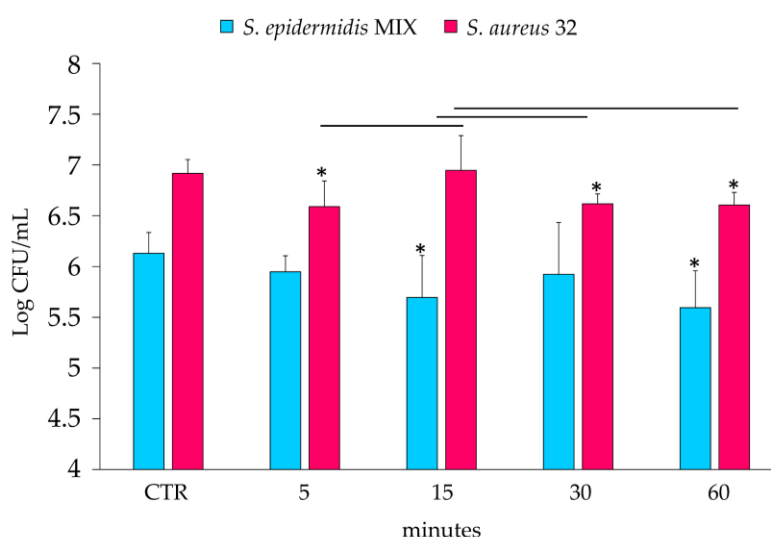


Figure 39. Anti-biofilm activity of PAW against dual-species biofilm of *S. epidermidis* MIX and *S. aureus* 32. The antibacterial effect was evaluated during the time (5, 15, 30 and 60 minutes). CTR represent the non-treated sample (control). *Statistically significant in respect to the control. The lines indicate the intragroup significance.

5.4 Discussion

The outcomes of this study confirm the already existing research regarding the anti-biofilm properties of plasma activated water. The observed reduction in colony-forming units of *S. aureus* in mono-species biofilms after PAW treatment supports the recent findings of Xia et al, who reported a significant biofilm reduction due to the degradation of exopolysaccharides, extracellular DNA and protein of EPS, compromising bacterial membranes and causing the intracellular accumulation of ROS and RNS, both in gram-positive and gram-negative. Moreover, the disruption of EPS, resulted in an exposure of bacterial cells to PAW, leading to a significant increase in membrane permeability and disruption in membrane structures. Anyway, this study showed a more pronounced effect probably because PAW treatment was applied *in situ* [305]. Also Xu et al demonstrated that PAW exhibit an inhibitory effect on *S. aureus* biofilm, mainly due to ROS and RNS accumulation and also that this treatment is effective against time-dependent long-term regeneration of *S. aureus* biofilm. Interestingly in this work it was highlighted that different inducement time and treatment time of PAW, and their combination, can influence the outcomes. These results underline the importance to find the best inducement-treatment time combination [306].

However, this study identified *S. epidermidis* (in mono-species biofilm) as more resistant to PAW treatment. Probably it is due to the behaviour of antioxidant biofilm-related system, that act in different way in the two analyzed species. In particular, as observed by Olwal et al, *S. epidermidis* is able to increase the release of eDNA in respect to the non-treated control, representing a biofilm resistance quality against oxidants [307]. Moreover, it was found that the biofilm of this bacterium is able to produce higher levels of catalase and therefore probably is able to counteract oxidative stress produced by ROS and RNS

[308]. Probably for this reason, *S. aureus* biofilm results more sensible to oxidants exposure, like H_2O_2 , leading to a more pronounced modification of biofilm structure than *S. epidermidis*. These mutations are reduced after the addition of catalase enzyme [309]. The lack of significant CFU reduction for *S. epidermidis* in mono-species biofilms suggests structural differences in the biofilm matrix, which protect embedded cells from reactive species. This resilience emphasizes the importance of optimizing PAW treatment parameters, such as exposure time and reactive species concentrations.

In dual-species biofilms, the moderate reduction in CFU for both *S. aureus* and *S. epidermidis* indicates a complex interaction between the species within the biofilm matrix. In fact, for example Bottery et al, similarly observed that interspecies interactions could alter biofilm composition and susceptibility to antimicrobial agents [310]. The diminished PAW efficacy in dual-species settings compared to mono-species biofilms underscores the need to consider microbial diversity in designing PAW-based applications.

Compared to chemical disinfectants, PAW has an excellent antibacterial activity, is chemical residue-free and environmentally friendly, that provides a clear advantage for cleaning and sterilization, which emphasized its application potential in food safety and healthcare [311]. However, the challenges of the species-specific efficacy observed in this study highlight gaps in existing methodologies that require further exploration. Moreover, another challenge link to the use of PAW, is the stability in terms of antimicrobial activity that can be influenced from several factors. For example, storage temperature can cause a decrease in antimicrobial potential, probably due to the modifications in the amount of the long-term reactive species [312].

To address these limitations, future studies could investigate the synergistic use of PAW with natural bioactive compounds or its combination with mechanical biofilm

disruption techniques. This approach is supported by research suggesting enhanced antimicrobial efficacy when PAW is integrated with complementary strategies like natural extract with antimicrobial activity [313], or eco-sustainable technologies like ultrasound assisted extraction [314].

Generally, the inhibitory potential of PAW against microorganisms, depends on several factors including the exposure time of the water to the plasma, as it has been seen that longer activation times generate more reactive species and consequently offer greater antimicrobial activity [295]. Conductivity and oxidation-reduction potential also increase with increasing activation time, while pH decreases [315]. However, it must take into account that longer exposure times and higher electrical voltages imply higher energy expenditure. In my opinion, since it is an eco-sustainable technology, PAW should be generated considering these aspects, looking for the best combination of parameters to effectively reduce the microbial load while preserving the environment.

6. CONCLUSION

The worldwide prevalence of skin disorders associated with microbial dysbiosis highlights the urgent need for innovative, sustainable and effective therapeutic strategies.

This thesis explored the potential of eco-sustainable antimicrobial approaches to affect skin eubiosis with a focus on natural extracts derived from food by-products such as pomegranate peel and almond hull, and eco-friendly technologies such as plasma-activated water, generally demonstrating significant antimicrobial activity against pathogenic species while preserving beneficial ones. Each chapter underscores the potential of these eco-sustainable approaches, demonstrating their effectiveness, safety, and alignment with circular economy principles.

The findings underline the biological value of waste materials from the food industry, not only to address global environmental challenges, but also to align dermatological treatments with One Health approaches. By reducing the overuse of synthetic antimicrobials, which often disrupt the balance of the microbiota and contribute to antibiotic resistance, these bioactive compounds offer a promising, effective and eco-friendly alternative.

In conclusion, the strategies studied in this thesis represent a convergence of scientific innovation and ecological responsibility. While research in this field continues to evolve, it is critical to further explore the synergistic potential of natural extracts (also with green technologies), optimize their formulations, and validate their efficacy in clinical settings. These innovations highlight the potential of microbiota-focused therapies to not only treat skin conditions but also promote long-term skin health. By prioritizing sustainability along with therapeutic efficacy, future advances can contribute not only to healthier skin, but also to a healthier planet.

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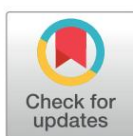
RESEARCH ARTICLE

Staphylococcus aureus/*Staphylococcus epidermidis* from skin microbiota are balanced by Pomegranate peel extract: An eco-sustainable approach

Sara D'Arcangelo¹, Paola Di Fermo², Firas Diban¹, Vincenzo Ferrone¹, Simonetta D'Ercole², Mara Di Giulio^{1*}, Silvia Di Lodovico¹

1 Department of Pharmacy, University "G. d'Annunzio" Chieti-Pescara, Chieti, Italy, **2** Department of Medical, Oral and Biotechnological Sciences, University "G. d'Annunzio" Chieti-Pescara, Chieti, Italy

* m.digiulio@unich.it



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Abstract

The imbalance in skin microbiota is characterized by an increased number of pathogens in respect to commensal microorganisms. Starting from a skin microbiota collection, the aim of this work was to evaluate the possible role of Pomegranate (*Punica granatum* L.) Peel Extract (PPE) in restoring the skin microbiota balance acting on *Staphylococcus* spp. PPE was extracted following green methodology by using n-butane and the Dimethyl Ether (DME) solvents and analyzed for phytochemical composition and antimicrobial activity. The PPE antimicrobial action was evaluated against Gram +, Gram – bacteria and yeast reference strains and the most effective extract was tested against the main skin microbiota isolated strains. PPE extracted with DME showed the best antimicrobial action with MICs ranging from 1 to 128 mg/mL; the main active compounds were Catechin, Quercetin, Vanillic acid and Gallic acid. The PPE in DME anti-adhesive effect was examined against *S. epidermidis* and *S. aureus* mono and dual-species biofilm formation by biomass quantification and CFU/mL determination. The extract toxicity was evaluated by using *Galleria mellonella* larvae *in vivo* model. The extract displayed a significant anti-adhesive activity with a remarkable species-specific action at 4 and 8 mg/mL against *S. epidermidis* and *S. aureus* mono and dual-species biofilms. PPE in DME could represent an eco-sustainable non-toxic strategy to affect the Staphylococcal skin colonization in a species-specific way. The innovation of this work is represented by the reuse of food waste to balance skin microbiota.

Introduction

The skin represents the largest organ of the human body with a surface area of about 2 m² that extends to 25 m² taking into account all the skin appendages (about 5 million), including sweat ducts and hair follicles [1]; it represents a shield against the environmental pollutants, chemicals and microorganisms [2]. Healthy human skin is colonized by numerous microorganisms which constitute the skin microbiota that lives in equilibrium establishing a microbial

Article

Almond Hull Extract Valorization: From Waste to Food Recovery to Counteract *Staphylococcus aureus* and *Escherichia coli* in Formation and Mature Biofilm

Sara D'Arcangelo ^{1,†}, Debora Santonocito ^{2,†}, Luciano Messina ³, Valentina Greco ⁴, Alessandro Giuffrida ⁴, Carmelo Puglia ², Mara Di Giulio ¹, Rosanna Inturri ^{3,*} and Susanna Vaccaro ³

¹ Department of Pharmacy, University "G. d'Annunzio" Chieti-Pescara, 66100 Chieti, Italy; sara.darcangelo@unich.it (S.D.); m.digiulio@unich.it (M.D.G.)

² Department of Drug Sciences and Health, University of Catania, 95125 Catania, Italy; debora.santonocito@unict.it (D.S.); capuglia@unict.it (C.P.)

³ Fidia Farmaceutici SpA, Local Noto Unit Contrada Pizzuta, 96017 Noto, Italy; lmessina@fidiapharma.it (L.M.); svaccaro@fidiapharma.it (S.V.)

⁴ Department of Chemical Sciences, University of Catania, 95125 Catania, Italy; valentina.greco@unict.it (V.G.); alessandro.giuffrida@unict.it (A.G.)

* Correspondence: rinturri@fidiapharma.it; Tel: +39-0931-812810

† These authors contributed equally to the work.



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Abstract: The increase in food waste accumulation needs innovative valorization strategies that not only reduce environmental impacts but also provide functional applications. This study investigates the potential of almond hulls, an abundant agricultural by-product, as a source of bioactive compounds. For the first time, almond hull extract (AHE), was evaluated in terms of anti-adhesive and anti-biofilm activity against *Staphylococcus aureus* ATCC 29213 and *Escherichia coli* ATCC 9637. The extract was obtained by an optimized eco-friendly green technique using ultrasound-assisted extraction (UAE), and it was characterized for its main compounds by high-performance liquid chromatography–mass spectrometry (HPLC-MS) and nuclear magnetic resonance (NMR) analysis. Antimicrobial activity was evaluated on planktonic cells by minimum inhibitory /bactericidal concentration (MIC/MBC) and by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assays. Afterward, AHE activity was evaluated against the bacterial sessile phase, both against in-formation and mature biofilm. Finally, the toxicity of the extract was tested on normal human adult cells (HDFa) by an MTT test. The principal active compounds present in AHE belong to the polyphenol group, in particular, the phenolic acid (Hydroxycinnamic sub-class) and, more significantly, the flavonoid class. The results showed that the extract has a relevant antimicrobial activity against the planktonic cells of both tested strains. Moreover, it significantly inhibited bacterial adhesion and promoted biofilm removal, highlighting its potential as a sustainable antimicrobial agent. The MTT test on human fibroblasts showed that the extract is not toxic for normal human cells. This research highlights how food waste valorization could have a high potential in the antimicrobial field.

Keywords: almond hull; eco-sustainability; food waste; biofilm; *S. aureus*; *E. coli*; food by-products; green extraction

1. Introduction

The food industry and food consumption generate around 40–60% of total municipal solid waste worldwide [1,2], with approximately 5×10^9 tons from agro-forest and 5×10^8 tons from food processing per year [3].

Conversely, by-products from the food-processing industry, including fruit pulp, peel, pomace, and seeds, as well as leaves, branches, flowers, and bark resulting from agro-industrial pruning operations, represent a significant source of bioactive compounds of considerable value, including proteins, lipids, and carbohydrates. These compounds have

8. Publications list during PhD period (from 2022 to 2024)

- **D'Arcangelo S**, Santonocito D, Messina L, Greco V, Giuffrida A, Puglia C, Di Giulio M, Inturri R, Vaccaro S. Almond Hull Extract Valorization: From Waste to Food Recovery to Counteract *Staphylococcus aureus* and *Escherichia coli* in Formation and Mature Biofilm. *Foods* **2024**, *13*, 3834. <https://doi.org/10.3390/foods13233834>.
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9. Conferences

9.1 International conferences

- Francés Á, **D'Arcangelo S**, Oliveira M, Cordero-García R, González-Raurich M, Álvarez-Ordóñez A, López M. Plasma-activated water as an innovative alternative for for the elimination of *Listeria monocytogenes* biofilms. XXIII National Congress of Food Microbiology, 09-12 Settembre 2024 Cartagena, Spain.
- Di Lodovico S, Nocera FP, Petrini M, Di Fermo P, **D'Arcangelo S**, De Pasquale V, De Martino L, Cellini L. *Staphylococcus pseudintermedius* an emerging antimicrobial resistant zoonotic strain for set up the Lubbock Chronic Wound Biofilm model. IC²AR Congress 2024, 08-12 Settembre 2024 Caparica, Portogallo. (**Premio best poster presentation**)
- **D'Arcangelo S**, Di Lodovico S, Diban F, Pinti M, Di Campli E, D'Ercole S, Di Giulio M. Pomegranate peel extract: an eco-sustainable tool to counteract skin resistant pathogenic Staphylococci. IC²AR Congress 2024, 08-12 Settembre 2024 Caparica, Portogallo. (**Premio best poster presentation**)
- **D'Arcangelo S**, Di Fermo P, Di Campli E, Pinti M, Janowicz AA, Di Giulio M. Staphylococci skin strains modulation by Pomegranate peel extract: an eco-sustainable approach. SCIAMI International workshop, 07-08 Giugno 2024, Chieti, Italia.
- Di Lodovico S, Nocera FP, De Pasquale V, Pinti M, **D'Arcangelo S**, Petrini M, Di Giulio M, D'Ercole S. Lubbock Chronic Wound Biofilm (LCWB) model: an *in vitro* *Staphylococcus pseudintermedius* and *Pseudomonas aeruginosa* dual-species biofilm. SCIAMI International workshop, 07-08 Giugno 2024, Chieti, Italia. (**Premio best poster presentation**)
- Di Lodovico S, Diban F, Di Fermo P, **D'Arcangelo S**, D'Ercole S, Cellini L, Di Giulio M. Graphene Oxide: an efficacious Challenge for the Chronic Wounds management. European Graphen Forum EGF 2023, Albufeira-Algarve, Portugal 25-27/10/2023
- Di Fermo P, Di Lodovico S., Ciociola T., D'ercole S., Petrini M., Diban F., Conti S., **D'Arcangelo S.**, Di Giulio M. Modulating action of antimicrobial peptide L18R against inter-kingdom biofilm in Lubbock Chronic Wound Biofilm model. IC2AR Congress 2022, 05-08 Settembre 2022 Caparica, Lisbona, Portogallo.
- Di Lodovico S., Di Fermo P., **D'Arcangelo S.**, Menghini L., Ferrante C., Castro-Amorim J., Gamberio P, Bessa L. J., Cellini L. Efficacious antimicrobial and antibiofilm action of Hop extract against multi-drug resistant *Staphylococcus* strains and *Cutibacterium acnes*. IC2AR Congress 2022, 05-08 Settembre 2022 Caparica, Lisbona, Portogallo. (**Premio best poster presentation**)

9.2 National conferences

- Di Lodovico S, Di Fermo P, Petrini M, D'Amico E, **D'Arcangelo S**, Diban F, Cellini L, Di Giulio M, D'Ercole S. Complex Electromagnetic Fields: eco-sustainable strategy to counteract the resistant *Candida albicans* infections. 51° Congresso Nazionale SIM, Cagliari, 24-27 /09/2023
- Di Giulio M, Di Fermo P, Diban F, **D'Arcangelo S**, De Filippis B, Di Lodovico S, D'Ercole S, Di Campli E, Cellini L. Resveratrol derivatives, promising strategy for innovative therapeutic schemes to tackle the *Helicobacter pylori* persistent infections. 51° Congresso Nazionale SIM, Cagliari, 24-27 /09/2023
- Di Lodovico S, **D'Arcangelo S**, Di Campli E, Dotta T C, Cellini L, Di Giulio M, D'Ercole S. Capparispinose: an aqueous eco-friendly extract to treat multi-drug resistant pathogens in chronic wounds. 14° Congresso nazionale Società Italiana di Microbiologia Farmaceutica, 23-24/06/2023, Roma. (**Premio best poster presentation**)
- Diban F, Di Fermo P, Ancarani E, **D'Arcangelo S**, Del Gaudio L, Cellini L, Di Giulio M. New commercial wipes inhibit the adhesion and dispersion of environmental biofilms. Nuova Società Italiana di Microbiologia Farmaceutica ETS 2023, Roma, Italia, 23-24/06/2023
- Di Fermo P., Di Lodovico S., Di Campli E., **D'Arcangelo S.**, Cellini L., Di Giulio M. Effect of Vitamin C on dormant states of *Helicobacter pylori*. Società Italiana di Microbiologia 2022, 18-21 Settembre 2022 Napoli, Italia.
- Di Lodovico S., D'Ercole S., Diban F., **D'Arcangelo S.**, Di Campli E., J. Bessa L., Cellini L., Di Giulio M. Antimicrobial and antivirulence effects of Hop extract against multi-drug resistant Staphylococci strains and *Cutibacterium acnes*. Società Italiana di Microbiologia 2022, 18-21 Settembre 2022 Napoli, Italia.

10. Awards

- **Best poster presentation** of the 37th PhD cycle in BFS. Participation in the first PhD day in Biomolecular and Pharmaceutical Sciences (BFS) on 14/11/2024 at Gabriele d'Annunzio University, Chieti-Pescara, with poster presentation entitled: “Innovative species-specific action formulations for balancing the skin microbiota.”
- **Best poster presentation** in IC²AR Congress 2024, September 08-12, 2024 Caparica, Portugal (Di Lodovico S, Nocera FP, Petrini M, Di Fermo P, **D’Arcangelo S**, De Pasquale V, De Martino L, Cellini L. *Staphylococcus pseudintermedius* an emerging antimicrobial resistant zoonotic strain for set up the Lubbock Chronic Wound Biofilm model).
- **Best poster presentation** in IC²AR Congress 2024, September 08-12, 2024 Caparica, Portugal (**D’Arcangelo S**, Di Lodovico S, Diban F, Pinti M, Di Campli E, D’Ercole S, Di Giulio M. Pomegranate peel extract: an eco-sustainable tool to counteract skin resistant pathogenic Staphylococci).
- **Best poster presentation** SCIAMI International workshop, June 07-08 2024, Chieti, Italy (Di Lodovico S, Nocera FP, De Pasquale V, Pinti M, **D’Arcangelo S**, Petrini M, Di Giulio M, D’Ercole S. Lubbock Chronic Wound Biofilm (LCWB) model: an *in vitro* *Staphylococcus pseudintermedius* and *Pseudomonas aeruginosa* dual-species biofilm).
- **Best poster presentation** in IC²AR Congress 2024, September 05-08, 2022 Caparica, Portugal (Di Lodovico S., Di Fermo P., **D’Arcangelo S.**, Menghini L., Ferrante C., Castro-Amorim J., Gamberio P, Bessa L. J., Cellini L. Efficacious antimicrobial and antibiofilm action of Hop extract against multi-drug resistant *Staphylococcus* strains and *Cutibacterium acnes*).
- **Best poster presentation** in 14th National Congress Italian Society of Pharmaceutical Microbiology, June 23-24 2023, Rome, Italy (Di Lodovico S, **D’Arcangelo S**, Di Campli E, Dotta T C, Cellini L, Di Giulio M, D’Ercole S. Capparis spinose: an aqueous eco-friendly extract to treat multi-drug resistant pathogens in chronic wounds)