SAPIENZA
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Atopic dermatitis: an inside look at the role of the skin microbiome.

Facoltà di Farmacia e Medicina, Ingegneria dell'informazione, informatica e statistica, Medicina e Odontoiatria, Scienze Matematiche, Fisiche e Naturali

Dipartimento di Medicina molecolare
Corso di laurea in Bioinformatics

Ilaria Coratella

Matricola: 1846271

Relatore

Prof. Enea Gino Di Domenico

Correlatore

Dott. Mauro Truglio

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A nonno Carlo

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Introduction

1.

Atopic dermatitis (AD) is a chronic inflammatory skin disease characterized by impaired epidermal barrier function and cutaneous inflammation. It is a prevalent condition, affecting approximately 15-20% of children and 1-3% of adults worldwide, with most cases occurring within the first year of life.

Several factors contribute to the pathogenesis of AD, including skin barrier defects such as increased trans-epidermal water loss and defective keratinocyte differentiation, as well as reduced levels of ceramides, filaggrin, antimicrobial peptides and a high degree of *Staphylococcus aureus* colonization in the affected skin lesions. In fact, several studies demonstrated that *S. aureus* is the main character in the subjects affected by AD. Furthermore, the dysbiosis of the cutaneous microbiota, with an altered balance of beneficial commensal microbes and the overgrowth of *S. aureus*, also plays a significant role in AD progression. In healthy individuals, a restricted collection of microbes typically colonizes the epithelial surface, while AD exhibits an altered skin bacterial microflora compared to non-AD individuals. Emerging research is uncovering the intricate association between distinct microbial classes, inflammatory skin diseases like AD, and their impact on the host genome.

The overabundance of this bacterium contributes to immune dysfunction, reduced antimicrobial peptides (AMPs), heightened allergic reactions, and skin barrier disruption. In particular, the dominance of biofilm-growing *S. aureus* in AD lesions is directly correlated with disease severity, thus contributing to sweat duct occlusion, skin inflammation, and

pruritus. In addition to dysbiosis, other ecological factors like humidity, temperature, pH, and lipid content influence the regulation of the skin microbiome.

The prevalence of AD has increased globally, and environmental factors play a crucial role in its development. Protective environmental factors like having multiple siblings, pet ownership, and farm residence have been associated with lower AD risk. At the same time, clinically apparent infectious diseases in early infancy and antibiotic use have been linked to an increased risk. These associations strongly support the involvement of microbes in the etiology of AD. The current understanding of host-microbe interactions at the molecular level provides insights into the clinical associations with the environment and opens avenues for novel therapeutic approaches.

Diagnosis

1.1.

AD diagnosis is based on history, clinical manifestations, and morphology of skin lesions. In 1980, Hanifin and Rajka proposed major and minor criteria for AD diagnosis (Table 1), with three of four major criteria and three of 23 minor criteria required for diagnosis (Kulthanan et al., 2021).

Major features (3 of 4 required)
1. Pruritus
2. Typical morphology and distribution
2.1 Flexural lichenification or linearity in adults
2.2 Facial and extensor involvement in infants and children
3. Chronic or chronically-relapsing dermatitis
4. Personal or family history of atopy, such as asthma, allergic rhinitis, atopic dermatitis
Minor features (3 of 23 required)
1. Xerosis
2. Ichthyosis/palmar hyperlinearity/keratosis pilaris
3. Immediate (type 1) skin test reactivity
4. Elevated serum immunoglobulin E
5. Early age of onset
6. Tendency toward cutaneous infections (*S. aureus* and Herpes simplex virus)/impaired, cell-mediated immunity

Minor features (3 of 23 required) (Continued)
7. Tendency toward non-specific hand or foot dermatitis
8. Nipple eczema
9. Chelitis
10. Recurrent conjunctivitis
11. Dennie-Morgan infraorbital fold
12. Keratoconus
13. Anterior subcapsular cataract
14. Orbital darkening
15. Facial pallor/facial erythema
16. Pityriasis alba
17. Anterior neck fold
18. Itch when sweating
19. Intolerance to wool and lipid solvents
20. Perifollicular accentuation
21. Food intolerance
22. Course influence by environmental/emotional factors
23. White dermographism/delayed blanch

Table 1: Major and minor criteria for diagnosis of AD (Kulthanan et al., 2021).

Percutaneous skin or *in vitro* tests, such as specific IgE and patch testing, can identify potential allergic triggering factors and contact dermatitis. Food allergies, particularly in children under five years, are commonly associated with AD. Food allergy testing may be considered in children with moderate-to-severe AD who do not respond well to treatment, followed by an oral food challenge test for confirmation (Kulthanan et al., 2021).

The Scoring Atopic Dermatitis Index (SCORAD), Eczema Area and Severity Index (EASI), Investigator Global Assessment scale (IGA), and Six Area, Six Sign Atopic Dermatitis severity score (SASSAD) are widely used tools for assessing AD severity based on affected body areas and lesion characteristics (Frazier et al., 2020).

Distinguishing AD from other skin disorders can be challenging. Familial history of atopy, lesion scattering, and consideration of nutritional abnormalities, malignancies, and keratinization or immunodeficiency diseases aid in the differential diagnosis of AD (Mandlik et al., 2021). Skin barrier dysfunction, including filaggrin gene expression, skin ceramide levels, and epidermal proteases, contribute to the pathogenesis of AD (Mandlik et al., 2021).

Risk factors

1.2.

AD is influenced by various risk factors that can be categorized into environmental, genetic, immunological and microbial factors.

Environmental factors play a role in AD development. Climate, including temperature and latitude, has been linked to variations in AD prevalence. Urban living, characterized by pollution, stress, and social isolation, is associated with a higher risk. Dietary habits, such as the Mediterranean diet, may protect against AD. Breastfeeding and the timing of weaning could influence susceptibility to AD, but further research is needed. Obesity and lack of physical exercise contribute to chronic inflammation and impaired metabolism, negatively affecting the skin. Exposure to environmental factors like air pollution and tobacco smoke increases the risk of AD (Bonamonte et al., 2019).

Genetic factors play a significant role in AD, as the condition tends to run in families. Genome-wide analyses and candidate gene studies have identified the filaggrin gene (FLG) as a susceptibility gene for AD. FLG mutations impair skin barrier function and are found in a significant percentage of AD patients, particularly in Northern European populations. Other genes involved in the Th2 immune response, such as IL-4 and IL-13, have also been associated with AD. Monogenic diseases resembling AD have revealed potential candidate genes. Ongoing research aims to discover additional genes and factors contributing to AD (Brown et al., 2020).

In individuals with AD, there are notable changes in the skin microbiota. Lesional skin is frequently colonized by *S. aureus*, leading to reduced microbial diversity. Medical treatments for eczema only partially reverse these changes. *S. aureus* colonization increases during AD flare episodes and in the remission phase. Also, non-lesional skin and the nose of AD individuals exhibit altered microbiome composition. *S. aureus* and *S. epidermidis* become dominant species during AD flares, while they are less prevalent in healthy skin.

The increase in *S. epidermidis* may serve as a compensatory mechanism to limit *S. aureus* colonization. Specific clonal lineages of *S. aureus* are associated with the severity of AD, and their clonal expansion can persist within the host, indicating resistance to treatment (Di Domenico et al., 2019).

Main mechanisms of AD

1.3.

Structure of the epidermis and the role of the skin barrier

1.3.1.

Microbes contribute to the skin barrier by producing antimicrobial peptides (AMPs) and free fatty acids and stimulating the innate immune system. The physical barrier of the epidermis consists of corneocytes, desmosomes, claudins, and lipids, while the chemical barrier includes molecules that contribute to hydration and infection prevention. The immunological barrier involves components of the innate and adaptive immune systems. The neuro-sensory system functions as a danger sensor, including keratinocytes and ion channels on cutaneous nerves (Luger et al., 2021).

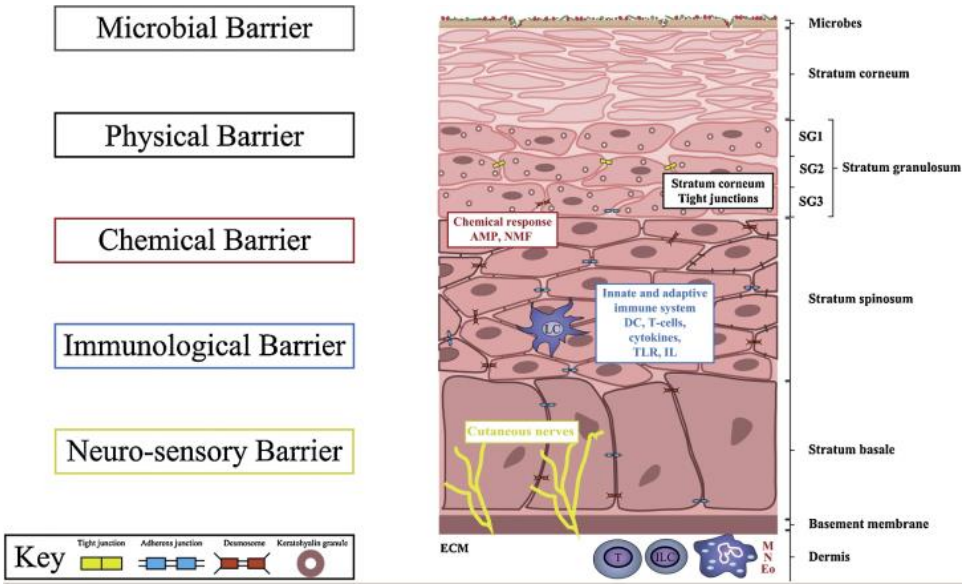

Figure 1: epithelial structure, (Luger et al., 2021).

Keratinocytes, the predominant cell type in the epidermis, differentiate from the deepest layer towards the skin's surface. The epidermal layers include the stratum basale (SB), stratum spinosum (SS), stratum granulosum (SG), and stratum corneum (SC). Keratinocytes produce lamellar bodies in the SS, which deliver precursors of SC lipids into the intercellular space. The SC, composed of corneocytes, provides physical protection and is surrounded by a cornified cell envelope and extracellular lipids that create a barrier against water loss and penetration of exogenous molecules. The SC lipids, including ceramides, free fatty acids, and cholesterol, play a critical role in permeability and barrier function. Tight junctions form a barrier in the living epidermal layers, and Langerhans cells interact with the SG2 layer of keratinocytes. Disruption of the barrier can lead to pathogen entry, triggering immune responses. Keratinocytes and neutrophils produce AMPs and participate in innate immunity. Additionally, keratinocytes produce pro-inflammatory cytokines and interact with T cells, while Langerhans cells play a role in adaptive immunity (Luger et al., 2021).

Filaggrin mutations in AD

1.3.2.

Filaggrin is a large protein (37-kD) that plays a crucial role in the structure and function of the SC, which is the outermost layer of the skin. It is named for its ability to aggregate keratin intermediate filaments. The precursor of filaggrin, called profilaggrin, is a larger and inactive polymer. Structurally, profilaggrin consists of a calcium-binding motif and 10 to 12 filaggrin monomers (Drislane et al., 2020).

Profilaggrin is expressed late in epidermal differentiation and stored in keratohyalin granules. It undergoes post-translational modification, including dephosphorylation and cleavage by various endoproteases, resulting in the formation of filaggrin monomers. Filaggrin monomers contribute to the mechanical strength of the cytoskeleton by binding to and collapsing keratin filaments. These filaments are anchored in the SC by corneodesmosomes, which contain specific proteins and adhesion molecules. The degradation of filaggrin releases amino acids and derivatives, which, along with other components, form the natural moisturizing factor (NMF). The NMF is essential for maintaining skin pH, hydration, UV protection, and the integrity of the epidermal barrier. (Drislane et al., 2020).

The FLG gene is located on chromosome 1q23.3 in the epidermal differentiation complex (Drislane et al., 2020). It consists of three exons, with exon 3 coding for the entire profilaggrin protein. Diagnostic sequencing of exon 3 is challenging due to its highly repetitive DNA sequence. Different alleles of the FLG gene encode 10, 11, or 12 filaggrin monomers, leading to varying levels of filaggrin protein in the epidermis. Low copy number variations (CNVs) have been associated with an increased risk of AD, independent of classic FLG LoF mutations. FLG LoF mutations are strongly associated with AD. These mutations cause a complete loss of the expressed protein on the affected allele, leading to a deficiency of processed filaggrin. The most common LoF variants, such as p.R501X and c.2282del4, are

0prevalent in northern Europeans, while East Asian populations exhibit a more diverse range of FLG mutations. AD associated with FLG mutations presents a distinct phenotype known as AD FLG. This phenotype is characterized by palmar hyperlinearity, an increased risk of asthma, more severe eczema herpeticum, heightened allergic sensitization, and a persistent clinical course. FLG null mutations are particularly associated with the early-onset persistent form of AD. While FLG LoF mutations are significant genetic risk factors for AD, only a portion (10% to 40%) of AD patients have these mutations. Other factors, such as the overexpression of type 2 cytokines like IL-4 and IL-13, contribute to acquired filaggrin deficiency in AD patients. This down-regulation of filaggrin expression affects the integrity of the epidermal barrier. AD patients often exhibit a skin barrier defect, as indicated by increased transepidermal water loss (TEWL) in both lesional and non lesional skin. The levels of the natural moisturizing factor (NMF), important for skin hydration, are reduced in AD patients, suggesting systemic down-regulation of filaggrin. FLG null mutations influence immune responses in AD. They increase levels of circulating thymic-derived Tregs and affect the balance between Th-1, Th-2, and Th-17-like Tregs. Filaggrin degradation products can modulate dendritic cell expression and promote regulatory T-cell production (Drislane et al., 2020).

Microbiome

1.3.3.

Healthy microbiome

1.3.3.1

The skin harbors the most diverse commensal communities in the body, with over 1000 different bacterial species from different phyla. Each person may have a personalized skin microbiome, although some shared features of skin microbial communities reflect common skin physiology. For instance, *Cutibacterium acnes* (previously known as *Propionibacterium acnes*) is commonly found in sebaceous skin sites. Interactions between the skin and its microbiota play a crucial role in maintaining a balance between health and disease. The skin's unique innate immune system, coupled with its physical barrier, regulates resident microbial communities. The presence of indigenous microbes is essential for optimal immune cell function in healthy skin, and their absence can lead to immune defects. Maintaining microbial diversity is beneficial for supporting the immune milieu of the skin, as specific microbes can induce specific types of immune cells and drive certain immune responses. Furthermore, commensal bacteria display chemical moieties that interact with innate immune receptors, influencing immune responses (Pothmann et al., 2019).

AD microbiome

1.3.3.2.

AD is characterized by an altered skin microbiota, with a high prevalence of *S. aureus* colonization and secondary infections. *S. aureus* exhibits host-specific factors, including adhesion mechanisms and proinflammatory mechanisms, that contribute to the inflammatory component of AD (Di Domenico et al., 2019). During an AD flare, the diversity of the skin microbiome decreases, leading to a reduction in the presence of genera such as *Streptococcus*, *Corynebacterium*, and *Cutibacterium*. This shift is accompanied by an increase in the prevalence of *Staphylococcus*, particularly *S. aureus*. Analysis through shotgun metagenomic sequencing has demonstrated that patients with AD often exhibit colonization by a single strain of *S. aureus* during severe flares. However, with effective treatment and recovery, the microbiome composition tends to restore to a more diverse and normal state (Pothmann et al., 2019). The prevalence of *S. aureus* colonization on AD lesional skin is higher compared to healthy control subjects, and it is positively correlated with disease severity. Furthermore, specific *S. aureus* strains isolated from AD patients differ from those found in unaffected carriers (Paller et al., 2019; Di Domenico et al., 2018).

AD microbiome is also characterized at variable titles by the presence of other species. Indeed, *Staphylococcus epidermidis,* commonly considered a commensal organism on the skin that aids in immune system maturation and pathogen defence, can, under certain circumstances, exacerbate the inflammatory response in AD (Hrestak et al., 2022). More recently, *S. lugdunensis* emerges as a player in AD, with different studies highlighting its overexpression during AD flares. Nevertheless, the exact role of this species in AD pathogenesis remains unclear (Edslev et al., 2021).

In addition to *S. aureus*, other microbes may play a role in AD exacerbation. The *Malassezia* genus of fungi, commonly found on the skin, is associated with AD, and antifungal therapy targeting *Malassezia* can benefit AD patients (Pothmann et al., 2019). Candida and

dermatophytes, along with viral skin diseases such as herpes simplex virus (HSV), may also contribute to AD flares. The interaction between these pathogenic microbes and the skin's immune cells contributes to inflammation and disease progression (Pothmann et al., 2019).

Role of *S. aureus* in AD

1.3.4.

S. aureus

1.3.4.1.

S. aureus is a gram-positive widespread bacterial pathogen and a leading cause of skin and soft tissue infections, surgical site infections, respiratory tract infections, and bacteremia. *S. aureus* produces virulence factors, including toxins, enzymes, and antigens, which help it evade the host's immune system. These toxins include superantigens, cytotoxins, adhesins, and other enzymes which play specific roles in the inflammatory and immune response. These molecules contribute to the bacteria's virulence by targeting different aspects of the host's immune response and providing essential nutrients for bacterial growth and survival (Yoshikawa et al., 2019).

One of the key toxins produced by *S. aureus* is α-hemolysin, which damages various host cells and compromises endothelial barrier function. Another toxin called Panton-Valentine leukocidin (PVL) targets white blood cells and is primarily associated with skin and soft tissue infections. Phenol-soluble modulins (PSMs), categorized into PSMα and PSMβ, are aggressive virulence factors that cause red and white blood cell lysis, induce inflammatory responses, have antimicrobial activities and play a role in epithelial surface spreading and biofilm detachment. Epidermal cell differentiation inhibitor (EDIN) exotoxins enter host cells and compromise the integrity of the endothelium barrier. *S. aureus* infections pose a

significant problem due to the emergence of multidrug-resistant strains, such as methicillin-resistant *S. aureus* (MRSA). These strains have limited treatment options and are prevalent in hospitals and communities. The bacteria's ability to produce antibiotic-neutralizing enzymes further complicates antimicrobial therapy and contributes to the spread of antibiotic resistance. The rise of antibiotic-resistant bacteria necessitates discovering new effective solutions, and alternative treatments targeting toxins or toxin-regulator genes are being investigated (Yoshikawa et al., 2019).

S. aureus in AD and biofilms

1.3.4.2.

The colonization of AD skin by *S. aureus* has been extensively studied, and it is closely associated with various factors such as previous hospitalization, the use of topical calcineurin inhibitors combined with topical steroids, and early-life skin colonization of *S. aureus*. The presence of *S. aureus* on the skin of AD patients can lead to a vicious cycle of infection and exacerbation of AD symptoms through the induction of inflammatory responses. Recent advancements in next-generation sequencing techniques have provided valuable insights into the changes in bacterial composition on the skin. While healthy skin is characterized by a diverse community of commensal bacteria, severe AD skin is predominantly colonized by *S. aureus*. Commensal bacteria, such as *S. epidermidis* and *Staphylococcus hominis*, play a protective role by modulating T-cell development, inhibiting inflammation, and producing antimicrobial peptides (AMPs). In contrast, the absence of commensal bacteria is associated with increased colonization of AD skin by *S. aureus*. Studies have shown that microbial diversity is decreased on *S. aureus*-colonized skin compared to methicillin-sensitive *S. aureus* (MSSA)-colonized skin, and MRSA colonization is associated with more severe inflammation in AD patients. Early colonization with commensal staphylococci has been found to lower the risk of developing AD, highlighting

the potential of commensal bacteria in modifying skin immunity and preventing AD development. Several factors contribute to the increased susceptibility of AD skin to *S. aureus* colonization. *S. aureus* has a stronger affinity for deformed corneocytes, which are generated when the levels of filaggrin and filaggrin degradation products (FDPs) are reduced in the SC of AD skin. Fibronectin-binding proteins and clumping factor B facilitate the adherence of *S. aureus* to AD skin. Moreover, decreased levels of antimicrobial peptides, such as cathelicidin (LL-37) and human beta-defensin (HBD)-3, due to the overexpression of Th2 cytokines on AD skin, contribute to chronic colonization by *S. aureus*. The reduced levels of FDPs in the SC are associated with more severe AD symptoms and stronger adhesion of *S. aureus* to corneocytes. Additionally, microbial dysbiosis and altered lipid profiles further exacerbate *S. aureus* colonization on AD skin. *S. aureus* colonization on AD skin involves the initial adhesion to the skin followed by biofilm formation. The severity of AD is significantly associated with the prevalence of *S. aureus* biofilms on the skin. Biofilm-growing *S. aureus* strains exacerbate AD severity, leading to refractory and recurrent infections. The presence of *S. aureus* biofilms occludes sweat ducts in AD skin lesions, contributing to the pathogenesis of the disease. Antibiotics and antiseptics can temporarily reduce *S. aureus* colonization, but recolonization occurs frequently, limiting clinical improvement. Biofilm-related infections pose challenges in treatment due to the higher concentrations of antimicrobials required to eradicate the bacteria compared to planktonic cells (Kim et al., 2019).

Biofilm formation by *S. aureus* is a complex process mediated by various factors. The adhesion of *S. aureus* to the skin is facilitated by host factors such as fibrinogen, fibronectin, and collagen, which interact with microbial surface components recognizing adhesive matrix molecules (MSCRAMMs). The maturation of biofilms requires the production of an extracellular polymeric matrix composed of host factors, polysaccharide intercellular adhesin (PIA), proteins, extracellular DNA (eDNA), and lipids. The biofilm matrix protects bacteria from host immune responses and antimicrobial agents, making eradication challenging (Di Domenico et al., 2019).

Dupilumab

1.4.

Dupilumab, a human monoclonal antibody, is known to bind to the IL-4 receptor alpha (IL-4Rα) subunit and inhibit IL-4R signaling induced by both IL-4 and IL-13. This mechanism of action has been shown to down-regulate Th2 inflammation in various allergic disorders, including AD, asthma, and potentially other allergic diseases. However, there is limited available data regarding the precise mechanism of action of Dupilumab, both in vitro and in vivo. In patient applications, Dupilumab has been found to inhibit the production of IgE by *ex-vivo* B cells when induced by IL-4 treatment. In preclinical mouse models, it was observed that Dupilumab inhibits IL-25-induced allergic airway inflammation and eosinophilic esophagitis (EoE) (Harb et. al., 2019).

Additionally, in a peanut allergy-associated EoE model, Dupilumab was able to suppress IL-25- and peanut-induced IgE production. These findings suggest Dupilumab has therapeutic potential in targeting Th2 inflammation in various allergic conditions. The antibody's impact on the receptor complexes can be influenced by the abundance of IL-4Rα and IL-13Rα1 subunits in the target cells. It is postulated that an IL-4Rα subunit antibody like Dupilumab could inhibit the binding of IL-4 to the type I receptor complex or prevent the assembly of the type II receptor complex by impeding the recruitment of IL-4Rα subunit by IL-13Rα1 upon binding of IL-13. The effects of an IL-4Rα subunit antibody on these receptor complexes may differ depending on the specific circumstances (Harb et. al., 2019).

After a 16-week treatment with dupilumab applied to AD patients, notable alterations in the microbiome composition were observed, accompanied by an increase in microbial diversity and a decrease in the presence of *S. aureus*. The enhanced diversity was noticeable as early as week 4, with the most significant impact observed in the affected skin lesions. However,

even the clinically normal-appearing skin demonstrated a reduction in *S. aureus* levels. In contrast, the placebo-treated group exhibited no significant changes in microbial diversity or *S. aureus* abundance. These findings indicate that targeting IL-4/IL-13 signaling through IL-4Rα blockade is potentially feasible to counteract *S. aureus* colonization (Callewaert et al., 2020).

Aim of this study

1.5.

This research aims to characterize microbiome variations in patients afflicted with severe AD, enabling a deeper comprehension of the primary bacterial constituents associated with this condition. Furthermore, it will investigate the therapeutic effects of Dupilumab administration on the skin microbiome of AD patients. Through these analyses, the research intends to shed light on the role of the microbiome and the potential effects of Dupilumab in managing severe AD.

Results

2.

Demographic and Initial Profile of Study Participants

2.1.

The study comprised 16 subjects (10 males and 6 females) diagnosed with severe AD and 14 healthy control subjects (HS). Two samples were collected from AD patients: lesional tissues (LE) and non-lesional tissues (NL) (Figure 2). The two groups were matched for age and sex.

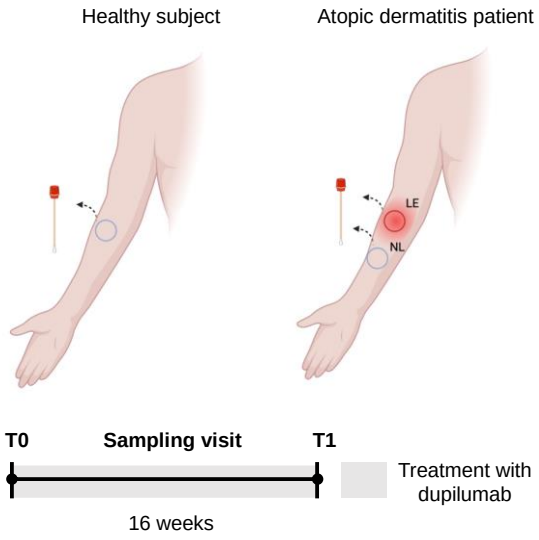

Figure 2: Sampling procedure in adults with severe atopic dermatitis (AD): This study sampled 16 adults with severe AD, determined by the Eczema Area and Severity Index (EASI). Sampling occurred at both the lesion site on the antecubital fossa and a non-lesional site within the same area but without inflammation at the time of enrolment (T0) and after 16 weeks of treatment with dupilumab (T1). Additionally, 14 healthy individuals served as controls and were sampled at the antecubital fossa during a single visit (T0).

Table 2 summarizes the demographic and clinical characteristics of the participants.

Factors	AD cases
Total number	N=16
Age in years, median (range)	37 years (21-62)
Sex	10 males/6 females
EASI score, median (range)	33.5 (24-58)

Table 2: demographic and clinical characteristics of individuals diagnosed with atopic dermatitis (AD) upon enrolment into the study. Eczema Area and Severity Index (EASI).

Alpha and Beta diversity

2.2.

Analysis of 4079297 reads (min. number of reads: 6703; max. number of reads: 252045) grouped into 215 taxa. The alpha diversity of the skin was assessed in ten healthy subjects (HS) as well as non-lesional (NL) and lesional (LE) tissues from sixteen AD patients (Figure 3). Specifically, The Shannon diversity and Pielou evenness index were computed to quantitatively determine species diversity. Compared to HS, a statistically significant increase in Pielou's evenness index was observed in both NL and LE ($P = 0.05$) samples. Specifically, HS exhibited an interquartile range (IQR) of 2.5 with a median of 0.5, NL displayed an IQR of 1.2 and a median of 0.58, while LE demonstrated an IQR of 2.80 and a median of 0.58. Similarly, a significant ($P = 0.01$) difference in the Shannon diversity index was observed in both NL and LE samples compared to HS. In detail, NL and LE exhibited an IQR of 0.7 with a median of 2.7, whereas HS displayed a IQR of 1.4 and a median of 2.3. Bray-Curtis beta diversity, represented as principal coordinate analysis (PCoA), confirmed these findings by illustrating a distinct ($P = 0.001$) spatial clustering of HS samples, while NL and LE samples displayed less well-defined clusters.

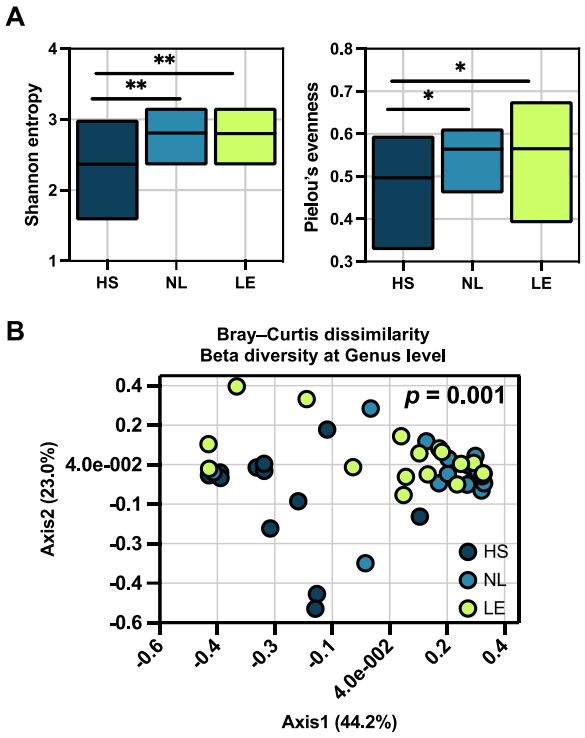

Figure 3: A, Alpha diversity was calculated using the Shannon entropy and Pielou evenness index. Statistical differences were determined using the Kruskal–Wallis test. B, Bray-Curtis dissimilarity beta diversity was calculated at the genus level and represented as principal coordinate analysis (PCoA). PERMANOVA test was used to assess significance. *, P<0.05; **, P<0.01; ***, P<0.001, ****, P<0.0001

The relative abundance of different phyla and genera

2.3.

Variations in the relative abundance were observed among healthy subjects (HS), non-lesional (NL) and lesional (LE), area of AD patients (Figure 4). The predominant bacterial phylum across all sample types was Proteobacteria. However, it is worth mentioning that the relative abundance of this phylum increased in AD patients. Specifically, the HS group exhibited a relative abundance of 0.53, whereas it increased to 0.61 in LE patients and remarkably rose to 0.81 in NL subjects. Another prominent bacterial phylum in this study was Firmicutes, which displayed a relative abundance of 0.19 in HS and LE (0.21) but noticeably decreased in NL subjects (0.09). Additionally, Actinobacteria also exhibited differences in the relative abundance among the studied groups. It appears to be more

prevalent in healthy subjects (0.29), whereas its abundance declined in the other two categories.

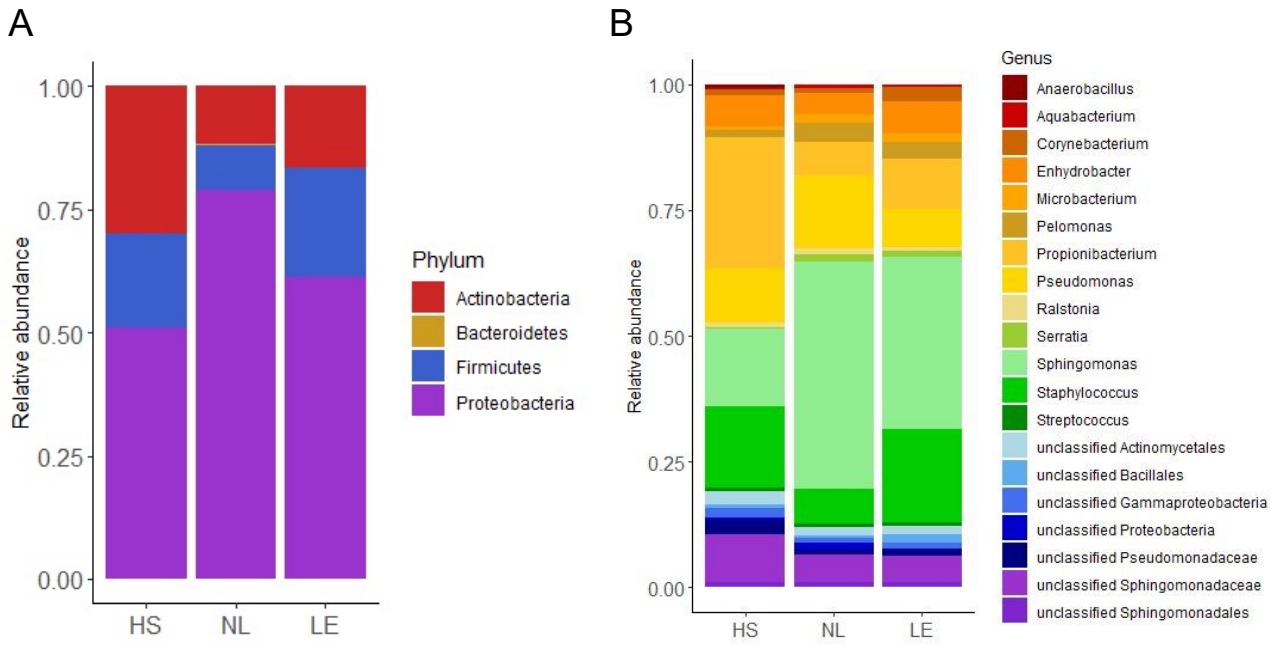

Figure 4: Microbiota variation between healthy subjects and atopic dermatitis (AD) patients. Skin microbiome was evaluated on samples collected from the skin of 14 healthy subjects (HS) and non-lesional (NL) and lesional (LE) of 16 AD patients. Relative abundances at the phylum level (A) and top twenty genera (B) were represented in a stacked bar plot.

In HS, the most prevalent bacterial genus was *Propionibacteria*, with a relative abundance of 0.31. This was followed by *Sphingomonas* (0.17) and *Staphylococcus* (0.15). Notably, the relative abundance of *Propionibacteria* significantly decreased in both the NL group (0.06) and the LE group (0.09) when compared to HS. This reduction in *Propionibacteria* was accompanied by an increase in the relative abundance of *Sphingomonas* in both NL (0.44) and LE (0.31). Lastly, the relative abundance of the *Staphylococcus* genus remained relatively stable in NL (0.07) and LE (0.18) compared to HS.

Differences in the microbiome of Healthy versus diseased patients (Lesional and Non lesional)

2.4.

The linear discriminant analysis (LDA) combined with effect size (LEfSe) algorithm was assigned to robustly identify abundant microbial taxa with a log LDA score above 3.0 that were statistically different between biological classes in this study. LEfSe analysis at genus and species level revealed the presence of Genus *Propionibacterium* (genus, LDA score = 3.6), and the *Cutibacterium granulosum* (species, LDA score = 4.2), was found to be significantly higher in the skin of HS compared to NL and LE. *Sphingomonas* (genus, LDA score = 5), *Pelomonas* (genus, LDA score = 4), *Serratia* (genus, LDA score = 3.7), *Pseudomonas panacis* (species, LDA score = 3.9), are the most abundant bacteria on NL tissues. Notably, *S. aureus* (log10 LDA score = 4.2) and *S. lugdunensis* (log10 LDA score = 3.9) were significantly more abundant on LE than NL and HS.

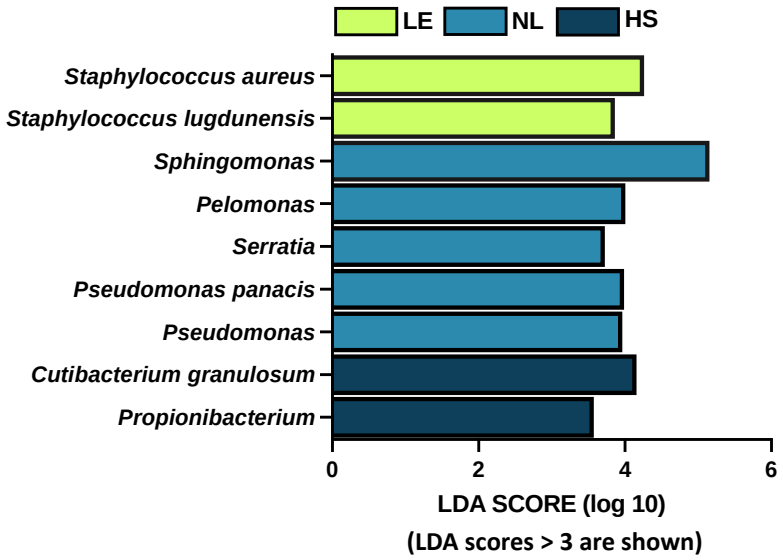

Figure 5: Linear discriminant analysis (LDA) combined with effect size (LEfSe) analysis identifying taxonomic differences on the skin microbiome of healthy subjects (HS) and from the non-lesional (NL) and lesional (LE) area of atopic dermatitis.

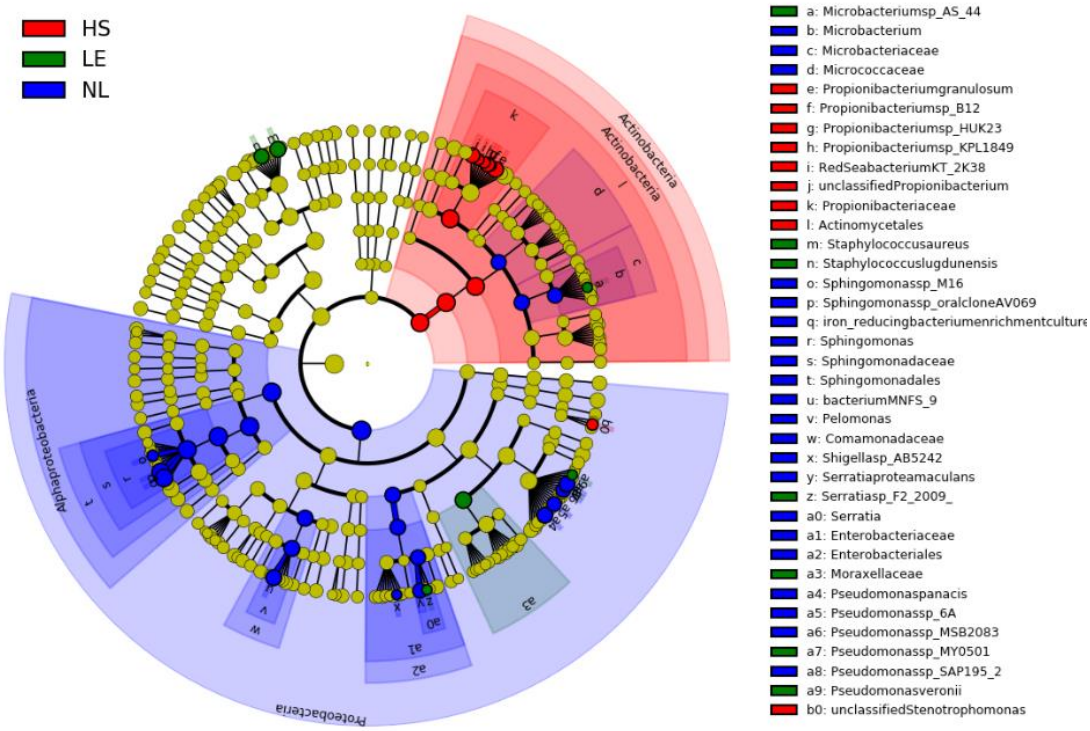

Figure 6: This cladogram, derived from LEfSe analysis, illustrates differential microbial taxa. The central point represents the root of the bacterial tree, with each subsequent ring denoting the next lower taxonomic level, ranging from phylum to genus. The diameter of each circle corresponds to the relative abundance of that taxon within the skin microbial community.

As shown in the biological clades (Figure 6 Cladogram), taxonomic distributions further confirm the association of specific skin microbial taxa, ranging from phylum to genus. Notably, among all the species, *S. aureus* and *S. lugdunensis*, closely placed in the graph as they belong to the same genus, are among the most prevalent in lesional tissues of AD

patients. Conversely, the *Propionibacterium* genus appears to be overexpressed in the skin of HS compared to AD. These findings strongly suggest host-specific alterations in the skin microbiome in AD compared to HS.

In general, AD patients exhibited a notable increase in *S. aureus* and *S. lugdunensis* populations within the affected skin. In contrast, Gram-negative genera showed a significant association with the unaffected skin of AD individuals. The presence of Genus *Propionibacterium*, specifically *C. granulosum*, was found to be higher in the skin of healthy subjects.

Relative abundance for sample type of: *P. granulosum, S. aureus, S. lugdunensis*

2.5.

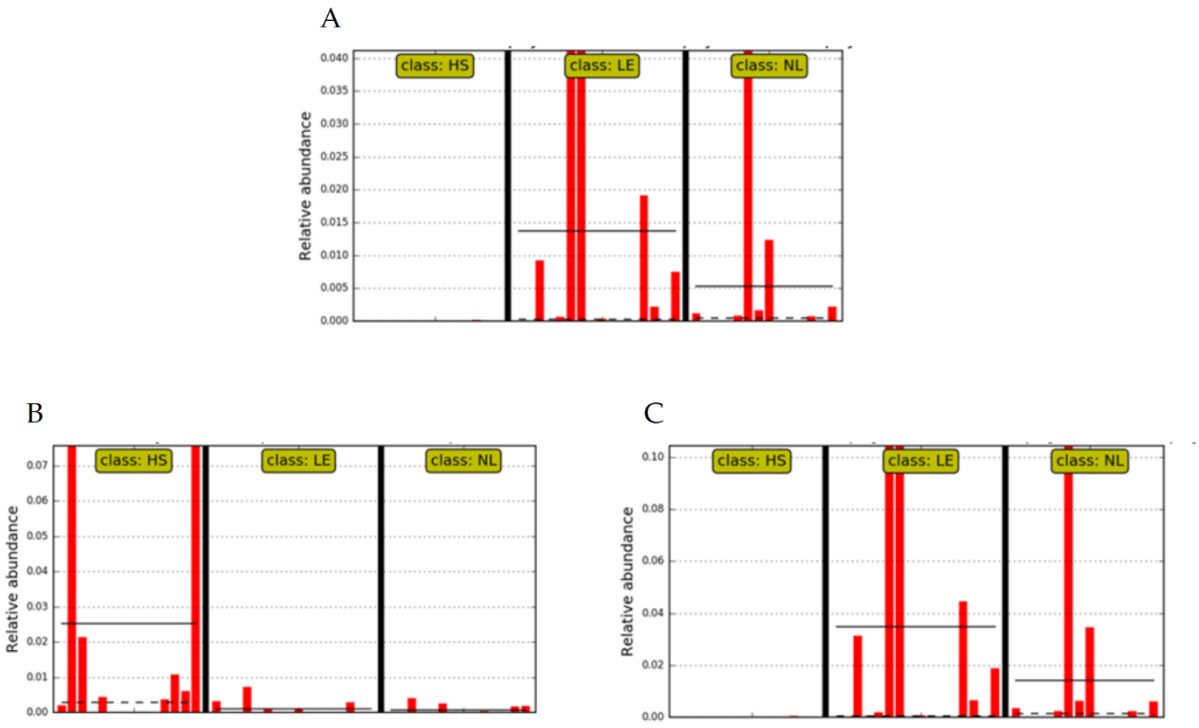

Figure 7**:** relative abundance for *P. granulosum* (A), *S. aureus* (B), *S. lugdunensis* (C).

In the charts above (Figure 7), relative abundances of the most representative bacterial species are shown. These graphs represent the difference in relative abundance between the three samples. *S. aureus* and *S. lugdunensis* are absent in HS, while they show a high relative abundance in LE and NL: 0.038 and 0.02 for *S. aureus*, 0.018 and 0.005 for *S. lugdunensis*, showing coherency with the other graphs. Interestingly, *P. granulosum* seems to be highly present in HE (0.025), and they disappear in the samples of LE and NL.

Skin microbiome variations after the treatment with Dupilumab

2.6.

Changes in the cutaneous microbiome profiles of AD patients were examined after 16 weeks (T1) of treatment with Dupilumab. While not statistically significant, both the NL and LE skin samples demonstrated a decrease in microbial diversity at T1 as assessed by the Shannon diversity index, compared to baseline samples (T0). Following Dupilumab treatment, bacterial diversity in both NL and LE skin samples diminished, aligning with levels seen in the HS (Figure 9). Furthermore, considering Pielou's evenness index, there was a significant decrease from T0 to T1 in both NL ($P = 0.0310$) and LE ($P = 0.0131$) areas.

The impact of Dupilumab in the cutaneous microbiome profiles was also confirmed the Bray-Curtis beta diversity analysis. Notably, the beta diversity, as per the Bray-Curtis dissimilarity, revealed no significant difference between NL and LE and the HS control group.

Preliminary data on the relative abundance of *S. aureus* highlighted a significant reduction between T0 and T1 on the LE of AD patients (Figure 8C). These findings suggest that Dupilumab treatment can modulate the cutaneous microbiome profile, thus improving the clinical condition of AD patients (Figure 8D).

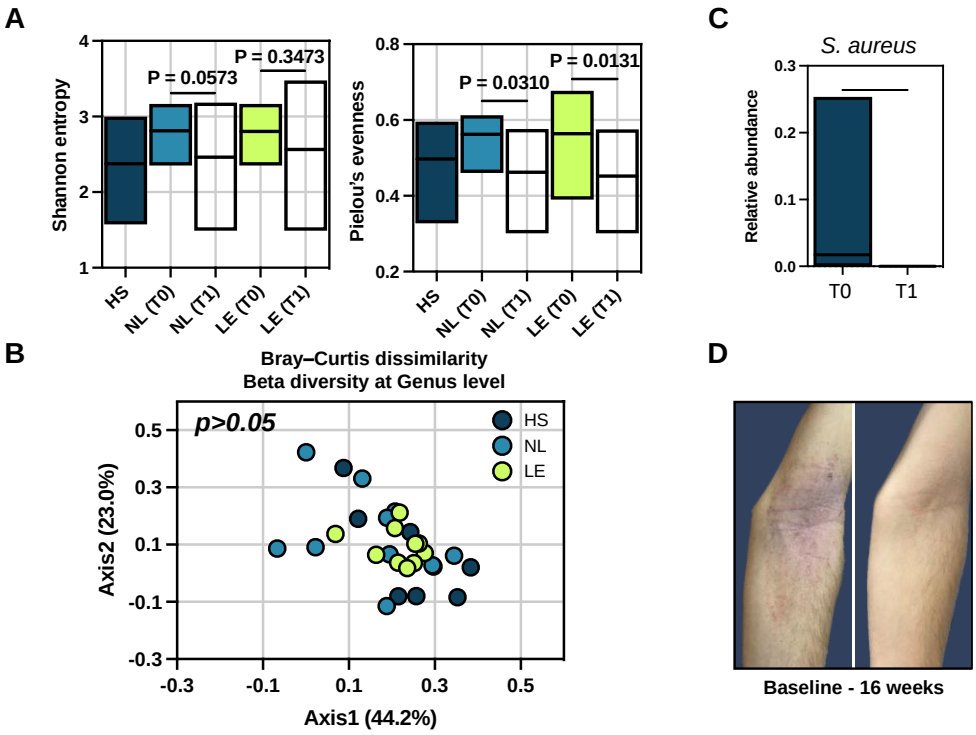

Figure 8: A, Alpha diversity was calculated using the Shannon entropy and Pielou evenness index. B, Bray-Curtis dissimilarity beta diversity was calculated at the genus level and represented as principal coordinate analysis (PCoA). C, Relative abundance was evaluated at the species level for *Staphylococcus aureus*. D, Representative image showing clinical findings before treatment with Dupilumab and after 16 weeks of therapy. Statistical differences were determined using the Kruskal–Wallis test and PERMANOVA test. *, P<0.05; **, P<0.01; ***, P<0.001, ****, P<0.0001.

Discussion

3.

AD is a multifactorial disease that involves abnormalities in the immune and epidermal barrier of the skin and a microbial dysbiosis and an overrepresentation of pathogenic *S. aureus* (Paller et al., 2019). This study demonstrated a global skin dysbiosis in AD at the antecubital fossa compared with HS. The Shannon entropy and Pielou's evenness increased in diseased subjects, indicating a higher number of bacterial species in individuals with AD compared to healthy individuals. This finding contrasts with the majority of previous literature, which reported a negative correlation between Shannon entropy and severe AD, primarily attributed to *S. aureus* (Nakatsuji et al., 2017). Nevertheless, these variations can

be attributed to the specific skin site analysed. Indeed, our data are consistent with previous findings showing an increase in Shannon entropy, and a higher bacterial richness in AD subjects compared to healthy controls (Han et al., 2018; Bjerre et al., 2021). Notably, following the treatment with Dupilumab, no significant differences were observed in both alpha and beta diversity between AD patients and HS. In the context of AD, these measures reflect the complexity and variability of the skin microbiome. The lack of significant differences in alpha and beta diversity post-treatment suggests a more homogeneous microbial composition, indicating that Dupilumab treatment may potentially help restore the microbiome in AD patients, bringing it closer to that observed in healthy individuals. This data is consistent with other studies revealing restored in alpha and beta diversity to levels found in healthy controls (Hartmann et al., 2023). The administration of Dupilumab to AD patients for a duration of 16 weeks resulted in a significant reduction in the relative abundance of *S. aureus* by the end of the treatment period. This finding aligns with numerous other studies that have investigated the impact of Dupilumab on AD. For instance, in a study by Callewaert et al. in 2020, the therapeutic role of Dupilumab in AD was extensively explored. The results from this study indicated that blocking IL-4/IL-13 signalling through IL-4Rα blockade had the potential to decrease *S. aureus* colonization, which is known to be associated with the severity of AD in humans. This underscores the strong correlation between *S. aureus* and the activity of Th2 cells. This relationship has also been investigated in an interesting mouse study conducted by Castillo et al. in 2020, which demonstrated a mutually reinforcing interaction between allergic skin inflammation and *S. aureus* skin colonization. Specifically, topical application of *S. aureus* led to an increase in inflammation, as well as elevated levels of IL-4 and IL-13.

S. aureus has been extensively implicated in the development of AD in scientific literature, which is consistent with the widespread presence of this bacterium among the patients in our study. Remarkably, *S. aureus* is a minority on the skin of healthy individuals. Previous studies (Di Domenico et al., 2019) have identified specific clonal complexes (CC1, CC5, CC8, CC15, and CC45) that appear to be closely associated with AD pathogenesis. The alteration of the skin barrier is linked to AD development, as highlighted in the aforementioned article.

However, an important question arises regarding whether changes in the skin microbiota composition, particularly the overgrowth of *S. aureus*, precede the onset of AD by stimulating the immune system or if the chronic inflammatory condition in individuals with AD contributes to the disruption of skin microbiota homeostasis, leading to AD.

S. aureus were demonstrated to promote AD infection by facilitating biofilm formation, which is challenging to eliminate, and by neutralizing antimicrobial peptide production (Di Domenico et al., 2019). Several studies (Chung et al., 2022) have identified various attributes associated with *S. aureus* phenol-soluble modulins (PSMs), which contribute to the bacterium's enhanced survival and colonization within the host. Upon exposure to *S. aureus*, keratinocytes exhibit increased production of thymic stromal lymphopoietin (TSLP) and interleukin-33 (IL-33). The role of TSLP signalling in initiating skin inflammation is well-established, as higher levels of TSLP induce inflammation associated with Th2 immune responses. Th2 immune response is characterized by elevated levels of IL-4, IL-13, IL-17, and thymic stromal lymphopoietin (TSLP), as well as reduced expression of antimicrobial peptides (AMPs). Additionally, IL-33 expression has been found to be upregulated in the skin of individuals with AD.

In a study conducted by Liu et al. in 2017, it was observed that epicutaneous exposure of mouse skin to *S. aureus* led to IL-36 production and promoted MyD88-dependent skin inflammation. The signalling pathway involving IL-36R and MyD88 was found to stimulate T cell production of interleukin-17 (IL-17), which plays a pivotal role in driving skin inflammation. Although we have gained valuable insights, further investigation is warranted to better understand the role of *S. aureus* in AD, enabling a more comprehensive characterization and identification of potential treatment targets.

The overabundance of *S. lugdunensis*, as observed in our research, has been extensively discussed in previous studies. However, the role in AD pathogenesis of this species is still not discussed. Several studies, (Di Domenico et al., 2019) discussed the potential inhibition of *S. aureus* by this species. Therefore, additional research on the role of *S. lugdunensis* in AD is warranted.

Regarding the genus level analysis, it is noteworthy that *Staphylococcus* exhibited similar relative abundance in both healthy skin and lesional tissues. This suggests that the pathogenicity of *S. aureus* may be attributed to specific characteristics of the species itself rather than the entire genus. In other words, *S. aureus* and *S. lugdunensis* are the implicated species for AD development, while the *Staphylococcus* genus does not show a correlation with AD.

In line with a study by Luu et al. in 2021, which identified *Sphingomonas* as a major overexpressed genus in AD patients, the relative abundances of *Sphingomonas* in this study demonstrated a higher expression in non-lesional (NL) tissues rather than lesional tissues, as observed in both LEfSe analysis and abundance plots. This observation, not yet reported in the literature, presents an interesting aspect that warrants further investigation. Additionally, the prevalence of *P. panacis* species was significantly higher in NL tissues. This species is known to produce bacterial extracellular vesicles (BEVs) and has been frequently associated with the pathogenesis of Type 2 diabetes (T2D) due to the involvement of its BEVs in blocking the host organism's insulin signalling pathway. However, there is currently no evidence regarding the involvement of *P. panacis* and its BEVs in non-lesional tissues of AD subjects or their potential contribution to AD development.

A significant decrease was observed in the *Propionibacterium* genus and the species *C. granulosum*. In relation to the *Propionibacterium* genus, an intriguing study by Francuzik et al. demonstrated a negative correlation between a species called *C. acnes* and *S. aureus*. This finding may explain the overall decrease of the *Propionibacterium* genus in AD patients. The study revealed that *C. acnes* inhibits the growth of *S. aureus* through the production of propionic acid, which was confirmed through culture experiments. The inhibitory effect of propionic acid was also observed for *S. epidermidis*. Although *C. acnes* is one of the prevalent species in AD tissues, it was not detected in the present study. Another study conducted in 2011 by Kitagawa et al. on mice demonstrated that vaccination against *C. acnes* can be effective for AD prevention, as the immune response against *C. acnes* appears to have a protective effect against AD.

The reduction of the *Propionibacterium* genus in diseased tissues aligns with findings from other studies, such as Bjerre et al. in 2017, corroborating the current observations. Furthermore, *C. granulosum* exhibited a significantly diminished presence in diseased tissues, suggesting a potential role in protecting the skin barrier. Numerous publications have described the antimicrobial activity of this species, which should be further investigated to better understand the mechanisms involved in AD development.

Materials and methods

4.

Study Design and Patient Enrolment

4.1.

A control group consisting of healthy subjects (HS) undergoing routine mole checks at the same institution was also included. In order to ensure ethical conduct, informed consent was obtained from all participants as well as their legal representatives. To assess the severity of AD, the Eczema Area and Severity Index Score (EASI) was utilized. This scoring system enabled the evaluation of clinical manifestations, specifically on the antecubital fossa area, and provided a comprehensive assessment of disease severity. The EASI score ranges from 0 to 72, wherein higher scores indicate more pronounced symptoms. A score of 0 signifies a state of clear or no eczema, while scores ranging from 0.1 to 1.0 suggest almost clear conditions, 1.1 to 7 correspond to mild disease, 7.1 to 21 indicate moderate disease, 21.1 to 50 reflect severe disease, and scores exceeding 51 represent very severe disease. All participants enrolled in the study exhibited EASI scores ranging from 24 to 58, with a mean score of 33.5 (Table 1). Consequently, all individuals were classified as having AD based on

the established scoring criteria. The samples were collected from the forearm, which is typically one of the most affected regions in AD. The non-lesional region was consistently chosen from the same area but free from inflammation. For healthy subjects (HS), samples were obtained from the same region. All of the HS had no concurrent skin inflammatory disorders, pityriasis versicolor, or other disease history. AD patients or HS with previous topical antifungal, antibiotic and/or steroid treatment for AD within 1 month prior to sampling were excluded. The recruitment period for all subjects took place at the San Gallicano Dermatological Institute, spanning from September 2021 to January 31, 2022. For the control group, we selected HS whose age, gender, and skin sampling area closely matched those of the AD patients. The study adhered to the principles set forth in the Helsinki Declaration, and ethical approval was granted by the Central Ethics Committee I.R.C.C.S. Lazio, a distinguished division of the Istituti Fisioterapici Ospitalieri in Rome (Protocol 7679—21.06.2016, trials registry number 821/16).

Sample collection

4.2.

The samples were collected by dermatologists with commercially available sterile swabs (COPAN swabs, Brescia, Italy) from the skin of 10 HS and the unaffected skin and lesional area of 16 AD patients for microbiome analysis.

Sequencing and analysis

4.3.

Extracted DNA was amplified by PCR with dual-index primers targeting the V1-V3 regions of the bacterial 16S rRNA gene, using the ARROW for NGS Microbiota solution A kit (ARROW Diagnostics) according to the manufacturer's instruction. A sterile sample tube that had undergone the same DNA extraction and PCR amplification procedures was used for quality control (Cavallo et al., 2022). Before sequencing, amplicons were purified using the Agencourt® AMPure XP PCR purification system (Beckman Coulter, Milan, Italy), and equal amounts (10 nM) of the sample's DNA were pooled and diluted to reach a 4-nM concentration. Finally, a 5 pM of the denatured libraries was used to generate sequences using the 2 × 250 cycles MiSeq Reagent kit (Illumina) on an Illumina MiSeq instrument. Sequencing data were analysed using the MicrobAT system (Cavallo et al., 2022). During MicrobAT processing, demultiplexed sequences showing reads of length less than 200 nucleotides, an average Phred quality score below 25, and at least one ambiguous base was discarded. The resulting sequences were aligned at a 97% sequence similarity and assigned to taxonomic (e.g., species) levels at an 80% classification threshold using the Ribosomal Database Project (RDP) classifier (release 11.5). Species that did not meet these criteria were assigned to the corresponding group, "unclassified [genus]". The Biological Observation Matrix (BIOM) was obtained, and the following analysis was carried out in R studio (https:// www.rstud io. com/; version 4.0.2) using the phyloseq package. Microbial community differences were measured in terms of alpha and beta diversity after reading depth rarefaction. Shannon index and Pielou index were used to evaluate alpha diversity, and significance was assessed by the Kruskal Wallis test. Bray Curtis beta diversity was calculated, and the distance matrix was represented as Principal coordinate analysis (PCoA). Significance was assessed by Permutational multivariate analysis of variance (PERMANOVA). Bacterial relative abundances at phylum and genus level between selected groups were examined (Cavallo et al., 2022).

FastQC has been used to assess the quality of reads

4.4.

Sequencing was applied in order to obtain DNA sequences from the 46 samples, including HS, NL and LE. Subsequently, a statistical analysis of the goodness of reads obtained was performed. To reach this goal, FastQC was used. This tool is designed to facilitate efficient quality control assessments on raw sequence data obtained from high-throughput sequencing pipelines. FastQC enables users to swiftly evaluate the quality of their data and identify any pertinent concerns that may warrant attention before proceeding with further analysis.

MultiQC was used to perform further statistical analysis

4.5.

This is a powerful tool that exploit the output of FastQC to consolidate quality indicators from the obtained reads, enabling inter-readset comparisons. Serving as a comprehensive reporting tool, MultiQC efficiently analyses results and statistics generated by diverse bioinformatics tools. Its primary function is to summarize experiments encompassing multiple samples and multiple analysis steps, providing researchers with a consolidated overview of their data. By integrating information from various sources, MultiQC streamlines the process of data interpretation and facilitates a comprehensive understanding of complex bioinformatics analyses.

Alpha diversity

4.6.

The objective of conducting alpha diversity analysis is to assess the diversity within individual samples across various categorical variables. Several metrics, such as Pielou's evenness and Shannon Diversity, are employed for this purpose. Each metric illuminates a distinct facet of the internal complexity observed within the samples. In this study botch Pielou's evenness and Shannon Diversity were computed.

Shannon diversity can be computed using the following formula:

$$H = -\sum_{i=1}^{R} p_i \ln(p_i)$$

Where H is a quantification of the entropy (diversity) within a group (in our case the sample type). The higher is the Shennon index, the higher is the diversity of species that can be observed inside the sample. pi is the relative abundance of a species within a sample.

Pielou's evenness quantifies how evenly the individuals are distributed among the different species present.

$$J = \frac{H}{\ln(S)}$$

It is computed dividing the Shannon index H by ln(S), which is the total number of species in the individual community.

Richness refers to the sum of the number of species present in each sample. Higher is the number of species present, larger is the Richness index.

Beta diversity

4.7.

The primary objective of beta diversity analysis lies in quantifying the dissimilarity across samples. Although various methodologies exist to investigate beta diversity, this study has employed the Bray-Curtis dissimilarity metric. This measure assesses whether the proximities between samples within a specific condition demonstrate greater similarity relative to the distances observed between samples originating from distinct conditions.

$$BC_{ij} = 1 - \frac{2C_{ij}}{S_i + S_j}$$

Where BC_{ij} is the Bray-Curtis dissimilarity, C_{ij} is the sum of only the lesser counts for each species found in both samples, S_i is the total number of specimens counted on site i, while S_j is the total number of specimens counted on site j.

Python packages

4.8.

The generation of graphs depicting relative abundances for both phyla and species, specifically for the genera and phyla of bacteria, was accomplished using the Python programming language. To facilitate this analysis, several packages including matplotlib, seaborn, pandas, and NumPy were implemented. Initially, the original .xlsx files containing absolute counts for each sample were utilized. The data from each sample type (HS, NL, LE) was merged, resulting in three distinct datasets encompassing the absolute counts for each phylum and genus within each sample type. Subsequently, a new column was created within the datasets to represent the relative abundances. This was achieved by dividing the

absolute counts of each genus or phylum by the total counts of all genera or phyla within each respective sample type:

```
new_merged_df = merged_df.dropna()
sum_all = new_merged_df["Counts"].sum()
abb = new_merged_df["Counts"] / sum_all
```

Following the computation of relative abundances, the top twenty phyla and genera with the highest relative abundance values within the HS sample type were selected. These same phyla and genera were then chosen from the other two sample types. The selected data was subsequently consolidated into a single .xlsx file for both genus and phylum. Additionally, a new column specifying the sample type was incorporated into the dataset. Finally, plots depicting the relative abundances were generated based on the processed data, providing a visual representation of the distribution patterns within the different sample types.

MicrobAT for the taxonomic analysis

4.9.

Taxonomic analysis of the microbiota was performed. It was carried out using the MicrobAT software tool, which is specifically designed for the analysis of DNA samples derived from diverse biological matrices. The software enabled the determination of relative and absolute varieties and abundances of microbial taxa within the studied environment. To conduct the analysis, DNA samples were obtained from the target biological sources and subjected to sequencing. The resulting sequencing data were then processed using MicrobAT. The software facilitated the identification and classification of microbial taxa present in the samples, providing insights into the microbial diversity and abundance within the specific environment of the three groups.

Linear discriminant analysis Effect Size (LEfSe) for the bioinformatic analysis of the samples

4.10.

Statistical analysis of the taxa has been performed, in order to check differences in the HS, NL and LE. To this aim, the LEfSe tool has been used, which carried out the analysis taking into account the following parameters: Alpha value for the factorial Kruskal-Wallis test among classes = 0.05. Alpha value for the pairwise Wilcoxon test between subclasses = 0.05. Strategy for multi-class analysis = One against all (less strict). Finally, the LDA model parameters for K = 2, 3 ad 3.5 to identify, genera and phyla with the highest abundance on the skin microbiomes of the three categories of samples. Three different analysis with different values of K have been used or statistical significance.

Known as Linear discriminant analysis Effect Size, LEfSe is specifically designed to identify key features that are likely to explain differences between different classes or groups of samples. It achieves this by combining standard statistical significance tests with additional tests that encode biological consistency and assess the relevance of the observed effects. By leveraging this comprehensive approach, LEfSe enables researchers to pinpoint the discriminative features that have the most substantial impact in distinguishing between classes, providing valuable insights into the biological factors driving the observed differences. Our input was a file containing all the names of species (found in each sample) and their relative abundance.

LEfSe works following these steps:

Step 1: The Kruskal-Wallis test. It examines all features to determine if the values in different classes exhibit differential distributions. Features that deviate from the null hypothesis undergo further analysis in Step 2.

Step 2: The pairwise Wilcoxon test. It assesses whether all pairwise comparisons between subclasses from different classes align significantly with the overall trend observed at the class level.

Step 3: The selected subset of vectors obtained from Step 2 is utilized to construct a Linear Discriminant Analysis model. This model captures the relative differences among the classes, enabling the ranking of features based on their effectiveness in distinguishing between classes.

LEfSe provided us with different plots. More specifically, we obtained:

1) Plots illustrating the biomarkers identified by LEfSe, where they were ranked based on their effect size and associated with the class exhibiting the highest median.

2) Cladograms representing the LEfSe outcomes, obtained through the corresponding module, arranged according to the hierarchy derived from the label names.

3) Abundance histograms displaying the raw data of individual features.

4) Plots depicting the raw data of features detected by LEfSe as biomarkers, showcased as abundance histograms.

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5.

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