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16S rRNA Gene Sequencing Analysis of the Human Cutaneous Microbiome

Workflow setup and influencing factors

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Ma chi me l'ha fatto fare. .?

ABSTRACT

Complife Italia is a company specialised in consulting and testing services for the cosmetic, nutraceutical, and medical devices markets, and thrives to offer tailored, state of the art solutions to its clients. Our clients being particularly interested in the effect of their products on the cutaneous microbiome, a collaboration with the University of Eastern Piedmont (UPO) took shape at the end of 2019, and by summer 2020 the Complife Microbiome Laboratory was fully operational at the Centre for Autoimmune and Allergic Diseases of the same university (CAAD-UPO).

The human microbiome has long been recognised as a fundamental adjuvant to the innate and adaptive immune system and spurred even more interest since the advent of Next-Generation Sequencing technologies over the past 20 years. In fact, these approaches allowed to overcome the severe limitations of culture-based studies (slow-growing organisms, nutrient balance, environmental conditions…) and to finally observe all members of the microbiome (bacteria, fungi, viruses, mites…). While these new techniques brought much greater understanding of the microbial composition in health and disease, it also further evidenced the extreme variability of the cutaneous microbiota structure depending on the subject and its environment, but also within the same subject depending on the body site.

To satisfy our clients (and our curiosity), we first established a reliable, reproducible, and repeatable workflow based on the 16S rDNA sequencing approach, a method giving a broad understanding of the bacterial component of the cutaneous microbiota with excellent accuracy down to the genus level. We also tested some variations of the pipeline to try and have more flexibility in our protocols and prices. Then, we could repurpose about 1000 of the 3000 samples collected through client-ordered studies and analyse microbiome variations based on several factors (sex, age, seasonality, living area) and various body sites (face, armpit, chest, legs).

Long-term, the Complife Microbiome Laboratory should be able to offer similar analyses on the fungal members of the cutaneous microbiota (focusing on the ITS-1 gene), but also to better characterise the cutaneous microbiota of the central northern Italy inhabitants by targeting bacteria and fungi of interest also at the species level. Finally, we make our mission to use our resources to study the impact of different factors on the microbial communities' composition.

CONTENTS

ABBREVIATIONS

§	paragraph
°C	Celsius degree
3D	three dimensions
AD	Atopic Dermatitis
ADS®	Active Drying System®
AMP	Antimicrobial Peptides
ANOVA	Analysis of Variance
BMI	Body Mass Index
bp	base pair
C. acnes	*Cutibacterium acnes*
C. difficiles	*Clostridium difficiles*
CAAD	Centre for Allergic and Autoimmune Diseases
cm	centimetre
ddPCR	Digital Droplet PCR
DGGE	Denaturing Gradient Gel Electrophoresis
DNA	Desoxyribonucleic acid
FMT	Faecal Microbiota Transplant
G. Mil.	Garbagnate Milanese
GIT	Gastro-intestinal tract
h	hours
HGP	Human Genome Project
HMP	Human Microbiome Project
HQ	Headquarters
IBD	Irritable Bowel Disease
IBS	Irritable Bowel Syndrome
ITS-1	Internal Transcribed Spacer 1
K/mm^2	clusters by square millimetre, or cluster density
LDA	Linear Discriminant Analysis
LefSe	LDA Effect Size
M. globosa	*Malassezia globosa*
M. restricta	*Malassezia restricta*
mL	millilitre
NAO®	Nucleic Acid Optimizer®
NGS	Next-Generation Sequencing
NMF	Natural Moisturising Factor
OTU	Operational Taxonomic Unit
PCoA	Principal Coordinate Analysis
PCR	Polymerase Chain Reaction
PERMANOVA	Permutational Analysis of Variance
POC	Point Of Collection
RDP	Ribosomal Database Project
RNA	Ribonucleic acid
rRNA	ribosomal RNA
S. Aureus (or *S. au.*)	*Staphylococcus aureus*
S. epidermidis (or *S. ep.*)	*Staphylococcus epidermidis*
SD	Seborrheic Dermatitis
spp.	species
TGGE	Temperature Gradient Gel Electrophoresis
T-RFLP	Terminal Restriction Fragment Polymorphism
UPO	Università del Piemonte Orientale (University of Eastern Piedmont)
V	variable regions
WGS	Whole-Genome Sequencing
µL	microlitre

INTRODUCTION

I. The human microbiota

I.1. General introduction

I.1.1. Ecosystem, holobiont, and microbiome

The origin of the term “microbiota” can be dated back to the early 1900s and designates the set of all the microorganisms that colonized a given environment (Hou et al. 2022). In fact, all multicellular organisms live in close association with the surrounding microbes, and as such exist as “metaorganisms” (or “holobionts”) composed of a macroscopic host and its symbiotic commensal microbiota (Y. Belkaid and Segre 2014; Iebba et al. 2016). The human species is no exception (Sekirov and Finlay 2006; Iebba et al. 2016), and over the millennia we evolved in parallel with the bacteria, viruses, fungi, archaea and protozoans that inhabit us, establishing over time permanent, transmissible, and predominantly symbiotic interactions (Grice and Segre 2011; Turnbaugh et al. 2007; Jandhyala et al. 2015; Moskovicz, Gross, and Mizrahi 2020).

But should we talk about microbiota or microbiome? Since the beginning of the 2000s and the advent of new computational techniques, it is commonly admitted that the term “microbiota” refers to the collection of microbes living on and inside us, while the microbiome indicates the collection of genes in the microbiota (Turnbaugh et al. 2007; Hamady and Knight 2009; Castelino et al. 2017; Lederberg 2000; Kennedy and Chang 2020).

Every part of our body is colonized and the human microbiota is thought to represent about 100 trillion microbial cells, outnumbering our ‘‘human” cells 10 to 1 (Savage 1977; Hamady and Knight 2009; Iebba et al. 2016); and the microbiome to have a coding capacity a hundred-fold higher than that of our own cells (Ley, Peterson, and Gordon 2006). If the initiation stage of the human microbiome’s development remains obscure, it is generally admitted that microbial communities are acquired at birth: in fact, it was observed that vaginally delivered infants acquired bacterial communities resembling their own mother's vaginal microbiome, while C-section infants harboured bacterial communities similar to those found on the skin surface (Dominguez-Bello et al. 2010). Studies focusing on microbiota variation over the human lifespan also showed that the microbial communities were very dynamic during early years of life, as the immune system is maturing and being educated, before stabilising after puberty (Y. Belkaid and Segre 2014).

Each body district (mouth, throat and airways, stomach and intestine, urogenital system, skin, etc.) hosts a specific microbial population that has adapted to its very own microenvironment (temperature, humidity, exposure…)(Blum 2017; Hou et al. 2022), and the most populated and studied body district is the intestine: over the years, numerous studies have been conducted and, to date, we estimate the human gut microflora to

be composed of over 35000 bacterial species. (Jandhyala et al. 2015). If the gut microbiome is not only extremely abundant but also quite easy to collect, this is not the case for all body districts: the skin hosts particularly low quantities of microbes, which explains the relatively new interest and knowledge in the human cutaneous microbiota.

However, issues linked to the quantity or quality of bacterial are nowadays minor since the biggest challenges in microbiome studies remain the complexity of the microbiome and the unexpected variability between individuals (Hamady and Knight 2009). While diet, environment, host genetics and early microbial exposure have all been implicated, much of its role and diversity remains unexplained (Huttenhower et al. 2012) and microbiome studies should be designed with extreme attention to influencing factors.

I.1.2. Role of the microbiome

Microbes colonize the human body from birth and to death, and, in doing so, interfere with our anatomical, physiological, and immunological development (Iebba et al. 2016): on one hand the human microbiota provides a wide range of metabolic functions that we lack (S. R. Gill et al. 2006; Hamady and Knight 2009); on the other hand, the components of the microbiome may enter the general circulation and be transported to various organs affecting their functionality (Iebba et al. 2016). In fact, the development of defined arms of the immune system – particularly the adaptive immunity – has coincided with the acquisition of a complex microbiota, and a large fraction of the host complexity evolved to maintain the symbiotic relationship. In turn, the microbiota can regulate multiple aspects of the immune response: the highest number of immune cells is found in body sites colonized by commensals, illustrating both its beneficial presence and the extraordinary pressure it exerts on our defence system (Y. Belkaid and Segre 2014; Iebba et al. 2016).
But our microbial populations are also subjected to two strong selective pressures: i) by the microbiota itself which tends to diversify its members to lower the competition among them (= individual genomic diversity), and ii) by the host, which tends to homogenize the genomes (= promoting functional redundancy). An imbalance in these powers would lead to the dysbiosis of the ecosystem and potentially leading to diseased states of the host (Iebba et al. 2016).

The mechanisms by which genetic information specifies the structure and biological functions have been investigated since the 1950s (Olson 1993), but the microorganisms that inhabit human body were only recently recognized to be a major factor shaping human health (Abeles and Pride 2014).
The Human Genome Project (HGP) took shape in 1988 in the United States with the release of the *Mapping and Sequencing the Human Genome* report (Genome 1988) and eventually lead to the complete sequencing of the human genome by the International Human Genome Sequencing Consortium (2001). But the major breakthrough in the field of human microbiome studies was the launch in 2007 of the Human Microbiome Project (HMP, US National Institute of Health, 2007-2014) and the subsequent Integrative Human Microbiome

Project (iHMP, 2014-2016) (Ladizinski et al. 2014). This ten-year project allowed to collect fundamental information on the taxonomic composition of the vaginal, oral, skin and gut microbiomes of thousands of samples over the years (Samaras and Hoptroff 2020).

The HMP aimed at characterising the human microbiota and its role in health and disease by probing the nature and the extent of the microbial communities living in and on ''normal'' adult donors (Grice et al. 2009; Li et al. 2012). It was initiated to better understand the role of the microbiota in human health and disease: this unprecedented opportunity to examine the microbial diversity within and across body habitats/individuals became a critical baseline for future metagenomic studies of the human microbiome (Li et al. 2012). In fact, this ambitious project was instrumental in establishing reference sequences, building comprehensive multi-omic data sets, developing better computational and statistical tools, and creating reliable analytical and clinical protocols as resources for the broader research community (Proctor et al. 2019).

From the early years of the HGP, the development of genetic and physical maps of the euchromatic portions of the human genome (F. S. Collins and McKusick 2001; Abdellah et al. 2004) allowed scientists to identify the genes involved in diseases (F. S. Collins and McKusick 2001). Since then, researchers have amassed extensive evidence that many aspects of healthy bodily function are intimately dependent upon the particular assemblage of microbes (Kennedy and Chang 2020). For example, most of the microorganisms that compose the intestinal microflora are considered non-pathogenic and cohabit with enterocytes in a symbiotic relationship, thus helping the body to metabolise nutrients or drugs and acting as a barrier that prevents colonization by pathogenic bacteria (Jandhyala et al. 2015). However, the microbiota has also been shown to be associated with a state of disease of the subject, thus it is extremely clinically relevant to understand which microbes appear where in the body, and what are their regulation mechanisms (Blum 2017; Kennedy and Chang 2020).

I.2. Microbiome and human health

I.2.1. Eubiosis and dysbiosis

So, depending on its composition in a given location, the microbiome may have both beneficial and detrimental roles that affect human health, including improvements in microbial resilience to disturbances, immune evasion, maintenance of physiologic processes, and altering the microbial community in ways that promote or prevent pathogen colonization (Abeles and Pride 2014).

It is commonly admitted that, in stable ecosystems, the microbes tend to maintain a state of equilibrium ("eubiosis") and resist abrupt changes in community structure: this is also known as the Nash Equilibrium, where none of the components of an ecosystem is advantaged by changing strategy (Iebba et al. 2016;

Fredricks 2001). On the contrary, “dysbiosis” refers to an ecosystem where the bacteria no longer live together in mutual harmony. (Iebba et al. 2016)

If investigators long relied upon Koch’s postulates for causation between microorganisms and disease (Y. Belkaid and Segre 2014; Segre 2013), the correlation between microbiota dysbiosis and diseased states demonstrated that the latter may result from ecologic shifts in microbial inhabitants or community structure rather than only the presence of potential pathogens (Fredricks 2001; Hou et al. 2022; Iebba et al. 2016). Generally, most of the microorganisms that compose the microbiota are considered non-pathogenic and cohabit with enterocytes in a symbiotic relationship, thus helping the body to metabolise nutrients or drugs and acting as a barrier that prevents colonization by pathogenic bacteria (Jandhyala et al. 2015). The relationships between microbiota, host, and disease are however complex (Y. Belkaid and Segre 2014), and disease development and progression is now primarily associated with dysregulation of community composition, modulation of host immune response, and induction of chronic inflammation (Hou et al. 2022).

First, the host response to exogenous infectious agents was demonstrated to amplify and/or promote a dysbiosis status (Iebba et al. 2016).

Then, each inflammatory disease has specific genetic and biological mechanisms, and many inflammatory diseases also associated with shifts in resident microbiota from “healthy” to “diseased” state (Y. Belkaid and Segre 2014). In fact, the profound changes in the microbiota (and in the immune system as a direct result) are now believed to contribute to dramatic and rapid progression in chronic inflammatory and autoimmune disorders seen in high-income countries (Y. Belkaid and Segre 2014).

Similarly, the elucidation of the relationship between the composition of the intestinal microflora and a large number of GIT and metabolic diseases – inflammatory bowel disease (IBD), irritable bowel syndrome (IBS), obesity, diabetes (Jandhyala et al. 2015) – has given a greater understanding of the host-microbiota relationship and has now allowed the development of microbiota-based therapy (FMT, bacteria modulation) (Hou et al. 2022), and these strategies are well on the way to achieving the optimal clinical effect in the treatment of *C. difficile* infection, diabetes, and IBD. (Hou et al. 2022).

Finally, studying the composition of the microbiota in different skin districts may be useful to understand the aetiology of many skin disorders (rosacea, pityriasis), including the ones that have preferred locations: acne in sebaceous sites, eczema in humid habitats, psoriasis in drier areas (Paulino et al. 2006; Byrd, Belkaid, and Segre 2018; Kong et al. 2012).

I.2.2. Endo- and exogenous factors

Nowadays, the taxonomic analyses pioneered by the HMP are a standard in detecting disease-associated shifts in community composition and characterizing states of ecological dysbiosis: by obtaining detailed and precise microbiome information, the medical community aims at making a standard of microbiome-based therapies

(Noecker et al. 2017). But, over the years, human microbiome studies have revealed that even healthy individuals differ remarkably in the microbes that occupy habitats such as the gut, skin and vagina (Huttenhower et al. 2012). Essentially, the efforts to map the human microbiome in health and disease led not only to discovering disease mechanisms linked to microbiota imbalance, but has also increased the appreciation of the microbiome's role in human well-being (Huttenhower et al. 2012; Qin et al. 2012; Turnbaugh et al. 2006; Cox et al. 2014; Smith et al. 2013; Noecker et al. 2017).

However, the amazing degree of interpersonal variability complexifies also the studies on healthy volunteers: while it is possible (though not demonstrated) that all humans share the same microbial species, the abundance of individual species is highly inconsistent from one subject to another (Hamady and Knight 2009). Consequently, one should carefully consider a maximum of ecological and physiological parameters to understand and potentially interfere with the human microbial ecosystems (Iebba et al. 2016). Much of the interpersonal variability remains indeed unexplained, and if it could be demonstrated that host genotype, diet, or environment influenced our microbiota's composition, still very little is known on the physiological impacts of changes in microbial abundances (at any given taxonomic level) (Huttenhower et al. 2012; Hamady and Knight 2009; Iebba et al. 2016).

So, on one hand diseases with microbial involvement must be investigated within the context of their microbial community, host factors, and immunity (Y. Belkaid and Segre 2014). But, on the other hand, also studies on healthy subjects must take into account both endogenous and exogenous factors that can affect the composition of the microflora: age, lifestyle, founder effects such as mode of delivery and host genetics, lactation and nutrition in neonatal age, short- and long-term diet, drug intake, daily cycles, … (Figure 1)(Blum 2017).

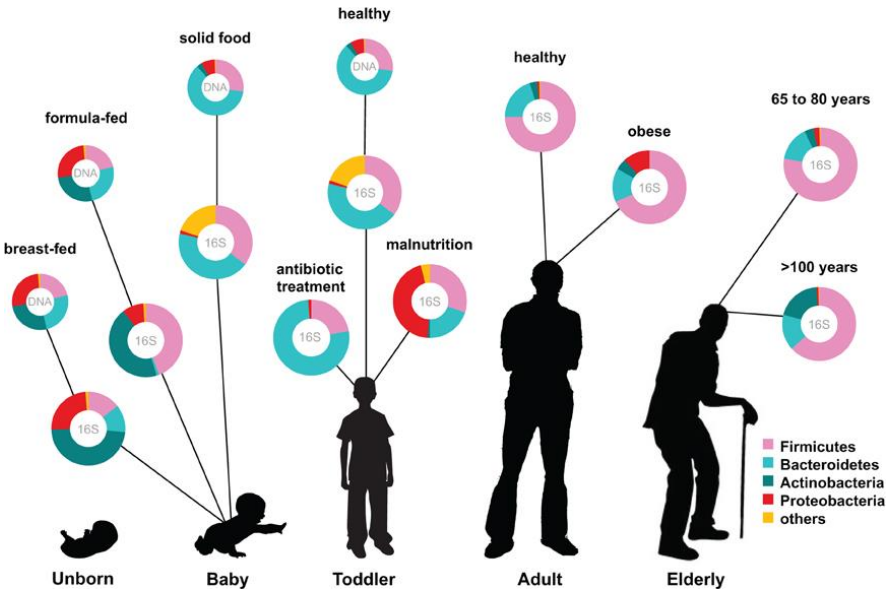


Figure 1. *The onset and shaping of the human microbiome through life stages and perturbations. (Ottman et al. 2012)*

II. Methods to study the microbiome

II.1. Early days

Microbiota studies heavily relied on culture-based methods until the end of the 20th century, but the main drawback was the difficulty to recreate a complex environment that would allow the growth of all the microbial communities (Grice et al. 2008; Hosomi et al. 2017). Our knowledge of microbial biodiversity was then severely limited, as cultured and culturable microorganisms represent only a tiny fraction of the microbial diversity in the environment (Hugenholtz and Pace 1996). In fact, the limitations of the *in vitro* methods in terms of individual microorganisms isolation (particularly anaerobic bacteria) and of recreating an appropriate environment (from a nutritional and physiological point of view) restricted the general view of the human microbiota composition (Staley and Konopka 1985; Bowler, Duerden, and Armstrong 2001; Grice and Segre 2011), as evidenced by the discovery of numerous bacterial sequence types that did not match cultivated members of the community (Fredricks 2001). The accurate and high-resolution mapping of microbiome by the HGP was therefore crucial for gaining a principled understanding of the microbial communities' behaviour, function, and impact on their host (Manor, Levy, and Borenstein 2014; Noecker et al. 2017).

Recombinant DNA and molecular phylogenetic techniques have provided methods for characterizing natural microbial communities without the need to cultivate organisms (Hugenholtz and Pace 1996) and these molecular-based, culture-independent techniques revealed that the vast majority of microbes remained yet uncultured (Rappé and Giovannoni 2003). Such new approaches gave important insights into the diversity of the microbial world across a variety of environments (Sogin et al. 2006; Campbell et al. 2010; Fortunato and Crump 2011; Gottel et al. 2011; Ramos-Padrón et al. 2011), and this increased knowledge in molecular biology helped overcome the severe limitations of culture-based methods, by identifying previously unknown members of the microbiota and improving the accuracy of the results, including in the human microbiome (Aas et al. 2005; Costello et al. 2009; Crielaard et al. 2011; Nasidze et al. 2011; Li et al. 2012; Cundell 2018).

Initially, fingerprinting techniques were used (T-RFLP, DGGE, TGGE), where a specific gene was amplified (typically 16S rRNA, but not always), then separated into different variants of the gene in the community sample by electrophoresis (Hamady and Knight 2009). Rapidly, the protocols relied almost exclusively on the amplification, sub-cloning, and Sanger sequencing of the highly conserved 16S rRNA gene: this method can provide sequence information over the entire length of the 16S rRNA gene in a single reaction, and is still one of the most comprehensive method of bacterial identification (Castelino et al. 2017).

It is based on the sequencing of the bacterial 16S ribosomal RNA gene, used as a phylogenetic and taxonomic marker to identify members of microbial communities (Pace 1997; Gevers et al. 2012), and allows for i) checking the stability in dominant members of a community, and ii) clustering communities according to changes in dominant members across large numbers of samples (N. Fierer and Jackson 2006). If genome

sequencing became especially useful for asking which specific genes or species contribute to differences among communities (Hamady and Knight 2009), the Sanger sequencing is famously expensive and time consuming (Castelino et al. 2017), and soon new techniques arose.

II.2. Metagenomic breakthrough

The introduction of Next-Generation Sequencing (NGS) technologies at the beginning of the 2000s has allowed to identify the members of a microbial community at much lower costs and with higher throughput (Castelino et al. 2017). Through the advent of advanced DNA sequencing and analysis systems, it became possible to precisely identify and quantify the members of the microbiota (Grice and Segre 2011), therefore making huge steps forward in characterising both the resident and transient microflora and the influence of their interaction with their host's physiology (Ederveen et al. 2020; Baldwin et al. 2017).

The new molecular techniques revolutionised the vison of the human microbiota (Grice and Segre 2011), and the typical approach for microbiome studies at a metagenomic level became to produce a heat map showing abundance of each function/taxonomic group in each metagenomic sample, and i) use standard (nonphylogenetic) clustering techniques to relate the samples to one another according to the function they contain (S. G. Tringe et al. 2005; Turnbaugh et al. 2006, 2008, 2009; Huson et al. 2007; Dinsdale et al. 2008; Schloss and Handelsman 2008; Hamady and Knight 2009), and ii) assess the taxonomic diversity, evenness, and richness of a given microbiota using ecological diversity stats (Grice et al. 2009).

The HMP was instrumental in this technologic breakthrough: in fact, not only it had the purpose of creating a basic vision of the healthy human microbiome in each of its five major areas (airways, skin, oral cavity, gastrointestinal tract, and vagina) (Gevers et al. 2012; Peterson et al. 2009), but it also aimed at characterizing the genome of all members of the microbiota, including the corresponding sequences of messenger RNA, proteins and metabolic products (Turnbaugh et al. 2007; Peterson et al. 2009). It generated an unprecedented scale of 16S profiles to investigate the microbial diversity human microbiome through the collection of samples from 18 body sites of over 230 donors, and this breadth and depth of sampling dramatically improved the assessments on variability of human microbiome diversity within and between individuals and body habitats (Li et al. 2012).

The huge amount of information obtained with the HMP highlighted the critical importance of precise clinical metadata documentation – commonly age, sex, antibiotic use, sampling sites – for both the downstream analyses of the results and because it may help explain differences within and between studies.. (Kong et al. 2017), as sequencing results allow researchers to delineate potential associations between the microbiome's composition and the host's health status (Kong et al. 2017). Naturally, high-quality, unbiased, and rigorous characterisation of the metagenome's gene content is equally important for characterising the normal

microbiome or pinpointing disease-associated shifts in its functional capacity (Manor and Borenstein 2015; Noecker et al. 2017).

Most microbiome analyses approaches are based on bioinformatics methods and are composed of five generic steps:

1) sample collection from the area of interest
2) microbial DNA extraction and 3) targeted DNA amplification
4) sequencing, followed by 5) taxonomic classification, achieved by identifying the species-specific hypervariable regions (Kong et al. 2017; Hugenholtz and Pace 1996).

Phylogenetic methods rely on accurate reconstruction of the phylogeny through gene identification and are particularly useful for rRNA surveys, but functional taxon-based methods are typically more useful for metagenomic surveys. In fact, they represent a larger range of functions while overcoming the difficulty of reconstructing phylogenies of small fragments of many gene families (Hamady and Knight 2009). Similarly, meta-transcriptomic assays also offer valuable info regarding expression of microbial genes during natural course of disease: the reverse transcription and cDNA sequencing of RNA material isolated from microbiome samples can provide important insights on how different species respond to each other and to environmental changes (e.g. antibiotic treatment (Pérez-Cobas et al. 2013), dietary perturbations (David et al. 2014)) (Noecker et al. 2017; Byrd et al. 2017).

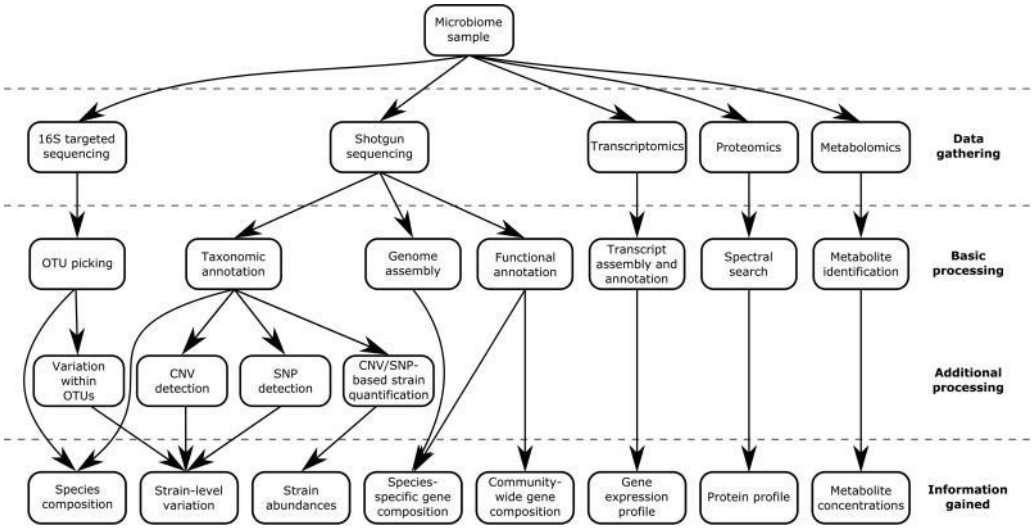


Figure 2. *Different approaches to microbiome analyses. (Noecker et al. 2017)*

High-throughput sequencing studies and new software tools fundamentally transformed the world of microbiota analyses, and the variety of experimental and computational methods available to choose from can be daunting (Hamady and Knight 2009). In fact, different sequencing platforms are available with various chemistries and technologies (Figure 2), and, depending on the study design, different amplicon size, sequencing depth, sequencing accuracy, and/or budget will be required (Kong et al. 2017). But the recent advances in technology, experimental techniques, and computational methods drew on a long tradition of

community analysis in large-scale ecological studies, and careful data analysis can lead to the uncovering of large-scale trends (Hamady and Knight 2009). Microbiome studies now typically employ either targeted sequencing of 16S rRNA gene, WGS sequencing, or other meta-omic technologies to characterise the microbiome's composition, activity, and dynamics (Noecker et al. 2017). In the past years, the number of studies exploring a wide range of microbial communities (including those that inhabit the human body) has exploded, confirming that the application of NGS technologies are indispensable tools to characterise the human microbiome in number settings: the analyses of sequencing data – an assortment of clustering, binning, annotation, and assembly algorithms – allows to obtain the profile composition of species in each sample or group of samples, the set of genes they collectively encode, or the genome sequence of specific member species (Figure 2) (Noecker et al. 2017).

II.3. Current NGS techniques

II.3.1. 16S rRNA gene sequencing analysis

Fingerprinting techniques were preferred until the end of the 2000s since they could provide info about the microbial community while being orders of magnitude cheaper faster to perform (Hamady and Knight 2009). However, sequencing costs have dramatically and continuously dropped since then while DNA sequencing speed significantly increased: coupled with the advances in the computational approaches in complex datasets analyses (Ludwig et al. 2004; Cole et al. 2005; Schloss and Handelsman 2005; Lozupone, Hamady, and Knight 2006; DeSantis et al. 2006), several research groups chose to focus on bacterial 16S rRNA gene sequence-based surveys for characterising the microbial communities that reside in and on our body (Turnbaugh et al. 2007).

The 16S rRNA gene is a small subunit ribosomal RNA gene, and remains the most widely used phylogenetic marker for identifying microbes (Fredricks 2001; Woese and Fox 1977): this gene exists in all bacteria and archaea but not in eukaryotes, its function has not changed over time, and its sequence is long enough for informatics purposes (Janda and Abbott 2007; Bjerre et al. 2019; Grice and Segre 2011). In fact, on one hand this gene presents highly conserved regions that serve as both molecular clocks and binding site for PCR primers (Hugenholtz and Pace 1996; Grice and Segre 2011; Fredricks 2001). On the other hand, the species-specific hypervariable regions allows the taxonomic classification of the microbiota members and can even infer phylogenetic relationships to known bacteria (Fredricks 2001; Grice and Segre 2011), and small subunit rRNA-based studies are usually considered ''metagenomic'' because it relies on the analysis of heterogeneous samples of community DNA (Hamady and Knight 2009).

Today, the most prevalent form of comprehensive microbiome taxonomic data is obtained via the targeted amplification and sequencing of the 16S rRNA gene, giving the genomic characterization of bacterial diversity

(Grice and Segre 2011; Noecker et al. 2017), and the obtention of growing numbers of 16S rRNA gene sequences spurred the multiplication of the number of tools available for microbiota studies (Schloss and Westcott 2011). Smaller fragments of 16S rRNA gene even proved sufficient as proxies for the full-length sequence for many community analyses, including those based on a phylogenetic tree (Zongzhi Liu et al. 2007; Z. Liu et al. 2008; Q. Wang et al. 2007; Hamady and Knight 2009). In fact, the main advantage of 16S rRNA gene is that it contains both fast- and slow-evolving regions and therefore can be used to resolve phylogenetic relationships at different depths (Hamady and Knight 2009). Additionally, nine distinct hypervariable regions have been identified as markers specific for different categories of bacteria species and genera (Castelino et al. 2017).

Since 16S rRNA sequencing studies gives the distribution of the various 16S rRNA gene types in a sample, it allows the investigation of the bacterial microbiota and proved particularly useful for characterising the human microbiome in a wide range of samples, and eventually led to the creation of a census of microbes without cultivation (Fredricks 2001; Hamady and Knight 2009; Castelino et al. 2017).

But the copy number of 16S rRNA gene varies across bacterial taxa, and determining which genes are present, absent, or vary in copy number across various strains in a microbiome sample is a crucial task in order to avoid overestimating abundances of taxa with multiple copies of the gene (Noecker et al. 2017). To this end, the 16S rRNA gene sequence analysis workflow is generally composed of three main axes:

- sequence pre-processing, to remove low quality sequences,
- constructing OTU tables, for clustering similar sequences based on a defined similarity threshold,
- and annotating tables based on the representative OTU taxonomic and phylogenetic relatedness, using specific databases (Conlan, Kong, and Segre 2012; Schloss 2010; Kong et al. 2017).

Metagenomics studies concerning human-associated microbes were initially performed with the Sanger sequencing platform (Ley et al. 2005; Steven R. Gill et al. 2006), the first non-culture analysis method that allowed to sequence the 16S rRNA gene in a single reaction. However, this approach is very time-consuming and expensive, and soon the pyrosequencing method was introduced (Castelino et al. 2017; Margulies et al. 2005).

This new technology not only rendered the sequencing itself orders of magnitude cheaper and faster, but it also eliminated the laborious steps of clone libraries preparation (Mostafa Ronaghi et al. 1996; M. Ronaghi 1998; Margulies et al. 2005; Hamady and Knight 2009). Pyrosequencing studies of 16S rRNA also demonstrated an interesting depth of coverage (about 1000 sequences/sample), which seems to provide a good balance between sample number and sampling depth (Hamady and Knight 2009; Castelino et al. 2017). This made the pyrosequencing of 16S rRNA amplicons an extremely popular approach in the scientific community, as longer reads could provide more reliable and specific matches, enabling in turn easier result analysis (Susannah G Tringe and Hugenholtz 2008; Ong et al. 2013). Until recently, much of the NGS work on human microbiome

was indeed performed on the Roche 454 NGS platform, but because clinical microbiome studies are often comprised of hundreds of samples collected at multiple time points, this approach can be prohibitively expensive (Ong et al. 2013; Castelino et al. 2017).

In fact, the number of microbiome studies increased dramatically only since the 2010s and, since then, it was demonstrated that more cost-effective platforms that rely on shorter read lengths gave similarly accurate results (Quail et al. 2012; Loman et al. 2012; Castelino et al. 2017). Among them, the first report of precise species-level identification was obtained using the Illumina MiSeq platform: if the longer read lengths of the Roche 454 cover larger region of the 16S gene (thus making taxonomic assignment easier), shorter read lengths are sufficient to analyse microbial communities (Zongzhi Liu et al. 2007; Klindworth et al. 2013; Ong et al. 2013; Castelino et al. 2017). It was indeed demonstrated that, when the read lengths are similar to the typical amplicon length, the computational analysis of resulting sequences gets simplified and no significant differences in diversity indices at phylum and genus level could observed between 454 and MiSeq platforms (Castelino et al. 2017; Ong et al. 2013). The deep sequencing of 16s rRNA gene on the Illumina platform therefore has the strong advantage of accurately quantify abundances (even for rare members of a microbial community), and since the data generated by the MiSeq benchtop sequencer is comparable to the Roche454 platform one, it is now replacing it in academy (Castelino et al. 2017; Ong et al. 2013).

Many empirical results highlight the MiSeq utility for precise (over 90% at species level) and high-resolution microbiome profiling, but microbial ecologists still grapple with how to interpret the genetic diversity represented by the 16S rRNA gene (Schloss and Westcott 2011; Ong et al. 2013). Essentially, 16S rRNA surveys revolutionised our knowledge of the human microbiome, but they often remain limited to a genus-level taxonomic identification: as it can fail to distinguish closely related taxonomic groups and cannot always discriminate rare or low-abundance taxa from noise, other approaches might be needed for the complete description of a microbial community (Yarza et al. 2014; Shakya et al. 2013; Noecker et al. 2017).

II.3.2. Whole-genome metagenomic sequencing or ”shotgun” sequencing

To analyse more in details the functional and taxonomic landscape of the human skin microbiome as shaped by the local biogeography, microbiome research moved beyond amplicon-based studies to the direct sequencing of all microbial DNA (or shotgun metagenomics) (Oh et al. 2014; Y. Belkaid and Segre 2014). In fact, common computational 16S metagenomic analysis tools are often limited in resolution and accuracy, and may fail to capture biologically and clinically relevant details on the composition of species and genes in the microbiome (e.g. strain-level variation, nuanced functional response to perturbation) (Noecker et al. 2017). Therefore, whole-metagenome sequencing offers the opportunity for multi-kingdom analysis and functional insights into diseases, and appears to be the only way to fully define the microbiota’s genetic diversity and to predict the gene functions associated (Chng et al. 2016; Grice and Segre 2011).

Furthermore, shotgun metagenomics sequencing allows, through the characterisation of eukaryotic and viral constituents of the microbiome, to study interkingdom interactions (e.g., bacterial-fungal) and to explore how these relationships exacerbate disease severity or facilitate a transition between opportunism and pathogenicity (Peleg, Hogan, and Mylonakis 2010; Y. Belkaid and Segre 2014; Chng et al. 2016). With growing concerns of antibiotic-resistant microorganisms, shotgun metagenomics also empowers the exploration of the reservoir of antibiotic resistance genes in the skin (Y. Belkaid and Segre 2014). Other advantages of whole-genome sequencing include reduced amplification bias, generation of multi-kingdom genetic info, strain identification, detailed genomic coverage for prediction of functional capacity, and higher internal consistency than 16S profiling (Chng et al. 2016; Huttenhower et al. 2012; Oh et al. 2014, 2016).

Similar to results from amplicon studies, shotgun metagenomics confirmed that microbial communities are primarily shaped by their microenvironment, and that the differential abundance of taxa (e.g. P. acnes, commensal Staphylococci and Corynebacteria) contributed most significantly to both the intra- and interpersonal variations (Y. Belkaid and Segre 2014). Generally, genome assembly from metagenomes consists in assembling the sequencing reads into contigs and then group them into multiple bins, with each bin ideally including contigs from the same taxon (Noecker et al. 2017). The functional annotation of shotgun metagenomics data actually occurs by mapping reads to genes or protein domains with known functional classifications, and read mapping is done either by aligning each read to a reference database (of gene or protein sequences), or by using probabilistic models (e.g. HMMs) to evaluate if a given read belongs to a specific protein family/domain (Noecker et al. 2017). But the analysis of shotgun metagenomic data is more challenging than that of 16s rRNA analysis: the amount of data generated is considerably higher, and also requires the filtering of high percentages of human sequences (Chng et al. 2016; Oh et al. 2014, 2016; Kong et al. 2017).

In fact, shotgun sequencing approaches have the substantial advantage over end-sequencing protocols to produce twice as many species-level OTUs that can be identified (on average), and it proved to be particularly useful in identifying the functional potential of gut microbiomes (Grice and Segre 2011; Ong et al. 2013). However, the challenges with WGS reside not only in data analysis complexity, but also in the difficulty of constructing sequencing libraries and the increased sensitivity to contamination with mammalian DNA, microorganisms from the environment, or laboratory reagents (Kong et al. 2017). Shotgun metagenomics analyses may ultimately fail to identify the taxonomic origins of a gene of interest and to produce accurate and unbiased estimates of gene families' abundances (Mande, Mohammed, and Ghosh 2012; Manor and Borenstein 2015; Noecker et al. 2017), as the method remains limited by the need for more reference genomes but also issues of costs, robustness, and sampling efficacy (Chng et al. 2016; Noecker et al. 2017; Kong et al. 2017). So, if whole metagenome analyses are more adapted to fundamental research approaches – as they have the potential to reveal functional triggers of diseases, gene function identification, or detection of strain-level variations (either gene copy number or gene content), 16S rRNA gene sequencing analyses might be

preferred for the general characterisation of a given microbiota (Chng et al. 2016; Kong et al. 2017; Noecker et al. 2017).

III. The skin microbiome

III.1. Skin structure and microbial communities

III.1.1. Generalities

The skin is the largest organ of the human body, and this 1.8 m2, multi-layered, three-dimensional structure presents an heterogeneous surface composed of an abundance of folds, invaginations and specialised niches that each have their own microenvironment (Grice et al. 2009; Grice and Segre 2011; Chen, Knight, and Gallo 2023). The skin acts first as a physical barrier to bar entry to foreign pathogens, and then as a substrate for an ecosystem of diverse life where microbial colonization is driven by the ecology of the skin surface: the various habitats create different growth conditions that support a myriad of commensals, which in turn may influence human health and disease (Grice et al. 2009; Y. Belkaid and Segre 2014; Grice and Segre 2011; Chen, Knight, and Gallo 2023).

The analogy with the ecosystem of the soil has long been relied on to explain the variety of microbial communities found on the skin: both matrices lack producer organisms, and their microbiota tend to group around structures that penetrate the surface to deeper layers (M. J. Marples 1969; Fredricks 2001). So, on one hand the skin is the primary interface of the human body with the environment and gets colonised by a very diverse collection of bacteria, fungi, viruses, and mites that may provide vital functions that the human genome has not evolved (Chiller, Selkin, and Murakawa 2001; Fredricks 2001; Roth and James 1988; Noble 1984; Roth and James 1989; Cogen, Nizet, and Gallo 2008; Grice and Segre 2011). These microbes indeed interact with the immune and the keratinized skin cells, cells over the entirety of a human's life, to maintain both the skin's physical and immune barriers under homeostatic healthy conditions against multiple stresses (wounding, infections) (Y. Belkaid and Segre 2014). On the other hand – and because of its compartmentalised structure – the skin microbiota is composed of a complex composite of microbes and host structural, hormonal, nervous, and immunological networks, and is generally thought to have coevolved with its host to finely tune this unique relationship (Y. Belkaid and Segre 2014).

As for any type of microbiome studies, the characterisation of the skin microbiota relied almost exclusively on culture-based methods until the end of the 20th century, but the improvements in DNA sequencing confirmed the suspicions of bias toward species that readily grow under standard laboratory conditions (particularly Staphylococci species) (Grice et al. 2009; Grice and Segre 2011). In fact, the number and type of microorganisms found in microbiome cultures is heavily determined by the chosen conditions, but no *in vitro*

system can exactly reproduce the skin microenvironment: only a minority of bacteria can flourish under isolation, and it is especially difficult when dealing with skin microorganisms that are inherently adapted to a cool, dry, and acidic environment (Fredricks 2001; Dunbar et al. 2002; Grice and Segre 2011). The other main limitation of culture-based studies was the traditionally low biomass of the skin microbiota: the overlapping cells of the stratum corneum forms some sort of armour, forming a cool, acidic (normal skin pH ≈ 5.5), and desiccated environment that is nutrient poor and less hospitable compared to the gut environment (Roth and James 1988; Grice and Segre 2011; Scharschmidt and Fischbach 2013; Y. Belkaid and Segre 2014). In the end, culture-based techniques were very selective towards species that thrive under diagnostic microbiology typical nutritional and physiological conditions, and are not representative of all the most abundant and/or influential organisms in the community (Grice and Segre 2011; Chen, Knight, and Gallo 2023).

With NGS technologies becoming more accessible, culture-independent methods such as amplicon-based sequencing became the standard for profiling the skin microbiome (Figure 3) (Chen, Knight, and Gallo 2023). Early works rapidly demonstrated that, despite its overall relative dryness which limits the growth of organisms requiring moisture, the skin actually had a greater richness at the genus level compared to other body regions (Roth and James 1988) (Li et al. 2012). The revolutionising work of the HMP even suggests that it already captured all of the most abundant genera, perhaps even the great majority of the human microbiome diversity (Li et al. 2012).

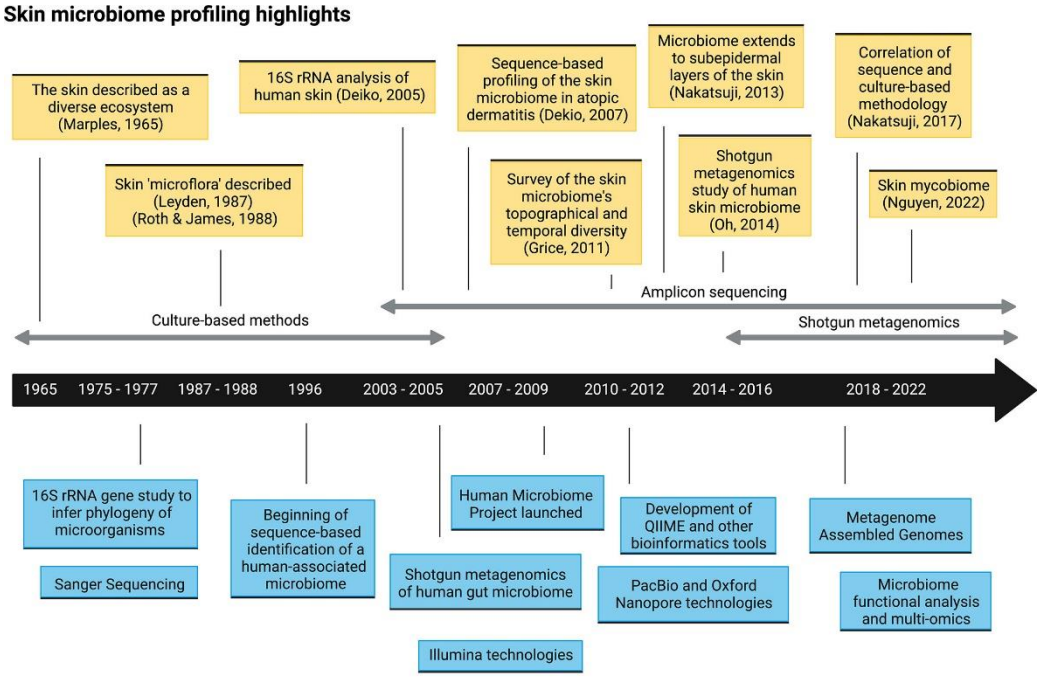


Figure 3. *Milestones and highlights of human microbiome studies. (Chen, Knight, and Gallo 2023)*

If the microbiome's characterisation heavily depends on choices of scientific focus (all microbes? One particular kingdom?), study design, costs, and result analyses, it is now universally accepted that microbial ecosystems

are not only comprised of bacteria but also of many other resident commensal microorganisms (Iebba et al. 2016; Kong et al. 2017). These bacteria, fungi, viruses, mites, bacteriophages, mycetes… all strongly contribute to maintaining the microbiome's balance, as they play a central role in cutaneous homeostasis and health through the utilisation of skin resources (Chen, Knight, and Gallo 2023; Iebba et al. 2016).

Nevertheless, the bacterial component of the microbiota remains largely superior to the others, and the extensive characterisation of the topographical diversity of the skin microbiome by Grice *et al.* (20 different skin sites on 10 healthy humans) detected 19 bacterial phyla, but with most of the sequences belonging to only four: *Actinobacteria* (51.8%), *Firmicutes* (24.4%), *Proteobacteria* (16.5%), and *Bacteroidetes* (6.3%) (Grice et al. 2009). Overall, the scientific community identified *Cutibacterium* (formerly *Propionibacterium*), *Staphylococcus*, and *Corynebacterium* as the major bacterial genera on the skin, while the fungi represented only a small fraction of the community, except in sebaceous areas like near the ears and the forehead (Figure 4) (Y. Belkaid and Segre 2014; Chen, Knight, and Gallo 2023; Grice and Segre 2011). It was already known that the fungal component of the skin microbiota was predominantly composed of yeasts, and modern approaches revealed *Malassezia* as the primary genus, particularly of the *M. globosa* and *M. restricta* (Roth and James 1988; Chng et al. 2016; Y. Belkaid and Segre 2014). On their end, the dominant members of the human virome include phages (mostly of *Staphylococcus* and *Cutibacterium* spp.), human papillomaviruses, and human polyomaviruses, but, depending on the body site, do not necessarily reflect the relative abundances of their host bacteria (Pride et al. 2012; Chen, Knight, and Gallo 2023).

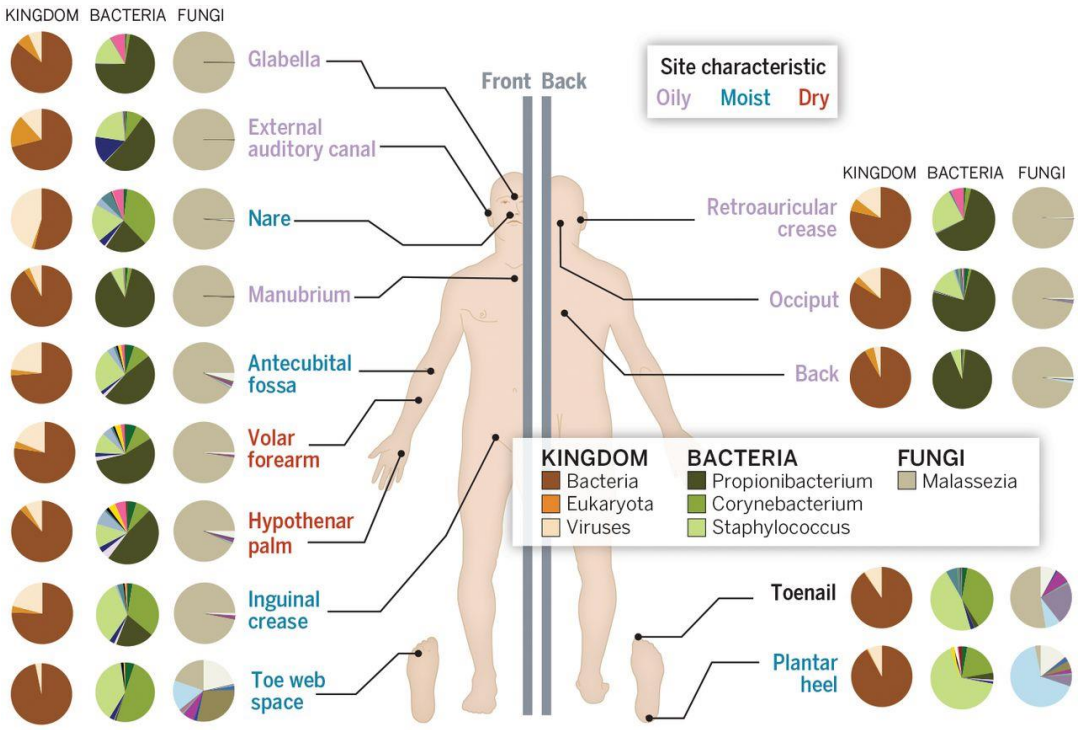


Figure 4. *Relative abundances of bacteria, fungi, and viruses of the skin microbiota, obtained by shotgun metagenomics. (Y. Belkaid and Segre 2014)*

III.1.2. The skin innate and adaptive immune response

As mentioned earlier, the skin is not simply a physical barrier that prevents loss of moisture and bars entry of pathogenic organisms, but it is also equipped with a variety of niches and appendages that provide a highly sophisticated system of immune surveillance (Segre 2006; Grice et al. 2009). The combined action of epithelial cells, lymphocytes, and antigen-presenting cells that populate the dermis and epidermis create a dialogue between the innate and adaptive arms of immunity that responds to wounding and infection in the most appropriate manner (Pasparakis, Haase, and Nestle 2014; Roth and James 1988; Y. Belkaid and Segre 2014). However, it was established early on that the skin humoral and cellular immune systems also plays a role in the composition of the cutaneous microbial communities (Roth and James 1988; Grice and Segre 2011). Later work confirmed that the cutaneous innate and adaptive immune responses can modulate the skin microbiota, but the same authors insisted as well on how the microbiota functions in educating the immune system (Grice and Segre 2011).

The healthy human skin is indeed one of the largest reservoirs of memory T cells in the body, and the microorganisms found on and in the skin educates these – about 20 billion – effector lymphocytes to respond to similarly marked pathogenic cousins, allowing the discrimination of harmless commensal against harmful pathogenic microorganisms (Clark et al. 2006; Grice and Segre 2011; Y. Belkaid and Segre 2014). But the role of the microbiota as an endogenous adjuvant of the skin immune system does not stop there. Some of its members also produce substances with antibacterial and antifungal properties, and therefore actively participate to tissue repair and battling infections (Noble 1984; Roth and James 1988; Y. Belkaid and Segre 2014). Additionally, certain commensals can influence the local pH, making their environment hostile to pathogen establishment (Turovskiy, Sutyak Noll, and Chikindas 2011). With ever more publications on the subject, it is now widely accepted that the microbiota plays a crucial role in efficient skin response to wounding and infection, and that humans and their microbial communities have coevolved to provide mutual benefits, with the commensals continuously adapting to specifically control the immunological network associated with their ecological niches (Y. Belkaid and Segre 2014; Roth and James 1988; Flowers and Grice 2020).

The microbiota members are generally classified into two categories: the resident and the transient flora. The first designates the organisms growing on the skin that are relatively stable in number and composition, while the second refers to microbes lying free on the skin surface, believed to be derived from exogenous sources and found primarily on exposed skin (Roth and James 1988). In the past years, the scientific community focused particularly on the relationship between skin diseases and cutaneous microbiota, and the realisation that the resident members were likely the primary drivers and amplifiers of skin pathologies suggested that transient partners may also influence the capacity of the microbiota to control the innate and adaptive immunities (Y. Belkaid and Segre 2014). Understanding the composition and structure of both resident and transient

communities in the various areas of the body – and how they modulate the local immunity – would therefore help elucidating the mechanisms controlling the cutaneous immune landscape (Y. Belkaid and Segre 2014).

III.2. A complex environment

III.2.1. Geography and physiology

Traditionally, members of the Actinobacteria phylum are more abundant on the skin surface, while *Firmicutes* and *Bacteroidetes* compose most of the gastrointestinal tract microbiota (Grice and Segre 2011). But the skin is a complex ecosystem composed of a wide range of physiologically and topographically distinct niches, each harbouring their own mixed representation of *Actino*- and *Proteobacteria*, *Firmicutes*, and *Bacteroidetes* phyla (Figure 5) (Z. Gao et al. 2007; Grice et al. 2009; Costello et al. 2009; Grice and Segre 2011).

It has long been postulated that the flora from any one area of the skin should not be considered representative of the entire cutaneous microbiome, and that samples should be taken from multiple sites to obtain a broad view of the microbiome (Roth and James 1988). The molecular approaches allowed in the 2000s to confirm the great variability of the skin microbiota within and between distinct topographical regions, and that the physiological differences between the individual habitats strongly influence the resident bacteria and fungi colonisation (Grice et al. 2009; Grice and Segre 2011; Findley et al. 2013; Y. Belkaid and Segre 2014).

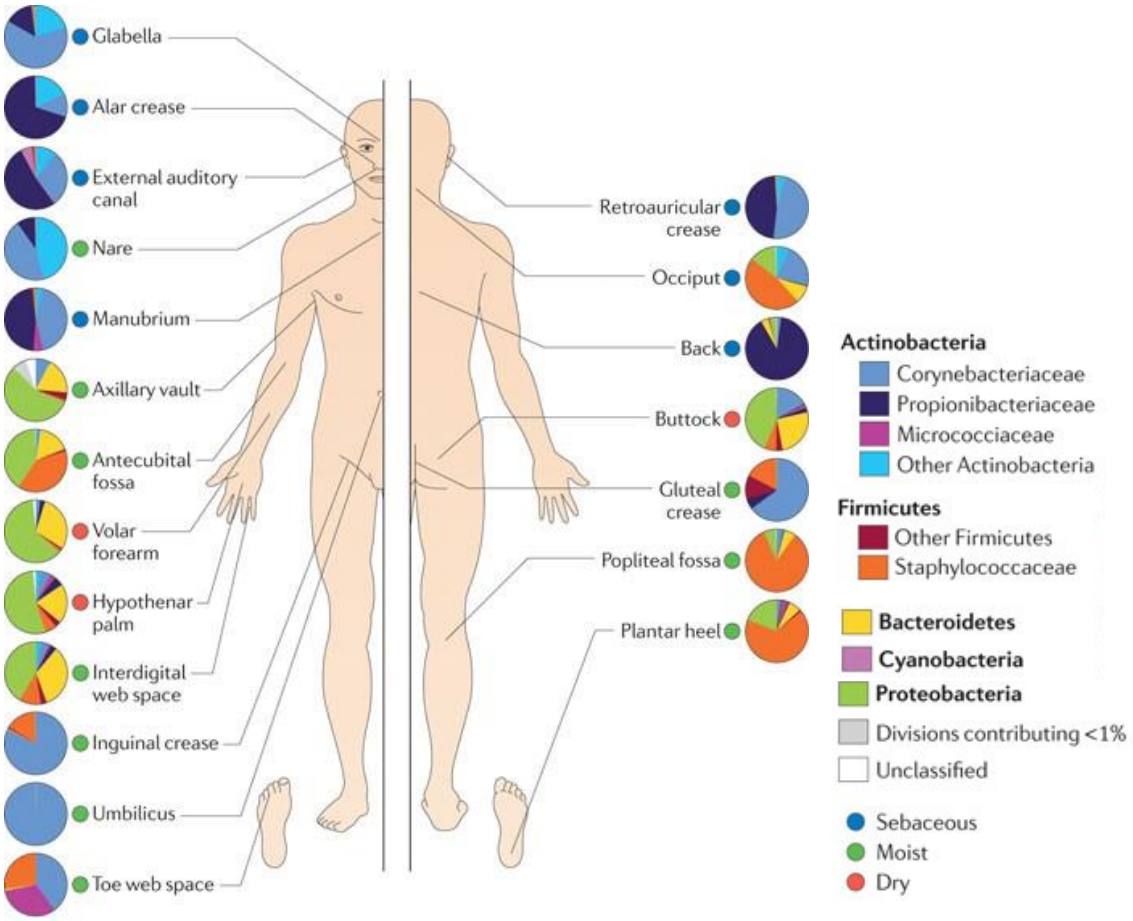


Figure 5. *16S rRNA analysis of bacteria distribution, depending on the skin site. (Grice and Segre 2011)*

First, the skin surface is home to approximately five million hair follicles and sweat ducts and their concave structure and depth significantly increases its complexity as a substrate (Chen, Knight, and Gallo 2023). Then, different areas of the body present different skin thickness, folds, and density of hair follicles and glands (Tagami 2008). Finally, many spatial factors and processes can affect the microbial community structure and composition (nutrient availability, communication, biofilm formation) (Donaldson, Lee, and Mazmanian 2016; Noecker et al. 2017).

The skin effectively produces nutrients in the form of lipids and protein (mostly keratin), and each of the stratum corneum, cellular layer, hair shaft and follicle, eccrine, apocrine, and sebaceous glands have their own associated microflora (Figures 4 and 5) (Fredricks 2001). Since their thermoregulating role provides water and salt through sweat, areas rich in eccrine glands are home to large proportions of *Staphylococci* and *Corynebacteria* spp. (Grice and Segre 2011). Similarly, the lipid-rich environment of areas with a high density of sebaceous glands is largely dominated by Cutibacteria species (Grice and Segre 2011). But despite the dramatic interpersonal differences in microbiome composition, the analysis of 16S ribosomal RNA gene sequences from 20 skin sites of healthy volunteers demonstrated that physiologically comparable sites are home to similar bacterial communities, and the microbiome of the different sites can be classified in three microenvironment categories: sebaceous, moist, and dry (Grice et al. 2009).

Sebaceous sites

The high density of hair follicles and sebaceous glands in certain regions (face, chest, back) leads to the production of large amounts of oily, lubricating sebum on the skin and hair that provides an antibacterial shield (Grice and Segre 2011). This hydrophobic coating creates an anoxic environments favourable to lipophilic, (eventually facultatively) anaerobic organisms such as *Propionibacterium* and *Malassezia* species (Roth and James 1988; Grice et al. 2009; Grice and Segre 2011).

Because anaerobes require special conditions for sample transport, processing, and growth (even having slow proliferation rates), their isolation through traditional culture-based approaches can be problematic (Bowler, Duerden, and Armstrong 2001; Davies et al. 2001; Grice and Segre 2011). For this reason, the early assumption was that the microbiota diversity of notoriously exposed and sebaceous areas was underestimated regarding the transient organisms (Roth and James 1988). Later studies however determined that sebum-rich sites are actually less diverse, even, and rich than moist and dry districts ($P < 0.05$, one-tailed t test), with the retroauricular crease (behind the ear) being the poorest area (Grice et al. 2009). The same author listed the other sebaceous skin sites as the glabella (between eyebrows), the occiput (back of the head), the external auditory canal (inside the ear), the alar crease (beside the nostrils), the manubrium (upper chest), and the back (Grice et al. 2009).

Moist sites

The moist areas regroup the nares (inside the nostrils), the axillary vaults (armpits), the antecubital fossae (inner elbows), the interdigital web space (between the fingers and/or toes), the inguinal crease (on the external side of the groin), the gluteal crease (topmost part of the fold between buttocks), the popliteal fossae (behind the knees), the plantar heels (bottom of the heels), and the umbilicus (Grice et al. 2009). These niches are dispersed all over the body, and despite the diversity of locations and hair follicle density (the armpits *vs.* the plantar heels, or the gluteal crease *vs.* the inner elbows or knees), they are more similar together than to geographically closer zones (the armpits *vs.* the top of the forearms, for example) (Figure 5) (Grice et al. 2009).

Dry sites

Surprisingly, the metagenomic approaches revealed that the dry areas were the most diverse skin sites, with a phylogenetic diversity greater even than in the gut or the oral cavity of the same individual (Costello et al. 2009). Another curious feature of their microbiota is the abundance of Gram-negative organisms, that were long thought to colonize the skin as rare contaminants from the GIT (Chiller, Selkin, and Murakawa 2001; Roth and James 1988; Grice and Segre 2011). But any microbe found in nature or in other of body areas (even non-cutaneous) may actually be found on the skin transiently, leading to a mixed bacterial population of bacteria in dry sites, with a greater prevalence of *Betaproteobacteria* and *Flavobacteria* compared to other regions (Roth and James 1988; Grice et al. 2009). The most diverse site on the body was in fact determined to be the volar forearm (inside of mid-forearm), while other dry areas include the hypothenar palm (the palm of hand, neighbouring the little finger), the front of the legs, and the buttock (Grice et al. 2009).

III.2.2. Endo- and exogenous factors

If many features of the skin microbiome are common in similar skin sites of different individuals, shotgun metagenomic studies suggested that the strain level diversity of dominant skin bacterial species was quite high, demonstrating the dual influence of both the host and the microenvironment on the microbiota's composition (Figure 6) (Y. Belkaid and Segre 2014; Kong et al. 2017). It was even showed that some body sites had a lesser interpersonal variation (alar crease, nares, back) than others (interdigital web spaces, axillae, umbilici), coincidentally corresponding to their microenvironment categories (nutrient-poor sebaceous vs. water-rich moist regions) (Grice et al. 2009), highlighting the role of many endo- and exogenous factors in shaping the composition and structure of the microbiota.

Naturally, the host genetic background is fundamental in shaping the bacterial attachment sites that welcomed the first colonising flora (Iebba et al. 2016), and the physiological and anatomical differences between males and females (hair follicles density, sweat and sebum production, hormonal influences) lead to gender-associated microbial differences (R. R. Marples 1982; Noah Fierer et al. 2008; Giacomoni, Mammone, and Teri 2009).

Similarly, the subject's age also has a great effect on the microenvironment of the skin and thus on the colonizing microbiota (Leyden et al. 1975; Somerville 1969; Grice and Segre 2011). It has long been established that the microbial flora is most varied in young children, but more recent investigation could demonstrate that, upon transition through puberty and sexual maturation, the cutaneous bacterial communities undergo major modifications (Y. Belkaid and Segre 2014; Roth and James 1988). In fact, and always depending on the studied skin site, the microbiome neonates, infants, and young children is unmistakably different from the one of in sexually mature children and adults, particularly at certain skin sites (Capone et al. 2011; Costello et al. 2013; Dominguez-Bello et al. 2010; Jo et al. 2016; Oh et al. 2012; Ying et al. 2015; Kong et al. 2017).

Other than genetic factors, the human cutaneous microbiome is also shaped by its exposome and lifestyle, eating habits, antibiotic exposure, hygienic practices, … all having the potential to selectively alter the cutaneous microbiome composition, and changes in these routines and patterns may increase the incidence or severity of dermatological disorders (Grice et al. 2009). Systemic and topical antibiotics for example may impair bacterial adherence to epithelial cells and suppress the normal flora, modifying he natural skin response to colonisation by harmful microorganisms increase colonization by other organisms. (Eisenstein, Ofek, and Beachey 1979; James and Leyden 1985; Roth and James 1988). In fact, the development of fungal infections after normal flora depletion demonstrates the inhibitory function of the microbiota (Roth and James 1988). Similarly, the use of cosmetics (soaps and shampoos, body/face moisturizers, make up, deodorants…) is also a potential factor that contributes to skin microbiome variations and might even alter the conditions of the skin barrier in some circumstances (Grice and Segre 2011; Perez Perez et al. 2016; Two et al. 2016; Kong et al. 2017).

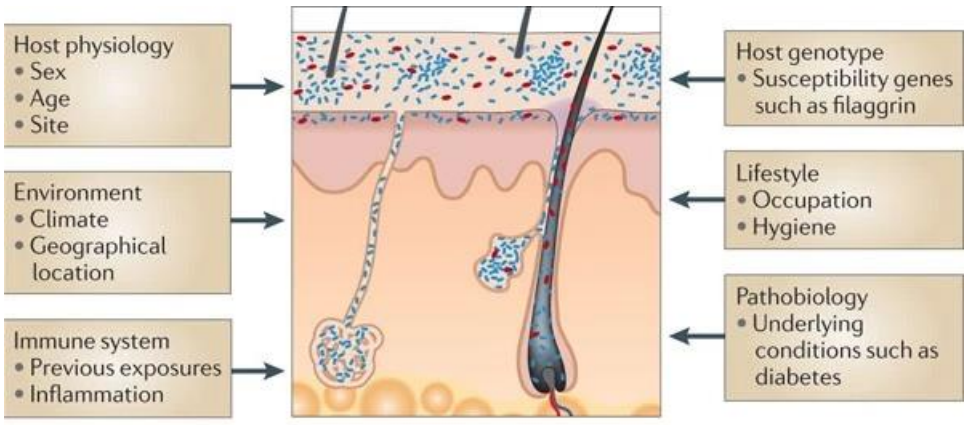


Figure 6. *Intrinsic and extrinsic parameters influencing the skin microbiota's composition. (Grice and Segre 2011)*

But the exposome comprises also the general environment's impact and most of the skin is particularly exposed to it. This can make the determination of transient and resident members of the community quite difficult, especially since transient organisms may become established as resident flora in areas that are consistently exposed (Price 1938) (Roth and James 1988) (Grice and Segre 2011).

This was first highlighted seeing how increases in temperature and humidity usually increase the density of bacterial colonization and may alter the relative ratios of the microbiome's components, while virulent infections tend to decrease when the microenvironmental temperatures are lower (Roth and James 1988). However, evidence that our built environments contribute to the cutaneous microbiome composition through correlations with surfaces that interface with the skin (including computer keyboards, phones, door handles, …) was only recently investigated (Umbach, Stegelmeier, and Neufeld 2021), and it is now considered that any modification of the microenvironment, whether extrinsic (occupation, clothing choice, pet ownership, cosmetic usage, season, time of day, birth country…) or intrinsic (sex, age, antibiotic usage, physical activities, race/ethnicity, delivery mode, diet…) strongly influence the skin microbiota, well beyond the sole genetic factors and environmental contaminations (Grice and Segre 2011) (Song et al. 2013), (Callewaert et al. 2013), (Meadow et al. 2013) (Kong et al. 2017).

III.3. The cutaneous microbiota composition

III.3.1. The "healthy" skin microbiota

Despite the relatively inhospitable habitats, the surface of the stratum corneum and the outermost layers of the epidermis are host to many small microcolonies from multiple kingdoms, but the microbiota remains largely dominated by a few bacterial genera: *Cutibacterium* (23.0%, formerly *Propionibacterium*; *Actinobacteria*), *Corynebacterium* (22.8%; *Actinobacteria*), and *Staphylococcus* (16.8%; *Firmicutes*) (Grice et al. 2009; Roth and James 1988; Chen, Knight, and Gallo 2023). Metagenomic analyses also revealed that the sebaceous sites had the least diversity and were principally populated by *Cutibacteria* spp. and *Staphylococci* spp., while moist areas preferentially host members of the *Corynebacterium* and *Staphylococcus* genera (Grice et al. 2009; Chen, Knight, and Gallo 2023).

Staphylococcus commensal species are highly abundant across all skin sites due to this genus' diversity: they thrive in humidity-rich environments, most have facultative anaerobic abilities, and certain tolerate high salt levels and can use the urea present in sweat as nutrient (Gallo and Hooper 2012; Grice and Segre 2011; Y. Belkaid and Segre 2014; Chen, Knight, and Gallo 2023). The lipophilic *Cutibacteria* species, on the contrary, are prevalently found in sebum-rich areas where they even largely dominate other lipophilic members (Y. Belkaid and Segre 2014). In fact, the third major member, *Corynebacteria* spp., are humidity-loving but do not produce their own lipids (similar to *Malassezia* fungi) and must obtain them from their environment (Y. Belkaid and Segre 2014), making them particularly well-suited to the skin surface with its lipid-rich sebum. However, if they are found in high proportions in moist area due to their preferred growing conditions, sebaceous sites are dramatically dominated by *Cutibacteria* species and *Corynebacteria* are poorly represented (Y. Belkaid and Segre 2014). Current data therefore suggests that, while the *Staphylococcus* presence does not impair the growth of other organisms, the overbearing of *Cutibacteria* explains why sebaceous body sites are the skin

sites with the least species diversity (Byrd, Belkaid, and Segre 2018; Chen, Knight, and Gallo 2023; Scholz and Kilian 2016). Other well-known members of the skin bacterial flora include the *Derma*- and *Micrococci*, but the high variability of the skin microbiome's composition and structure, depending on the individual and the tested areas, strongly hinders the conclusions on a "standard, healthy" cutaneous microbiota.

These considerable interpersonal, temporal, and geographical fluctuations are however fundamental for the holobiont symbiosis: in any ecosystem, the host actually ensures that any function that is exerted by the microbiota is codified by multiple, closely related microorganisms, rather by only one species. (Grice and Segre 2011; Chen, Knight, and Gallo 2023). So, beyond the sole host genetics- drive, the diversity within and between individuals is explained also by the myriad of factors that influence the microbiota's composition (lipid content, pH, sweat, sebum secretion…), since these parameters correlate with the predominant flora of a given area (Iebba et al. 2016; Grice et al. 2009) – for example, *S. epidermidis* reportedly prevents colonisation by other pathogenic bacteria, *C. acnes* retains the water in the stratum corneum, and *Corynebacteria* are involved in skin pH regulation (Sanford and Gallo 2013; Scheimann et al. 1960; Nodake et al. 2015; Ogai et al. 2018). But on the other hand, low genetic and metabolic diversities may lead usually commensal microbes toward pathogenesis, and overexpression of these pathobionts is also widely recognised as contributing to certain skin disorders (Grice et al. 2009; Chen, Knight, and Gallo 2023).

Similar to the gut microbiota, the skin microbiota present few distinct phyla but a high diversity at the genus and species levels and actively participates to colonisation resistance, notably competing for defined metabolites with harmful microbes and modulating the host innate immune response (Y. Belkaid and Segre 2014; Grice and Segre 2011; Costello et al. 2009). The resident and transient communities, together, in fact assist the skin innate and adaptive immune system in its development, nutrition, and resistance to pathogens (Bäckhed et al. 2004; Ordovas and Mooser 2006; Belda-Ferre et al. 2012; Kau et al. 2011; Mirmonsef et al. 2011; Li et al. 2012). But strongly perturbed, poorly diverse microbiotas are recurrently correlated to skin lesions associated to dermatological disorders, while sites exhibiting great microbial diversity generally are healthy, further evidencing the need for microorganism interdependency and diversity to maintain a healthy skin microbiome (Grice et al. 2009; Costello et al. 2009; Kong et al. 2012). The consensus in the field now is that, independently of its actual composition, any loss of microbiome homeostasis might lead to opportunistic microbial colonisation, sometimes in fact by the same commensals that inhabit it (Chng et al. 2016; Chen, Knight, and Gallo 2023). However, whether microbiome-associated skin lesions and dermatological diseases are triggered or amplified by an altered microenvironment remains under debate (Noecker et al. 2017; Chen, Knight, and Gallo 2023).

III.3.2. Diseased states

Because clinical improvement is seen upon administration of antimicrobial agents (topical emulsions, systemic antibiotics, ultraviolet light), underlying microbial contribution for many common skin diseases (eczema, psoriasis, acne, seborrheic dermatitis, dyshidrosis, rosacea) was long hypothesized (Fredricks 2001; Grice and Segre 2011; Roth and James 1988; Faergemann and Larkö 1987; Grice et al. 2009). The microbiome of patients with dermatitis such as psoriasis and atopic dermatitis (AD), in fact have a cutaneous microbiome often composed of up to 80% *Staphylococcus* spp. over both diseased and normal skin (Aly, Maibach, and Mandel 1976; Aly, Maibach, and Shinefield 1977; M. I. White and Noble W.C. 1986). Similarly, numerous studies reported early on that antibiotic treatments commonly leads to acne remission (Holland, Cunliffe, and Roberts 1977; Thomsen et al. 1980; Al-Mishari 1987; Till et al. 2000), but they relied on culture-based approaches and no constant correlation with changes in the cutaneous microflora could be established (Fredricks 2001). In fact, if many recent studies applying shotgun metagenomics to the skin microbiome are an important window into its various diseases, the cause/effect relationships inside the microbial communities and with their host are still difficult to untangle (Chen, Knight, and Gallo 2023; Kong et al. 2017).

Atopic dermatitis (AD) for example, also known as eczema: during flares, the lesioned areas see an increased colonisation of the known skin pathogen *S. aureus* and commensal *S. epidermidis*, suggesting that a disrupted barrier function can be exacerbated by *S. au.* and promote the overgrowth of opportunistic *S. ep.* strains (Chng et al. 2016; Nilsson, Henning, and Hjörleifsson 1986; Roth and James 1988; Kong and Segre 2012; Chen, Knight, and Gallo 2023). Metagenomic studies in fact demonstrated that AD lesions harboured similar ranges of organisms with respect to normal skin and that community membership was better preserved than the community structure, highlighting the importance of the microbiome's balance compared to its composition (Grice et al. 2009). But the role of the microbial communities in the pathogenesis of AD is still incompletely characterised, as both the strain-level functional differences and the many global and body-site-specific differences in bacteria, fungi, and viruses populations may contribute to disease complexities (Y. Belkaid and Segre 2014; Chen, Knight, and Gallo 2023).

On one hand, imbalances in *Malassezia* fungi, *Staphylococcus*, and *Streptococcus* bacterial species populations were identified in patients with AD, psoriasis, and seborrheic dermatitis (Zhan Gao et al. 2008; Y. Belkaid and Segre 2014; Chen, Knight, and Gallo 2023; Chng et al. 2016), but no actual common characteristic relative to the bacterial and fungal communities could emerge (Chen, Knight, and Gallo 2023; Zhan Gao et al. 2008; Y. Belkaid and Segre 2014; Owen et al. 2001; Grice and Segre 2011). On the other hand, older models of acne vulgaris believed an over-colonisation of *C. acnes* was the cause of the disease (R. R. Marples 1982; Leeming, Holland, and Cunliffe 1984; Roth and James 1988), when it is now understood that acne patients host the same proportions of *C. acnes* as the healthy populations, but have a higher strain diversity with certain members

presenting more virulence-associated factors (Chen, Knight, and Gallo 2023; Fitz-Gibbon et al. 2013; Noecker et al. 2017; Y. Belkaid and Segre 2014).

Time and time again, studies on the cutaneous microbiota have demonstrated that balance disruptions on either end of the host/microorganisms relationship can result in the onset or aggravation of skin disorders and infections, with both opportunistic and exogenous pathogens benefitting from dysbiotic statuses (Grice and Segre 2011; Chen, Knight, and Gallo 2023; Iebba et al. 2016). So far, the spotlight has mainly been put on the bacterial component of the microbiota for its ease of culture, but the role of other members (fungi, mites) in disease origin and progression is increasingly recognised (Darabi et al. 2009; Chng et al. 2016; Chen, Knight, and Gallo 2023; Flowers and Grice 2020). Any microbe might indeed exert a pathogenic effect, either by release of toxins, invasion of cells, alteration in host cell regulation, induction of allergic or inflammatory responses, or alteration in the microbial community (Fredricks 2001). However, the mechanisms involved remain largely unclear, and understanding the healthy microbiome and its typical and benign variations might actually be the key to elucidate dysbiotic disease states (Chen, Knight, and Gallo 2023).

IV. Aim of the project

All the work presented here is the fruit of a doctoral project stemming from the collaboration between Complife (Garbagnate Milanese, Italy) and UPO-CAAD (Centre for Allergic and Autoimmune Diseases of the University of Eastern Piedmont, Novara, Italy) and was conducted under the supervision of Prof. Elisa Bona, from the Department for Sustainable Development and Ecological Transition (University of Eastern Piedmont, Vercelli, Italy). Complife is a consulting and testing company for cosmetics, nutraceutics, and medical devices, and is particularly dedicated to offering state of the art efficacy analyses to its clients. In this vein, the increasing worldwide interest for creating cosmetics able to modulate the cutaneous microbiota led the firm to open, in 2019, a laboratory dedicated to skin microbiota studies.

The first objective of this PhD was to set up a reliable and robust protocol allowing to observe eventual variations of the cutaneous microbiota upon cosmetic use. Because most of the studies would be client-ordered, it was of crucial importance to have a cost- and time-effective protocol that would allow to observe both broad trends and particular features of the microbiota. Through the collaboration with UPO and thanks to the support of Dr Marta Mellai and Profs. Elisa Bona and Flavio Mignone, it was decided early on to base our workflow on the 16S rRNA gene sequencing analysis amplified from the V1-V3 hypervariable regions, and thus to focus on the bacterial component of the microbiota in its entirety. Reliability, reproducibility, and repeatability of the pipeline were assessed through a pilot and a preliminary study, and Complife could count on quality, dependable devices and protocols for sample collection by swabbing thanks to Santina Castriciano from Copan Spa (Brescia, Italy). This latter collaboration also gave us the opportunity to test different sampling

devices and consequently to possibly lower the cost of the specimen collection aspect of our studies, which later inspired us to test other variations of the base workflow.

The second objective was to try and understand the composition of the normal, healthy cutaneous human microbiota in central-northern Italy. In fact, the human microbiota is well known for its extreme variability, and the question still stands: is there a substantial core of abundant organisms/lineages we all share? There is indeed no possibility that any species at high abundance in all individuals (Hamady and Knight 2009) due to the multitude of potential influences (sex, age, lifestyle, environment…). In agreement with its clients, Complife could use over 1200 T0 and untreated samples from cosmetic-testing studies and analyse them based on a variety of factors: body site, sex, age, sampling season, and living area. Since most were facial specimens, the focus was first put on this area before extending the same reasonings to other areas.

The results presented here summarise the work conducted over the last four years, starting from scratch and now allowing to dig deeper into the factors that may direct the composition of the skin microbiota.

MATERIALS AND METHODS

Disclaimer: All studies conducted at Complife are designed in accordance with the Declaration of Helsinki, and all participants give their informed consent upon participating to said studies. All samples included in this work were collected by trained technicians from Complife Italia Spa (HQ: Garbagnate Milanese, Italy).

I. Sample Collection

I.1. Sampling method

Participants were asked to leave the tested area unwashed for 8–12 h prior to sampling and to avoid the use of cosmetics in this time period. Upon collection, the volunteers gently rinsed the area with clean hands and tap water before dabbing the area dry using a sheet of absorbent paper. Samples were retrieved by swabbing an area defined by a 3.5 by 5.5 cm adhesive paper template (Copan Spa, Brescia, Italy). Each tested device was equipped with a proprietary FLOQswab® (Copan Spa, Brescia, Italy), that was moistened on one side with a drop of water dispensed by a disposable Pasteur pipette and rubbed ten times horizontally, both from top to bottom and from bottom to top to cover the entire delimited area. The swab was turned 180° to its dry side, rubbed vertically ten times both from left to right and from right to left, then placed in a sterile tube equipped with a nucleic acid conservation system (Copan Spa, Brescia, Italy). Three different kits were used in this work – FLOQswab® + eNAt®, Smart eNAt®, and Active Drying System® (Copan Spa) – and the collection technique remained consistent throughout.

I.2. Sample Preparation

Upon arrival in the genomics laboratory, two procedures were used to prepare the samples before proceeding with the next steps:

- the swab-containing tubes were vortexed (with an added 1 mL of eNAt® solution in the ADS® device's tube) and spun (Corning® LSE™ Mini Microcentrifuge, AC100-240V), before pressing the swab against the walls and recovering the preservation medium,
- or eNAt® recovery was carried out using the NAO® Basket proprietary device, consisting in a perforated basket inserted in a 2 mL sealable collection tube (Figure 7, Copan Spa).

In this case, the head of swab was placed inside the basket and 500 µL of eNAt® medium were added, either from the initial collection device or from a new, sterile container. The device was vortexed and centrifuged at 10 000 rpm for 2 minutes, and the process was repeated with an additional 500 µL to give 1 mL of raw microbiota extract. All liquid handling was performed under a laminar flow hood and the specimen were stored at −20 °C until further use.

Figure 7. *NAO® Basket sample preparation device (courtesy of Copan).*

II. DNA Processing

II.1. Microbial DNA extraction

Three extraction kits were used in this project: QIAamp® DNA Microbiome Kit (Qiagen, Hilden, Germany), PureLink™ Microbiome DNA Purification Kit (Invitrogen, Waltham MA, USA), and ZymoBIOMICS™ DNA Miniprep Kit (ZymoResearch, Irvine CA, USA). The experimental procedures were obtained from the manufacturers' websites summarised in Figures 8, 9, and 10.

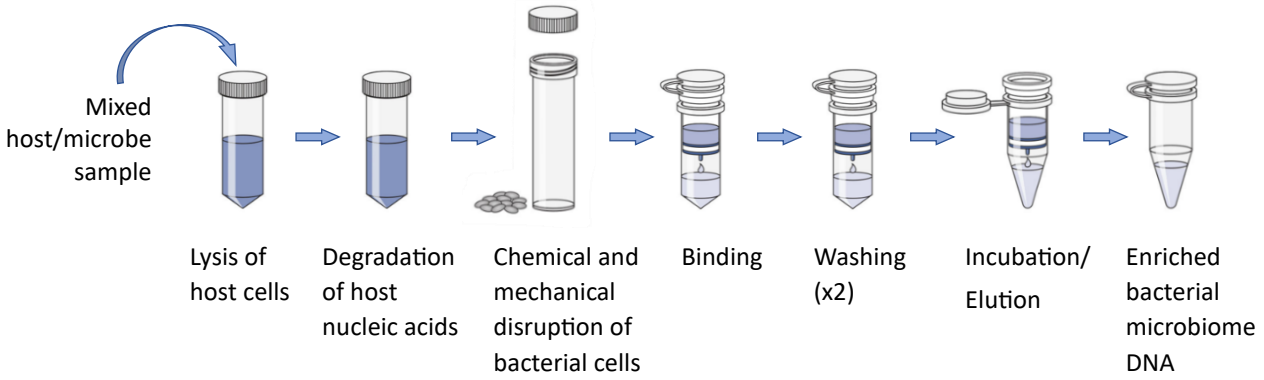


Figure 8. *Summary of DNA extraction with the Qiagen kit (adapted from www.qiagen.com).*

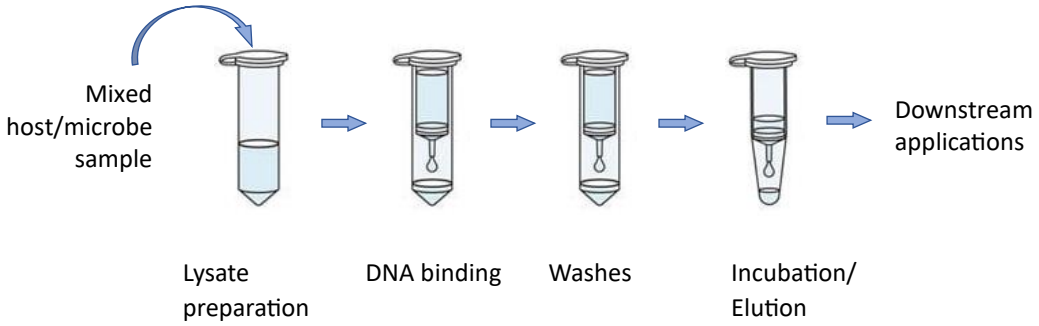


Figure 9. *Summary of DNA extraction with the Invitrogen kit (adapted from www.thermofisher.com).*

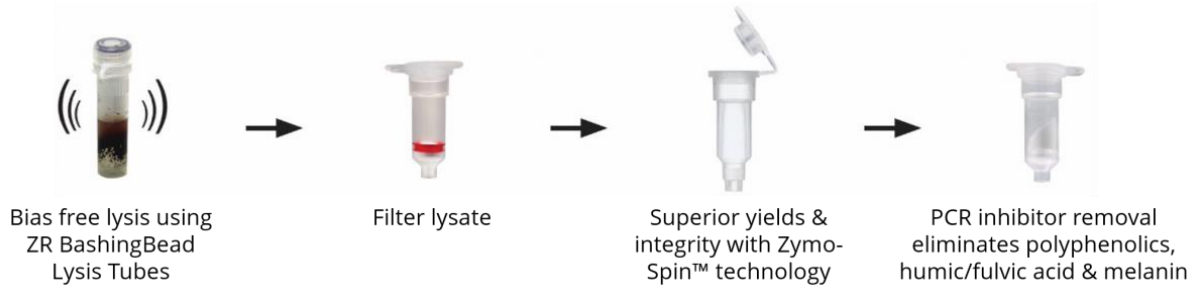


Figure 10. *Summary of DNA extraction with the ZymoResearch kit (https://www.bioscience.co.uk/cpl/zymobiomics-dna-kits).*

All samples were extracted on 500 µL independently of the manufacturers' guidelines, and the protocols were adapted accordingly: the volume of Qiagen buffer was halved in step 1 of the handbook, and the Invitrogen and ZymoResearch procedures were applied on the 500 µL sample volume instead of the swab or solid sample.

DNA quantity was measured for each sample using the Qubit® instrument (see § II.3.1.) and blank samples were tested when establishing the general extraction procedure.

II.2. Library preparation and sequencing

The bacterial 16S rDNA library was prepared in two rounds: the "target" PCR which amplified the V1-V3 hypervariable regions of the 16S rRNA gene, and the "index" PCR to label each sample and allow further identification (Microbiota Solution A, Arrow Diagnostics, Genoa, Italy). The procedure was obtained from www.arrowdiagnostics.it, a negative control was included to each PCR, and the absence of contamination was controlled via electrophoresis gel, along with the correct amplification of the bacterial DNA (see § II.3.2). The only modification brought to the original protocol was the slight increase of PCR cycles: because we expected low biomass samples, it was advised by the manufacturer to push the cycle number from 25 to 28 for the target PCR, and from 8 to 10 for the index PCR. The DNA quantity of each sample was measured by Qubit™ and normalised by dilution, the samples were pooled (3 µL each), the resulting mix was prepared for loading, and gene sequencing was conducted on the MiSeq platform (MiSeq® v2- or Nano v2-500 cycle Reagent Kits) with PhiX as an internal standard, both supplied by Illumina Inc. (San Diego, CA, USA) as reported in the literature (Torre et al. 2022).

II.3. Quality control

Quality control of the samples was conducted through (i) DNA quantification after extraction and during library preparation (Qubit™ Flex Fluorometer, Thermo Fisher Scientific, Waltham, MA, USA) and (ii) electrophoresis in agarose gel after each PCR round for control of the amplicon size and purity.

II.3.1. DNA quantification

The quantification of bacterial DNA was performed on the Qubit™ Flex Fluorometer with the Qubit™ 1X dsDNA HS Assay Kit (Invitrogen). The instrument was set up using two standardisation, 8-well strips containing 190µL of working solution and 10 µL of calibrating solution (Standard 1 or Standard 2, for each strip). Sample quantification was achieved by adding 1 µL of extracted sample to 199 µL of working solution, in Qubit Flex Assay Tube Strips where each well was attributed to a unique sample. The strips were shortly vortexed and spun for homogenisation, incubated in the dark for 3 minutes, inserted in the instrument, and the quantity of DNA (either extracted or amplified) was measured.

II.3.2. Purity and amplicon size

Quality control by electrophoresis was conducted on 1.5% agarose gels (in 50X TAE) supplemented with 2 µL of SYBR™ Safe DNA Gel Stain (Thermo Fisher). A ladder was introduced in the first well (GeneRuler® 100 bp

Plus DNA Ladder, Thermo Fisher), the samples – supplemented with 2X DNA Gel Loading Dye (Thermo Fischer) – loaded in the successive wells (with a blank in the last), and the electrophoresis ran for 15 min at 130 V. The gel was read via the ChemiDoc™ MP Imaging System (Bio-Rad, Hercules CA, USA) and the samples were considered pure if i) the negative control showed only the primers band after the target PCR, and ii) the samples presented only one band (other than the primers') at around 250 bp for both PCR rounds.

III. Microbiome and Statistical Analyses

The MiSeq sequencer generated two files per sample and these raw sequences were first processed using the MicrobAT software (Microbiota Analysis Tool – v. 1.1.0, SmartSeq Srl, Novara, Italy). As reported in the literature (Bona et al. 2021; Torre et al. 2022), this software is based on the RDP database (v.11.4) to assign bacterial taxonomy at each level (Phylum, Class, Order, Family, Genus, and Species)and does not produce OTUs (operational taxonomic units). In fact, the sequences are first filtered for length and quality (data quality evaluation), aligned against the RDP database, and then assigned to a specific species if query coverage ≥ 80% and similarity ≥ 97%.

From MicrobAT, samples lists were generated based on criteria of interest (device used, subject's sex, season…) to obtain three files processable by the Microbiome Analyst software. Features unlikely to be useful when modelling the data were identified and removed through data filtering: features that had low count and variance were pulled during the filtration step, and those with over four counts were filtered based on their median abundance levels across samples (prevalence).

Biodiversity within and between the different groups was assessed using the phyloseq package (J. H. Kim et al. 2021): alphadiversity was characterized via the Shannon (H', total number of taxa or richness) and Simpson indices (distribution of abundance or evenness) (Willis 2019), while the betadiversity allowed compare the microbial community composition of each group through generating a distance (or dissimilarity) matrix. Measurements were performed using Bray–Curtis's dissimilarity, graphical representation of the matrices was obtained using Principal Coordinate Analysis (PCoA), and the statistical significance of the clustering model of the sorting graphs was assessed using permutational analysis of variance (PERMANOVA). Statistical differences in taxa abundance between the groups were assessed by applying the Linear Discriminant Analysis (LDA) Effect Size (LefSe) method (Somboonna et al. 2017).

In Chapter II (§ II.1.1.), principal component analysis was performed using all the phyla and species considered most abundant (relative abundance > 0.2%), and two-way ANOVA was used to discriminate the effects of the "sex" and "age" factors and of their interaction. Both analyses were performed using R (v. 3.5.1) R Core Team (Team 2020), with FactoMineR and Factoextra packages. For each of the statistical analysis cited in this paragraph, differences between the study groups were considered relevant when *p*-values were below the 0.05 threshold.

CHAPTER I – Workflow design and improvement

I. General presentation of base pipeline

Based on the equipment available at UPO and thanks to the support of Prof. Elisa Bona, Prof. Flavio Mignone, and Dr. Marta Mellai, it was decided early on that the Complife microbiome analysis workflow should be based on the amplicon analysis of targeted regions of the 16S rRNA gene. First, the bacterial component represents most of the cutaneous microflora, and 16S analyses allow the accurate resolution of the microbiota to the genus level (Castelino et al. 2017; Zongzhi Liu et al. 2007). In fact, the 16S rDNA approach is considered very reliable to study the bacterial members of the microbiome and is vastly used in the scientific community to study even minor population variations (Byrd, Belkaid, and Segre 2018; Ederveen et al. 2020).

Second, this pipeline will be dedicated mostly to commercial applications, and must remain quick and cost-effective enough to attract clients looking to upgrade the formulas of high-margin cosmetics. But the quality of DNA can derive from any experimental step (material collection, DNA extraction, DNA amplification, sequencing preparation) (Leigh Greathouse, Sinha, and Vogtmann 2019). In order to obtain constant quality results and ensure moderate costs both from the client and the development point of view, we decided to focus on the sampling and DNA extraction steps.

I.1.Game plan

I.1.1. Sample collection

The human microbiota demonstrates a high spatial and temporal heterogeneity even in healthy habitats: material collection is therefore particularly crucial for studying the cutaneous microbiome and should follow a standardised protocol (Iebba et al. 2016; Grice et al. 2008). Different methods are available: swabs, biopsies, surface scrapes, cup scrubs, tape strips… (Figure 7, Grice et al., 2008); but the microbiota composition varies depending on the skin layer, and the sampling technique should be chosen according to the goal of the study (Costello et al. 2009; Findley et al. 2013; Kong et al. 2017).

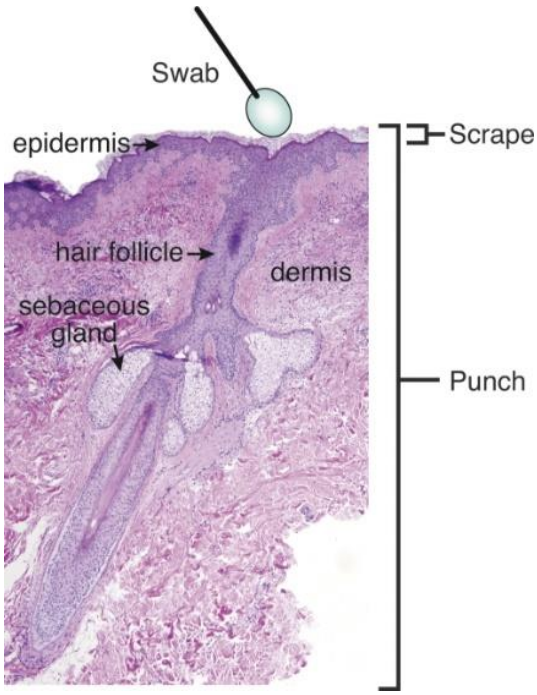


Figure 11. *Skin cross-section, and depth reached by various sampling methods. (Grice et al., 2008)*

The punch biopsy gives the best representation of the microbiota, as it collects bacteria from the outermost to the deepest layers of the skin but is rather invasive, but are not suited to visible areas such as the face or the legs (Kong et al. 2017; Grice et al. 2008). Tape-stripping and scraping methods allow to obtain high biomass quantities by collecting bacteria from both the surface and the stratum corneum, but requires extended areas and collect (like biopsies) high quantities of host DNA, eventually interfering with bacterial DNA isolation (Findley et al. 2013; Grice et al. 2008; Chng et al. 2016). Comparison of the tape-stripping and the sampling techniques demonstrated that both gave similar results, but where tape-stripping collected a greater number and wider variety of viable skin bacteria, swabbing gave more consistent results while being less invasive (Ogai et al. 2018; Bjerre et al. 2019).

Despite its inherent limitations, swabbing is now the most used method internationally because it is fast, non-invasive and very reliable for cutaneous microbiome applications, and even demonstrated that large-scale patterns can be observed and analysed (Ogai et al. 2018; J.-C. Lagier et al. 2015; Prast-Nielsen et al. 2019; Bjerre et al. 2019; Hamady and Knight 2009). The swab approach was the most obvious technique also in our case, since it is non-invasive and most of our studies will determine the effect of facial cosmetics on the skin bacterial population; because it is fast and simple, so the skills involved are easily transferable from one POC to another without losing reproducibility.

In this work, we developed a standardised method for the collection of skin microbiota samples across various cutaneous areas. For this project, we collaborated with Santina Castriciano from Copan and started by establishing a standard collection protocol, to be used routinely by Complife technicians and applicable to any cutaneous area of the body. We pursued our work with Copan and validated the efficacy of two new skin microbiome sampling devices for at-home, self-collection by the volunteer or the patient, hence relieving the POCs during studies that require many collection areas or experimental times.

I.1.2. Bacterial DNA extraction

Skin microbiome studies traditionally yields low biomass, and may be highly contaminated by the host and its environment, especially in exposed areas such as the face and the hands (Noah Fierer et al. 2008). The DNA extracted from each sample therefore must be of the highest quality and purity to be suitable for PCR amplification. Since most of the DNA extraction kit are the property of commercial suppliers, no comparative study between different approaches could be found in the literature, therefore we based our workflow on the ones already in place at the CAAD-UPO and start with the Qiagen QIAamp® DNA Microbiome kit. In fact, it is one of the only kits on the market which protocol presents a host DNA removal step, particularly crucial for skin samples where the bacterial biomass is low and human DNA presence is high. However, other kits from the same supplier or its competitor are reputedly also suitable for skin microbiome analysis, have much simpler protocols, and have lower costs despite not having the host DNA removal step. This market diversity pushed

us to evaluate the efficacy of various kits in obtaining good sequencing results and we tested two from two competitors.

I.1.3. 16S gene sequence amplification

16S analyses are based on taxa identification through sequence identification of DNA fragments, obtained after amplifying the extracted DNA by PCR. The quality of the resulting amplicons is crucial for getting exploitable results and depends particularly on two factors: the choice of primers and the number of cycles. First, and because of differential annealing during PCR cycles, choosing the wrong hypervariable region may induce a selective loss of bacterial diversity through either the over- or underrepresentation of certain members (V. Wintzingerode, Göbel, and Stackebrandt 1997; Kanagawa 2003). Then, the generation of chimeric sequences during PCR amplification hinders bacteria identification from the genomic sequence, leading to radically different biological conclusions (Kumar et al. 2011; Castelino et al. 2017; Chen, Knight, and Gallo 2023; Andersson et al. 2008; Z. Liu et al. 2008; Haas et al. 2011). Both form a phenomenon known as “primer bias”, and limiting as much as possible the number of PCR cycles reduces the overall number of amplification errors, avoiding the reduced biodiversity and the skewing of bacterial profiles (Acinas et al. 2005; M. D. Collins et al. 2002; Kong et al. 2017).

Nevertheless, the successful design of gene-specific primers able to target variable regions (V) and to yield determined amplicon sizes was reported early on (Human Microbiome Project Consortium 2012; Klindworth et al. 2013), and in the last years the V3-V6 regions of the 16S gene are used for gut microbiome studies, while the V1-V3 regions are preferred for skin microbiome applications (Kong et al. 2017; Chen, Knight, and Gallo 2023; Grogan et al. 2019; Meisel et al. 2016; Baldwin et al. 2017). In our case, the amplicons are 250 bp long and logically obtained from the V1-V3 regions (Robert et al. 2022; Hamady and Knight 2009) , and although such sequences are generally too short to achieve accurate species affiliation (Cosseau et al. 2016), accurate profiling down to the genus level can be achieved (Ederveen et al. 2020; Flowers and Grice 2020; Chen, Knight, and Gallo 2023; Robert et al. 2022). Because the downstream analysis is based on a propriety software developed by a SmartSeq/Arrow Diagnostics collaboration (Novara/Genova, Italy; see 1.1.4, §2), we did not have the opportunity to test different suppliers, which helped standardise our pipeline by reducing the number of possible variants.

I.1.4. Sequencing and computational analyses

Thanks to the collaboration with UPO, all sequencing could be performed on the MiSeq Illumina platform, admittedly one of the most cost-effective and accurate platform for in-depth analysis of 16S 250-bp amplicons (Kong et al. 2017; Castelino et al. 2017). For each sample, the instrument generates computer files from which the microbiome data can be extracted and taxonomic classification is subsequently achieved by identifying the

species-specific hypervariable regions (Hugenholtz and Pace 1996), and processing, analysing, and interpreting such data requires specific computational tools that filter, cluster, annotate, and quantify the obtained sequences (Noecker et al., 2017).

The number of available tools can be daunting (Kong et al. 2017; Caporaso et al. 2010; Noecker et al. 2017), but remain based on two general approaches: phylotyping (or similarity to reference sequences) and the OTU approach (similarity to other sequence) (Schloss and Westcott 2011). Both provide an accurate and interpretable taxonomic profile by identifying and estimating the abundance of the taxa present in each sample, and can be combined when, the sequencing capacities largely exceed the computational resources (Noecker et al. 2017; Schloss and Westcott 2011; Goodrich et al. 2014).

In our case, we could use the new software MicrobAT (Microbiota Analysis Tool) v. 1.1.0 provided by the UPO spin-off SmartSeq Srl (Novara, Italy) (Bona et al. 2021; Robert et al. 2022). It allowed the primary analysis of our data by directly matching the raw sequence data with the Ribosomal Database Project (RDP), therefore speeding up the alignment process and reducing the likelihood of reads from the same taxa being describe for different (but closely related) references (Noecker et al. 2017; Robert et al. 2022). Once sequences were grouped into operational taxonomic units (OTUs) with an identity threshold of >97% and subsequently assigned to taxonomy, three files per study could be generated. Those files were uploaded to the Microbiome Analyst open-access online tool, which allowed the secondary, statistical profiling of the bacterial community (and potential influencing factors) down to the genus level (Robert et al. 2022).

I.2. Workflow testing

I.2.1. Pilot study

The first objective of this project was to establish a reliable and reproducible pipeline for the analysis of the cutaneous microbiome. 18 samples were collected with two different devices, from three female subjects (V1, V2, V3) aged respectively 32, 36, and 43 years. Sample collection was performed at three different time points – T0, T1 (after 24h),and T2 (after eight weeks) – and each time on both cheeks, on skin unwashed from 8-12 hours prior to sampling, and with a wet swab to ensure maximal bacterial collection (Schowalter et al. 2010; Kong et al. 2017; Robert et al. 2022). Standardisation of the protocol was achieved thanks to Santina Castriciano and Copan, who provided us with area-defining templates and helped us define stroke number, directionality, and pressure (see Materials and Methods). The samples were sequenced in two different runs (MiSeq® Nano v2) and quality of the results was assessed through run parameters (Table 1) and individual sample resolution (Table 2).

	Cluster Density (600-800 K/mm²)	Clusters passing filtering	Q-score ≥ 30%
run 1	683	78.6%	85.2-83.6 %
run 2	896	82.0%	93.8-81.9 %

Table 1. *Sequencing data of the pilot study runs.*

The density of clusters on the flow cell remained within the desired range (600-800 K/mm2 ± 15%) for both runs, and their quality allowed a satisfying proportion to pass initial filtering. The Q-scores also confirmed the good run quality since the large majority of samples obtained a result ≥ 30%, thus indicating a nucleotide identification accuracy ≥ 99.9%.

	Total reads			Good quality reads			Unclassified		
	MAX	**MIN**	**MEDIAN**	**MAX**	**MIN**	**MEDIAN**	**MAX**	**MIN**	**MEDIAN**
run 1	75346	35902	58602	95.9%	91.8%	93.1%	15.0%	2.7%	5.3%
run 2	92428	59800	74964	95.4%	90.2%	92.8%	14.7%	2.7%	3.3%
ALL (median)	**83887**	**47851**	**66783**	**95.7%**	**91.0%**	**92.9%**	**14.9%**	**2.7%**	**4.3%**

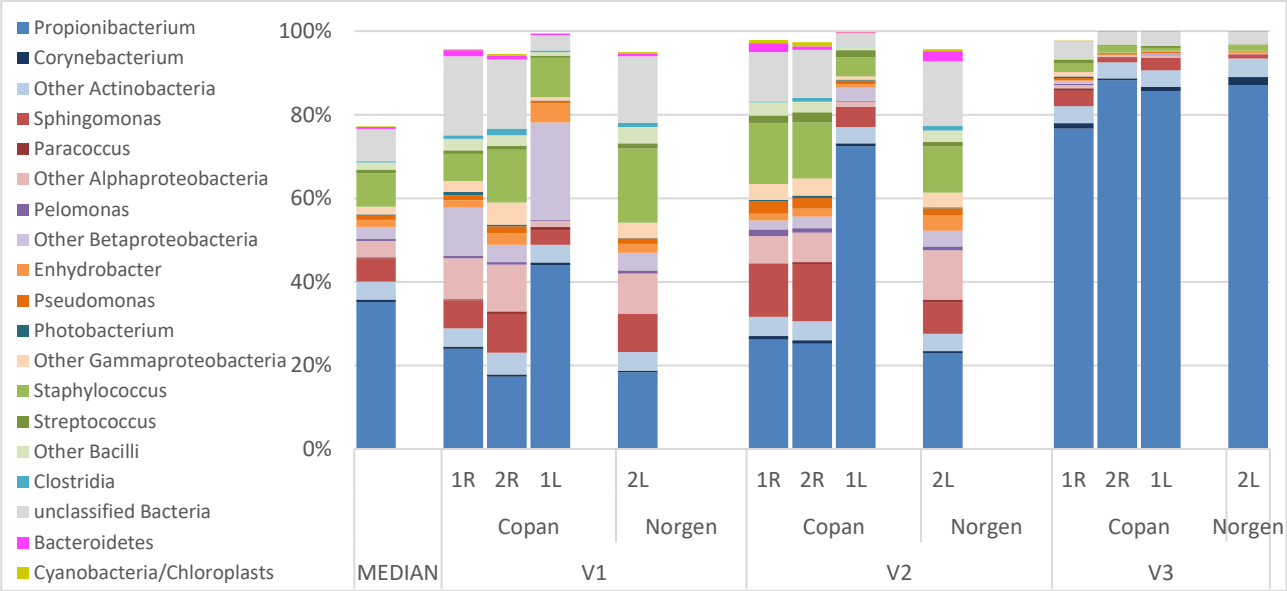
Table 2. *Run data of the pilot study samples.*

Individual sample quality was assessed by performing primary analysis of the samples' files with the MicrobAT software (Table 2), and we fixed a minimum of 20000 total reads to ensure good data analysis. All samples were sequenced with clearly higher numbers, while exhibiting excellent percentages of good quality reads and leaving on average only low proportions of unclassified sequences. The rarefaction curve of each sample clearly and rapidly reached a plateau (data not shown), further confirming the suitability of the raw data for further analysis. The sequencing results were then rearranged according to different variable (sampling device, collection time, sampled area, sequencing run) to allow the demonstration of the complete workflow's reliability, reproducibility, and repeatability.

I.2.1.1. Reliability

We started by demonstrating that the bacterial composition obtained through our protocols was in line with the literature. To this end, the 12 samples collected during the first sampling rounds were sequenced (MiSeq Illumina, run 1), and the resulting individual raw data was grouped then subjected to primary analysis (Graph 1, Table 3, and Annex 1).

Because the samples were collected on different days, with different devices, and on different areas, the results can practically be considered as randomised. Both the individual sample composition and the median microbiome composition are in line with the expected "normal" taxa distribution on the human face, independently of the device (Grice and Segre 2011; Byrd, Belkaid, and Segre 2018). The microbiome composition appears consistent for a given volunteer but some samples seem particularly different, highlighting the reported microbiota's susceptibility to temporal and topographical variability of the cutaneous microbiome (Oh et al. 2016; Byrd, Belkaid, and Segre 2018; Grice et al. 2009; Noah Fierer et al. 2008).



Graph 1. *Cumulated relative abundances of the 10 most abundant genera (according to median calculation) and organised by volunteer (V1-V3), sampling device (Copan or Norgen), collection time (first or second sampling), and side of the face (right or left).*

PHYLUM	CLASS	ORDER	FAMILY	GENUS	MEDIAN
Actinobacteria	Actinobacteria	Actinomycetales	Propionibacteriaceae	Propionibacterium	35.11%
			Corynebacteriaceae	Corynebacterium	0.63%
				Other Actinobacteria	*4.29%*
Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Sphingomonas	5.61%
		Rhodobacterales	Rhodobacteraceae	Paracoccus	0.25%
				Other Alphaproteobacteria	*3.93%*
	Betaproteobacteria	Burkholderiales	Comamonadaceae	Pelomonas	0.42%
				Other Betaproteobacteria	*2.93%*
	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Enhydrobacter	1.68%
			Pseudomonadaceae	Pseudomonas	1.03%
		Vibrionales	Vibrionaceae	Photobacterium	0.26%
				Other Gammaproteobacteria	*1.85%*
Firmicutes	Bacilli	Bacillales	Staphylococcaceae	Staphylococcus	7.96%
		Lactobacillales	Streptococcaceae	Streptococcus	0.89%
				Other Bacilli	*1.69%*
				Clostridia	0.33%
				unclassified Bacteria	*7.73%*
				Bacteroidetes	0.46%
				Cyanobacteria/Chloroplasts	0.19%
				TOTAL	**97.81%**

Table 3. *10 most abundant genera found on the face and their parent taxa, as determined by the pilot study data.*

Despite these differences, Graph 1 suggests that our approach gives overall consistent and reliable results for a given volunteer and independently of the device, making it suitable for the analysis of the cutaneous microbiome. Thanks to the great support from Santina Castriciano during the elaboration of sample collection and preparation protocols, Complife naturally accepted the scientific collaboration offered by Copan to test new devices and templates, and all following sample collection were performed using Copan devices.

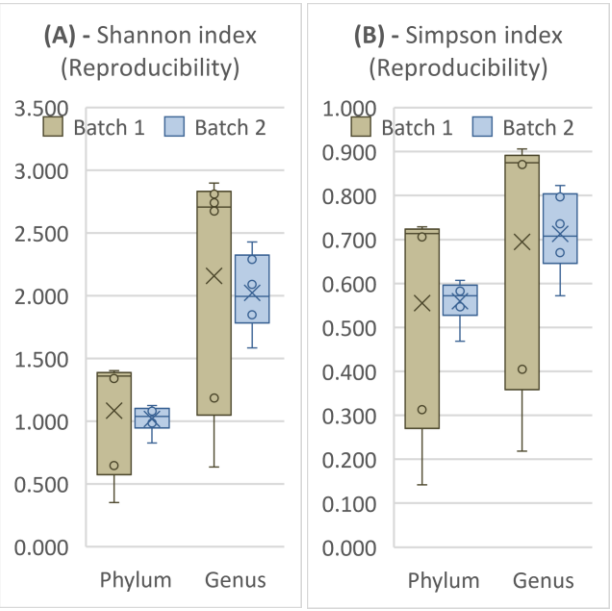
To further confirm the reliability of our pipeline, we decided to perform a second sample collection exactly eight weeks later to assess i) the reproducibility of our pipeline over time by comparing run parameters,

sequencing statistics, and microbiome composition for samples from run 1 and run 2, and ii) the repeatability of the workflow by preparing and sequencing duplicates of these new samples. Finally, the symmetry of the facial microbiota was established using the available suitable samples.

I.2.1.2. Reproducibility

The third sampling round (T2) was conducted eight weeks after the first one in the same conditions, with the same protocols, on the same volunteers, and by the same operator as rounds 1 (T0) and 2 (T1), and six samples were collected (one for each cheek of each volunteer). For the right cheek samples, the total volume of preservation medium recovered after sample preparation was homogenised and separated into two equal parts, adding 3 duplicates of the initial six samples for a total of 9 microbiome extracts. All nine (batch 2) were subjected to the same treatments as the samples collected at T0 and T1 (batch 1). Because of the eventual differences between left and right cheek suggested by Graph 1, this allowed to obtain an even number of samples collected on the right side of the face. In the end, we obtained a dataset with controlled and limited variables, which allowed us to follow-up on the primary analysis and perform a statistical analysis of the results (Tables 4, 5, and Graphs 2A, 2B, 3A, 3B, and 4).

The run parameters (Table 1) The alphadiversity analysis and associated statistics (Graphs 2A, 3A, and Table 4), calculated after the Shannon and Simpson indexes and indicative of taxa richness and evenness (see Materials & Methods), suggest a good similarity between the samples handled in the first batch and in the second batch.



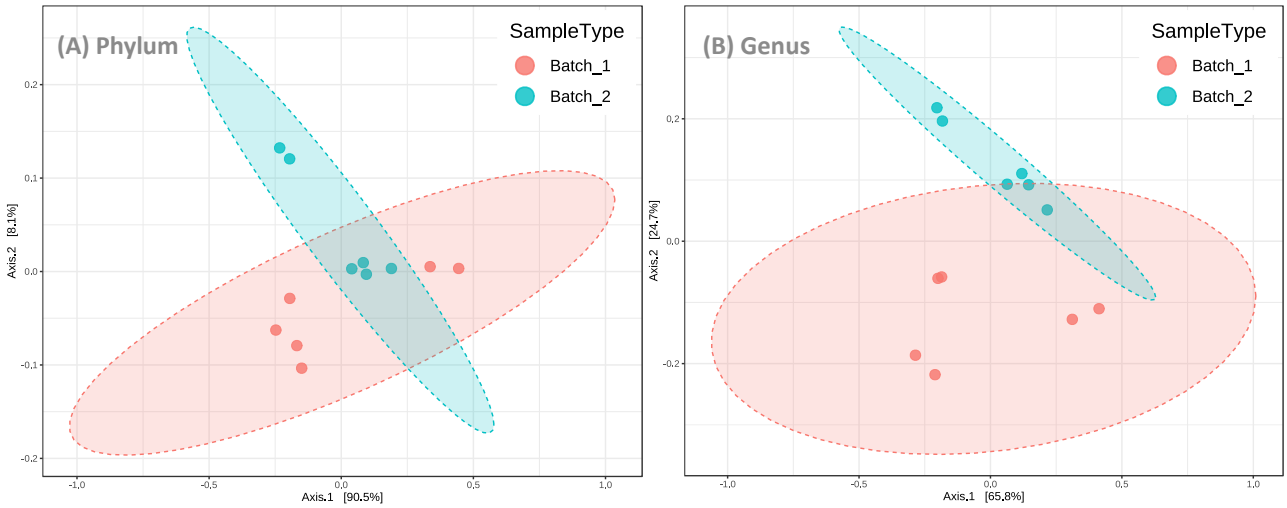
	alpha		beta
	Shannon	Simpson	
PHYLUM	3.94E-01	3.94E-01	0.624
CLASS	3.94E-01	3.94E-01	0.367
ORDER	3.94E-01	3.94E-01	0.263
FAMILY	3.94E-01	3.94E-01	0.156
GENUS	3.94E-01	3.94E-01	0.085

Table 4. *Statistical diversity values, at the sample level (alphadiversity) and the group level (betadiversity).*

Graph 2. *Visual representation of the distribution across samples of taxa richness (A) and evenness (B) at the phylum and genus levels.*

The *p*-values are all vell over the significance threshold of 0.05 (Table 4), suggesting that there are no differences from one batch to the other. Furthermore, the values are all the same independently of the taxonomic level or the calculation. Graphs 2A and 2B confirm this first assumption since the average

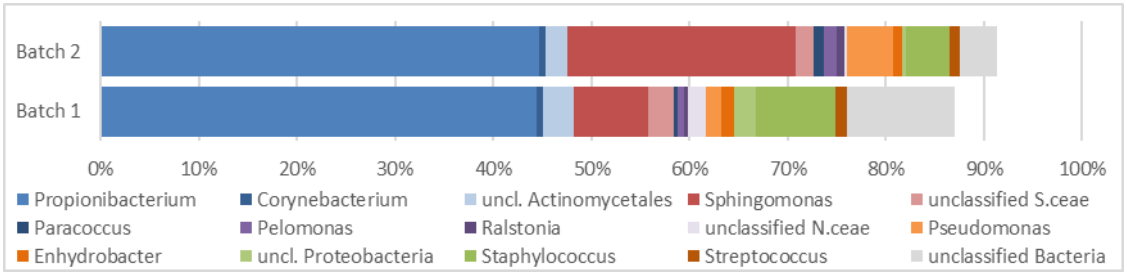
composition (x) is practically the same despite different distributions and medians (–). In fact, the samples from batch 2 seem more even to one another than for batch 1, indicating a probable good process repeatability.



Graph 3. *Visual representation of the distribution across samples of community composition at the phylum (A) and the genus levels (B).*

The betadiversity analysis (Graphs 3A, 3B, and Table 4) also yielded quite high *p*-values, and the graphical representation confirmed the overall reduced dispersion and distribution of samples from batch 2. However, the *p*-value at the genus level can be considered borderline relevant and the microbiome composition should be analysed further.

The analysis of the relative abundances of the identified taxa and the statistical analysis of eventual populations differences between batch 1 and batch 2 is summarised in Table 5 and Graph 4. The only statistically relevant results (LEfSe *p*-value, Table 5) regarded bacteria traditionally associated with environmental contaminations (*Sphingomonas* and *Pseudomonas*) while the proportions of the main and traditional members remained overall stable (*Propionibacterium*, *Corynebacterium*, *Staphylococcus*).



Graph 4. *Graphic representation of the 10 most abundant genera, and of the 10 most abundant unclassified parent taxa.*

On one hand these variations are indicative of environmental contamination during sample handling and contamination, but on the other hand the lower unclassified bacteria and diversities distribution in run 2 suggest a suggest an improvement of the experimental manipulations. In short, the statistical analyses of the sequencing results confirmed that our pipeline was reproducible, since the observed fluctuations might also

be attributed to the weather difference between the two experimental time (T0 vs. T2, February vs. May and the resulting increased contacts with the exterior).

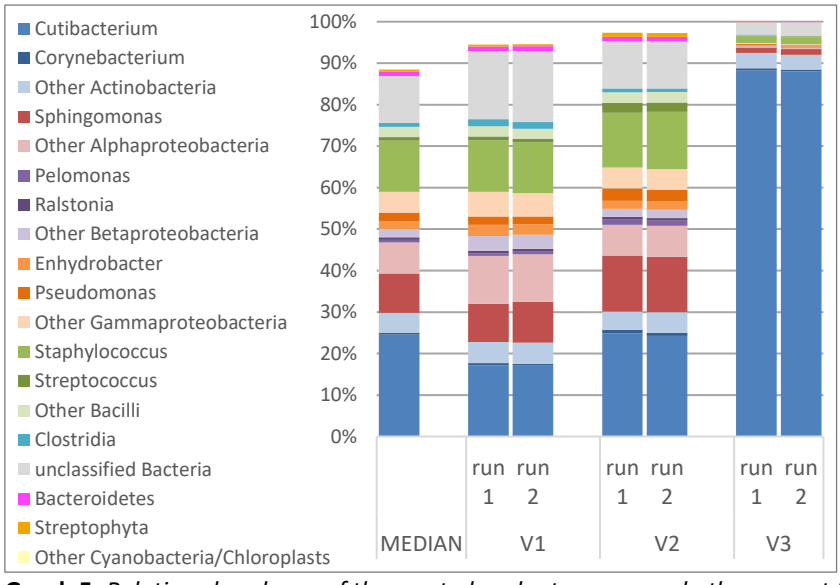
PHYLUM	CLASS	ORDER	FAMILY	GENUS	Batch 1	Batch 2	LEfSe *p*-value
Actino-bacteria	Actinobacteria	Actinomycetales	Propionibacteriaceae	Propionibacterium	44.34%	44.69%	1.00E+00
			Corynebacteriaceae	Corynebacterium	0.67%	0.58%	6.31E-01
				uncl. Actinomycetales	*3.20%*	*2.19%*	***3.95E-03***
Proteo-bacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Sphingomonas	7.52%	23.39%	**3.95E-03**
				unclassified S.ceae	*2.69%*	*1.81%*	*4.23E-01*
		Rhodobacterales	Rhodobacteraceae	Paracoccus	0.33%	0.99%	2.00E-01
	Betaproteobacteria	Burkholderiales	Comamonadaceae	Pelomonas	0.68%	1.30%	1.09E-01
			Burkholderiaceae	Ralstonia	0.33%	0.78%	7.82E-02
		Neisseriales	Neisseriaceae	*unclassified N.ceae*	*1.89%*	*0.29%*	*1.50E-01*
	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	1.51%	4.70%	**6.49E-03**
			Moraxellaceae	Enhydrobacter	1.38%	0.99%	5.22E-01
				uncl. Proteobacteria	*2.25%*	*0.29%*	*5.47E-02*
Firmicutes	Bacilli	Bacillales	Staphylococcaceae	Staphylococcus	8.07%	4.43%	2.00E-01
		Lactobacillales	Streptococcaceae	Streptococcus	1.12%	1.17%	8.73E-01
				unclassified Bacteria	*10.98%*	*3.74%*	*1.09E-01*
				TOTAL	**86.97%**	**91.33%**	

Table 5. *Relative abundances of the 10 most abundant genera and the 5 most abundant unclassified taxa across all samples, and statistical analysis of the differences between the two batches.*

I.2.1.3. Repeatability

Inter-run

The stability of the purified amplicons and the repeatability between runs was evaluated by re-loading the three samples obtained at T1 with the Copan device in the second sequencing run. The relative abundances of the 10 most abundant genera (calculated from the median abundances across samples), their relative parent class, and phyla – other than *Actino-*, *Proteobacteria*, and *Firmicutes* – are reported in Graph 5 and Annex 2.

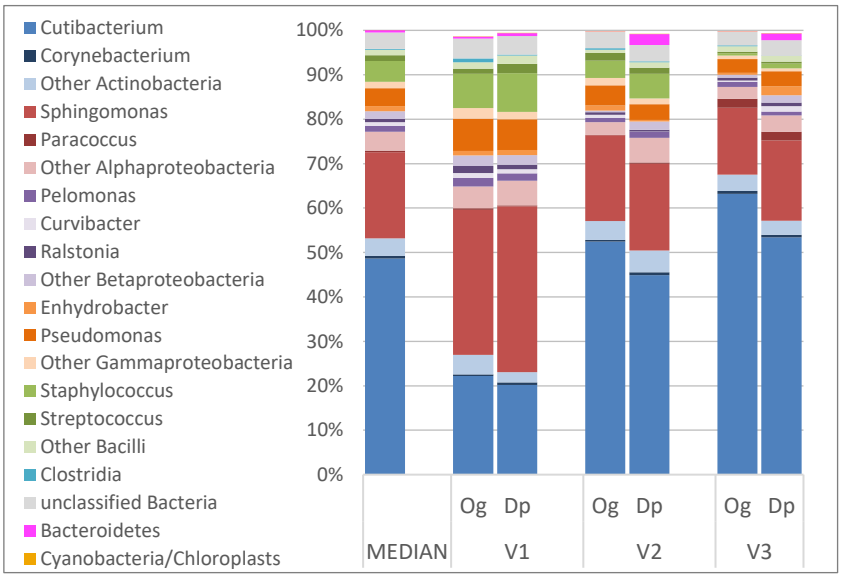


Graph 5. *Relative abundance of the most abundant genera and other parent taxa.*

Despite not being able to perform the statistical analysis of these results – as no definite conclusion could be drawn from only six samples – the graphical summary of the primary analysis shows a blatant similarity between the two runs and despite the relatively long storage time in-between. Interestingly, the major genera representation is consistent with our previous analysis, while the minor genera show various proportions and even different taxonomy depending on the analytical approach.

Intra-run

As mentioned earlier, the three samples collected from our volunteer's right cheek were duplicated and treated as "new" samples. from bacterial DNA extraction to computational analysis of the results, allowing to determine the repeatability of the complete pipeline within the same batch (Graph 6 and Annex 3).

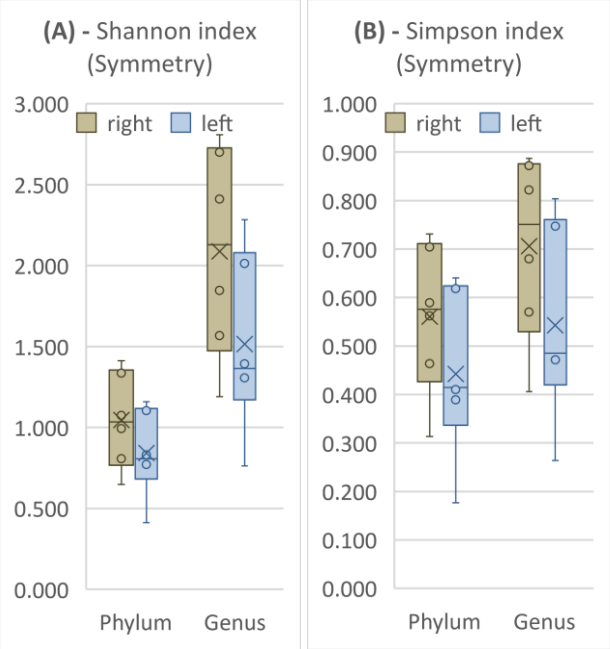


Graph 6. *Relative abundances of the 10 most abundant genera and other parent taxa.*

Similar to the inter-run results, no robust secondary analysis could be performed, but the observation of the relative abundances of the 10 most abundant genera and their parent taxa revealed very good correspondence between the original samples (Og) and their duplicates (Dp). In fact, this further confirms the quality of our whole workflow, and that samples treated and/or sequenced at different time points can easily be compared to one another.

I.2.2. Symmetry

Since we could demonstrate that samples treated at different time points and sequenced in different runs can be compared together, we decided to use the six samples collected at T0 and the six samples collected at T2 and assemble them into Right *vs.* Left groups to verify the symmetry between the two sides of our body (Graphs 7A, 7B, 8, 9A, 9B, and Tables 6, 7).

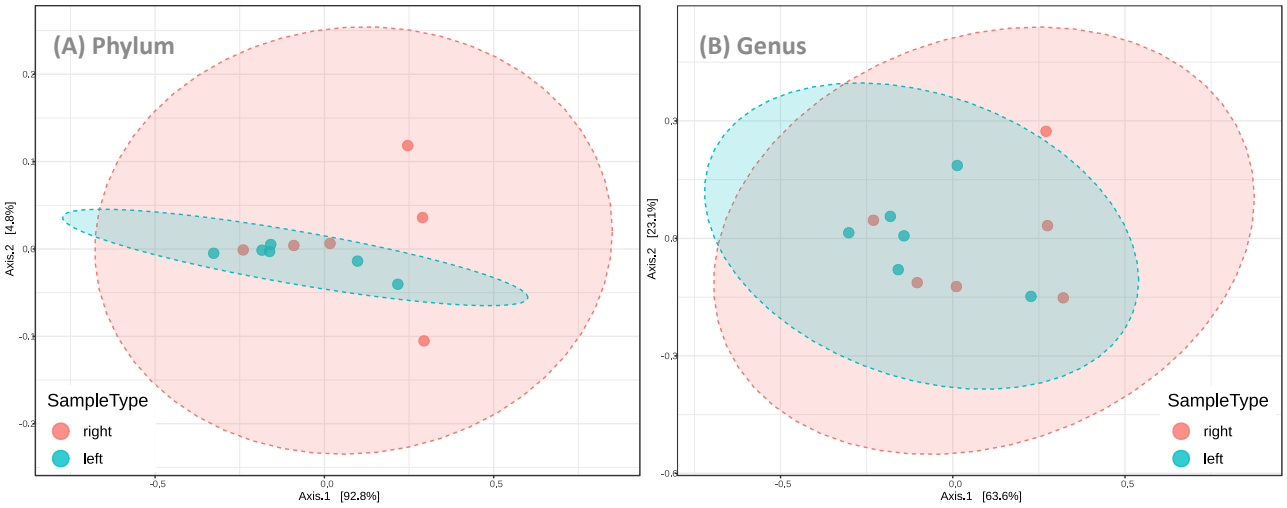


	alpha		beta
	Shannon	Simpson	
PHYLUM	3.94E-01	3.10E-01	0.215
CLASS	1.80E-01	1.80E-01	0.174
ORDER	1.80E-01	1.80E-01	0.212
FAMILY	1.80E-01	1.80E-01	0.190
GENUS	1.80E-01	1.80E-01	0.213

Table 6. *Statistical diversity values, at the sample level (alphadiversity) and the group level (betadiversity).*

Graph 7. *Visual representation of the distribution across samples of taxa richness (7) and evenness (7) at the phylum and genus levels, based on the side sampled.*

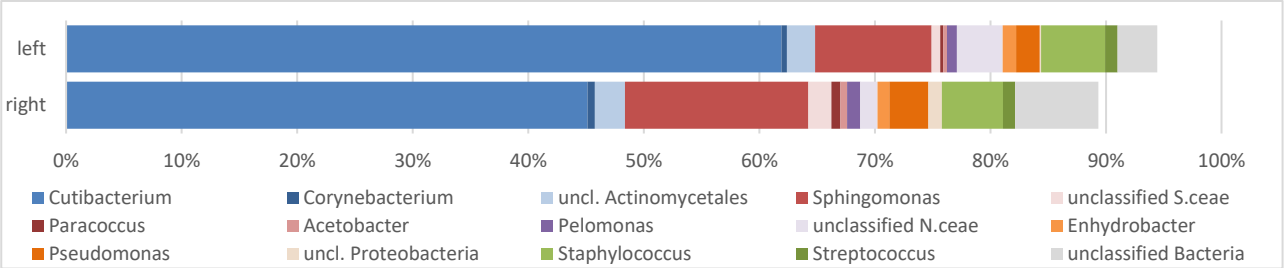
The statistical values in Table 6 suggest that no relevant difference is present between the two groups since they are well over the 0.05 threshold for all taxonomic levels, whether from the sample angle (alpha-diversities, similar distributions and dispersions for both categories in Graphs 7A and 7B) or the community angle (betadiversity, ellipses superimposition in Graphs 8A and 8B). In fact, the visual representation of the taxa richness, evenness, and repartition indicate a good similarity between the left and right sides of the face, in accordance with the literature (Noah Fierer et al. 2008).



Graph 8. *Visual representation of the distribution across samples of community composition at the phylum (A) and the genus levels (B), based on the side sampled.*

Some relative abundance differences are visible on Graph 9 for *Cutibacterium* and *Sphingomonas*, but the statistical analysis of the differences between the left and the right only revealed relevant fluctuations for

unclassified *Proteobacteria* and *Sphigomonadaceae* (Table 7). The analysis of the relative abundances further evidenced the symmetry betwwen the left and right cheek, and we could even demonstrate the same similarities between the elbows (whether sampled on the inside or the outside), the armpits, the thighs, and the groin (data not shown).



Graph 9. *Relative abundances of the 10 most abundant genera and their parent taxa of interest, depending on each side of the face.*

PHYLUM	CLASS	ORDER	FAMILY	GENUS	right	left	LEfSe *p*-value
Actinobacteria	Actinobacteria	Actinomycetales	Propionibacteriaceae	Cutibacterium	45.14%	61.92%	1.50E-01
			Corynebacteriaceae	Corynebacterium	0.64%	0.46%	1.50E-01
				uncl. Actinomycetales	*2.60%*	*2.45%*	*5.22E-01*
Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Sphingomonas	15.88%	10.06%	2.00E-01
				unclassified S.ceae	*1.97%*	*0.77%*	*5.47E-02*
		Rhodobacterales	Rhodobacteraceae	Paracoccus	0.79%	0.25%	1.50E-01
		Rhodospirillales	Acetobacteraceae	Acetobacter	0.57%	0.32%	6.31E-01
	Betaproteobacteria	Burkholderiales	Comamonadaceae	Pelomonas	1.13%	0.88%	2.00E-01
		Neisseriales	Neisseriaceae	*unclassified N.ceae*	*1.50%*	*3.94%*	*6.31E-01*
	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Enhydrobacter	1.02%	1.18%	4.23E-01
			Pseudomonadaceae	Pseudomonas	3.40%	2.04%	3.37E-01
				uncl. Proteobacteria	*1.14%*	*0.12%*	***2.50E-02***
Firmicutes	Bacilli	Bacillales	Staphylococcaceae	Staphylococcus	5.29%	5.54%	8.73E-01
		Lactobacillales	Streptococcaceae	Streptococcus	1.08%	1.07%	8.73E-01
				unclassified Bacteria	*7.18%*	*3.45%*	*1.09E-01*
				TOTAL	**89.33%**	**94.44%**	

Table 7 - *Relative abundances and LEfSe* p*-values of the 10 most abundant genera and their parent taxa of interest*

In fine, this pilot study demonstrates the robustness of our pipeline depending on several variables and the suitability of our computational approach to analyse the cutaneous microbiome's composition. The overall composition of our samples was in line with the literature, and that only a few members compose the vast majority of the microbiota (Grice and Segre 2011; Byrd, Belkaid, and Segre 2018; Chen, Knight, and Gallo 2023). We chose to focus our result analysis on the 10 most abundant genera (and their 5 most abundant parents): in fact, these 15 "major" taxa almost always composed over 80% of the bacterial population and were more or less consistently present across the various analyses (except for a few low-abundance members).

I.2.3. Preliminary study

We pursued the evaluation of our pipeline by conducting a preliminary study on 60 samples, collected on the right cheek and forehead of 15 men and 15 women, on volunteers usually participating to Complife studies and samples in our regular POC. The objectives of this preliminary study were to i) establish the "basal"

microbiome of our regular cohort, ii) evaluate the extent of the microbiota difference between two geographically close areas, and iii) determine if our workflow is easily adaptable to bigger numbers of sample.

For this study, we had the opportunity to test new area-defining templates from Copan (Figure 8). Initially, the 20 cm^2 collection area was delimited by a hollowed, 2mm-thick, plastic device: if it allowed the consistent collection of microbiota, its use was not so straightforward. The new template isolates the same surface but is made of adhesive paper: its fixed position allows to maintain the skin immobile during sampling, and that and the reduced thickness facilitated the stroking movement. Thanks to this evaluation, the area-defining, adhesive template for skin microbiome applications has recently been added to the Copan catalogue.

The samples were sequenced in two different runs: 12 in run 1 with the MiSeq® v2 Nano reagent kit as previously used, and 48 in run 2 with the regular MiSeq® v2 cartridge (which can hold up to 96 samples). The run parameters (Table 8) and sequencing statistics (Table 9) demonstrated that both runs were successful and that all the samples were correctly analysed.

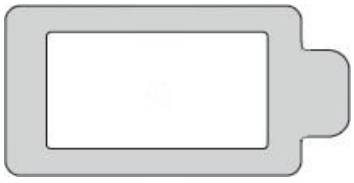


Figure 12. *Hollowed, adhesive paper template (courtesy of Copan).*

	Cluster Density: 600-800 K/mm2	Clusters passing filtering	Q-score >=30%
run 1	884	80.0%	92.1%
run 2	910	78.9%	77.6%

Table 8. *Sequencing data of the preliminary study runs.*

	Total reads			Good quality reads			Unclassified		
	MAX	**MIN**	**MEDIAN**	**MAX**	**MIN**	**MEDIAN**	**MAX**	**MIN**	**MEDIAN**
run 1	92675	59251	73731	93.3%	86.3%	90.4%	4.3%	2.9%	3.3%
run 2	670742	107554	233131	93.5%	87.5%	91.4%	6.7%	2.8%	4.0%
ALL (median)	**381709**	**83403**	**153431**	**93.4%**	**86.9%**	**90.9%**	**5.5%**	**2.9%**	**3.7%**

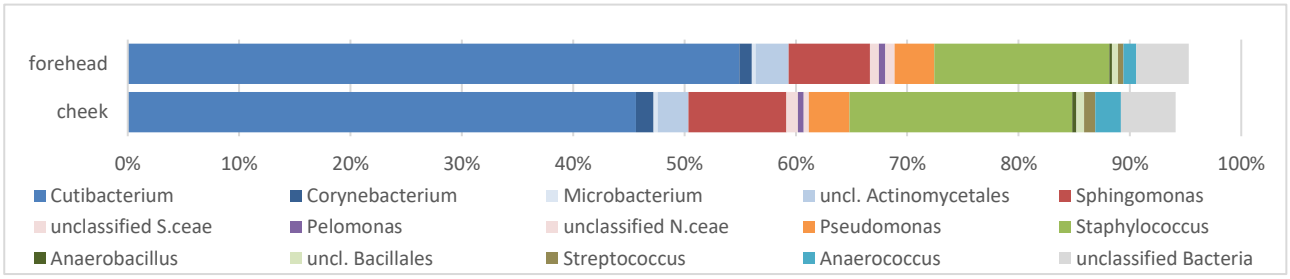
Table 9. *Run data of the preliminary study samples.*

The cluster densities (Table 8) are slightly high, but the rest of the data demonstrates that the results are still qualitative and may be used for microbiome analysis. The number of reads is remarkably higher in the second run (Table 9), which is a consequence of loading "only" 48 samples on a cartridge that can handle up to 96 16S samples. In fact, in the absence of other samples, more clusters of each sample could form on the flow cell, increasing the overall read numbers for a given sample. Still in Table 9, we can observe that the other values are very close together, indicating that the read number is not the sole parameter to consider when evaluating the sequencing results' suitability.

I.2.3.1. Geographical variations

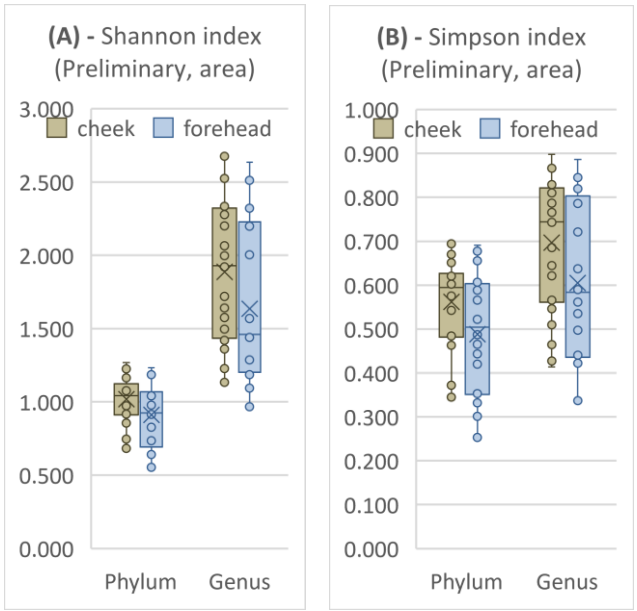
Skin thickness, folds, and density of hair follicles and glands determine distinct habitats on the body, leading to different microbial populations depending on the physiology of the skin site (Grice and Segre 2011; Tagami

2008). Because the cheek and the forehead present such anatomical differences, we wanted to evaluate eventual differences in community structure and composition between the two areas. The median microbiome composition each zone was established (Graph 10 and Table 10), and the data subjected to statistical analyses (Graphs 11A, 11B, 12A, 12B, and Tables 10 and 11). Since our previous reasoning allowed to give a good overview of the microbiome composition, we decided to apply the same approach to all further result interpretation: focus on the 10 most abundant genera (and 5 parent taxa), estimation of taxa richness and evenness through the Shannon and Simpson alphadiversity indexes, comparison of the community structure thanks to the betadiversity PERMANOVA analysis, and LEfSe analysis of the highlighted genera to pinpoint the cause of demonstrated fluctuations.



Graph 10. *Relative abundances of the 10 most abundant genera and their parent taxa of interest, depending on the sampled area.*

Again, we can notice that the type and proportions of the major genera remain comparable to what was observed in the pilot study, while the minor genera are less stable in percentages and type. Visually, it appears that only Cutibacterium is different between the two zones, as expected since it thrives in oily, anaerobic environments and the forehead traditionally produces more sebum (Grice et al. 2009). In fact, the LEfSe analysis gives a borderline p-values for Cutibacterium, while almost all other taxa exhibit similar proportions. The only statistically different value concerns the Anaerobacilli spp., more prevalent on the cheek. Such Bacillaceae are quite hydrophilic, and the reduced sebum proportion on the cheek indeed allows the water to be more available as a nutrient.



	alpha		beta
	Shannon	Simpson	
PHYLUM	3.71E-02	3.71E-02	0.092
CLASS	4.62E-02	6.76E-02	0.088
ORDER	5.14E-02	5.14E-02	0.104
FAMILY	5.14E-02	5.32E-02	0.171
GENUS	5.71E-02	5.14E-02	0.161

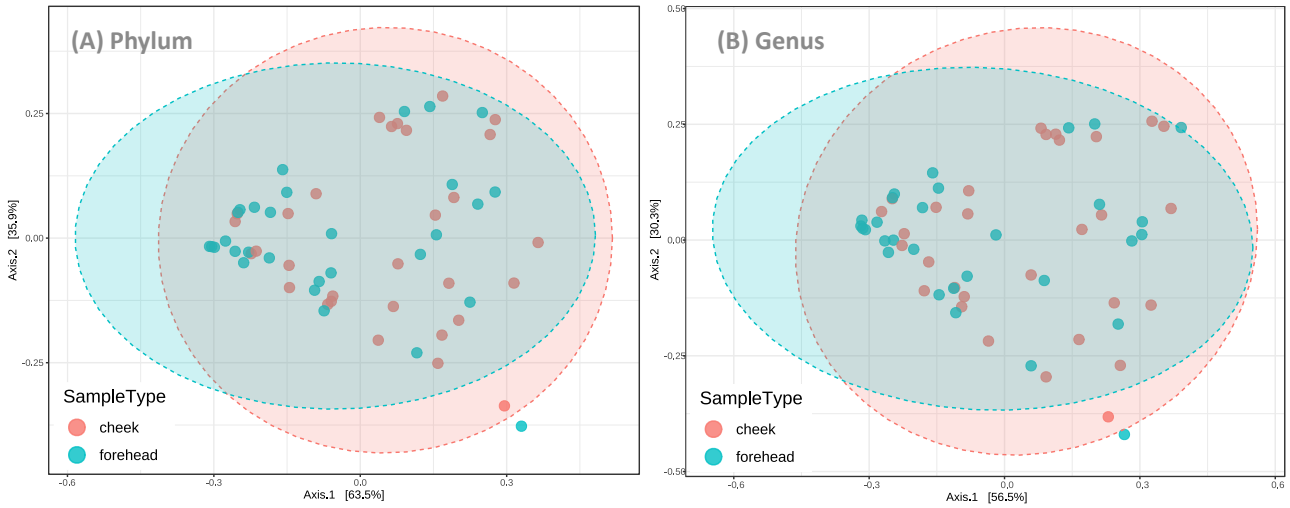
Table 11. *Statistical diversity values, at the sample level (alphadiversity) and the group level (betadiversity).*

Graph 11. *Visual representation of the distribution across samples of taxa richness (B) and evenness (B) at the phylum and genus levels, depending on the sampled area.*

The box and whiskers graphs of the alphadiversity (11A and 11B) indicates that the overall taxa richness and evenness are similar in both groups, but the *p*-values in Table 11 are either significative or borderline for all taxonomic levels. However, these should be attributed to the individual genera fluctuations (Table 10, LEfSe analysis) rather than to fundamentally different microbiota composition since the betadiversity analysis (Graphs 12A, 12B, and Table 11) did not detect structural differences between the two groups.

PHYLUM	CLASS	ORDER	FAMILY	GENUS	cheek	forehead	LEfSe *p*-value
Actinobacteria	Actinobacteria	Actinomycetales	Propionibacteriaceae	Cutibacterium	45.64%	54.92%	5.65E-02
			Corynebacteriaceae	Corynebacterium	1.59%	1.13%	1.32E-01
			Microbacteriaceae	Microbacterium	0.36%	0.32%	5.44E-01
				uncl. Actinomycetales	*2.75%*	*2.98%*	*1.98E-01*
Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Sphingomonas	8.80%	7.30%	1.56E-01
				unclassified S.ceae	*1.02%*	*0.80%*	*2.31E-01*
	Betaproteobacteria	Burkholderiales	Comamonadaceae	Pelomonas	0.52%	0.59%	6.05E-01
		Neisseriales	Neisseriaceae	*unclassified N.ceae*	*0.46%*	*0.81%*	*9.65E-01*
	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	3.66%	3.57%	4.08E-01
Firmicutes	Bacilli	Bacillales	Staphylococcaceae	Staphylococcus	19.99%	15.72%	3.75E-01
			Bacillaceae 1	Anaerobacillus	0.39%	0.23%	**3.99E-02**
				uncl. Bacillales	*0.68%*	*0.56%*	***3.99E-02***
		Lactobacillales	Streptococcaceae	Streptococcus	1.03%	0.48%	1.69E-01
	Clostridia	Clostridiales	C. Incertae Sedis XI	Anaerococcus	2.29%	1.15%	2.94E-01
				unclassified Bacteria	*4.92%*	*4.71%*	*3.52E-01*
				TOTAL	**94.11%**	**95.28%**	

Table 10 - *Relative abundances and LEfSe analysis results of the 10 most abundant genera and their parent taxa of interest.*

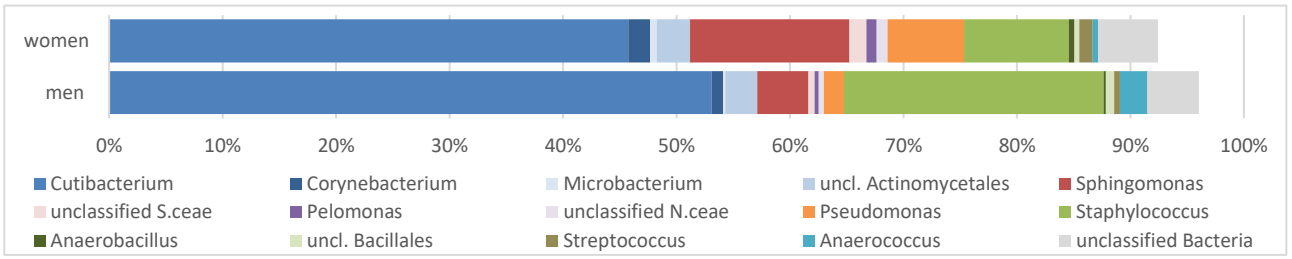


Graph 12. *Visual representation of the distribution across samples of community composition at the phylum (A) and the genus levels (B), depending on the sampled area.*

In conclusion, we could determine that even areas similarly exposed to the external environment and geographically close can still display statistically significant differences. Because most of the client-ordered works will focus on the impact of facial products on the skin microbiome, and because the high levels of sebum in the forehead might skew the community composition, we decided to perform studies for microbiome-friendly claims exclusively on the cheek.

I.2.3.2. Physiological variations

Despite being from the same species, men and women present certain anatomical and physiological differences, and this is particularly true for the face. In fact, the male facial skin is characterised by higher densities of sweat glands and pilosebaceous units, leading to higher pilosity, sweat levels, and sebum production, each potentially influencing the microbiota's composition. Therefore, we rearranged the 60 samples of the preliminary study based on the volunteers' sex and conducted a primary and secondary analyses of the bacterial component of the microbiome (Graphs 13, 14A, 14B, 15A, 15B, and Tables 12 and 13).



Graph 13. *Relative abundances of the 10 most abundant genera and their parent taxa of interest, depending on the volonteers' sex.*

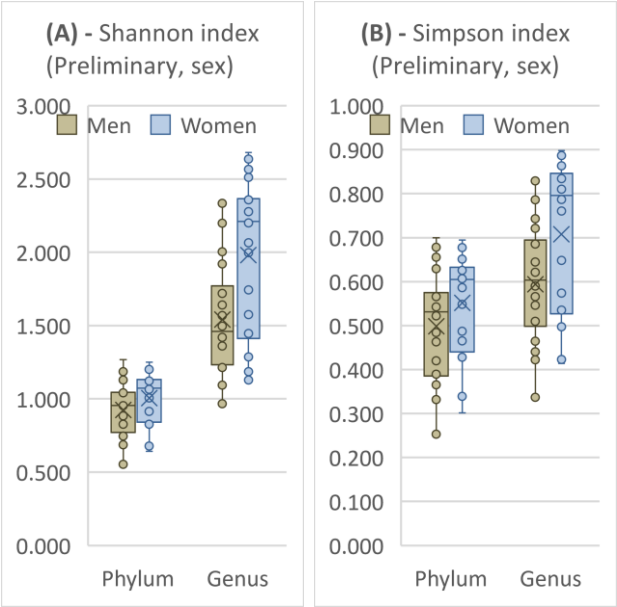
Cutibacterium percentages are higher in men – as expected because of the supposedly higher sebum content on the skin surface – but we saw earlier that such visual fluctuations do not necessarily translate into statistical relevancy. In fact, the LEfSe analysis of the highlighted genera (Table 12) determined that most of the *Actinobacteria* population was statistically similar between men and women while all *Proteobacteria* were higher in women, and that Firmicutes members showed contrasted affinities (sometimes even within the same order, as for *Actinobacteria*).

PHYLUM	CLASS	ORDER	FAMILY	GENUS	Men	Women	LEfSe *p*-value
Actinobacteria	Actinobacteria	Actinomycetales	Propionibacteriaceae	Cutibacterium	53.07%	45.74%	7.60E-02
			Corynebacteriaceae	Corynebacterium	1.03%	1.91%	2.87E-01
			Microbacteriaceae	Microbacterium	0.19%	0.60%	**1.21E-04**
				uncl. Actinomycetales	*2.82%*	*2.95%*	*1.00E+00*
Proteo-bacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Sphingomonas	4.50%	14.04%	**9.18E-07**
				unclassified S.ceae	*0.56%*	*1.49%*	***4.99E-07***
	Betaproteobacteria	Burkholderiales	Comamonadaceae	Pelomonas	0.35%	0.90%	**1.81E-05**
		Neisseriales	Neisseriaceae	*unclassified N.ceae*	*0.45%*	*0.96%*	*2.49E-01*
	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	1.78%	6.73%	**1.53E-07**
Firmicutes	Bacilli	Bacillales	Staphylococcaceae	Staphylococcus	22.89%	9.23%	**8.40E-05**
			Bacillaceae 1	Anaerobacillus	0.19%	0.51%	**1.81E-05**
				uncl. Bacillales	*0.73%*	*0.44%*	***1.60E-02***
		Lactobacillales	Streptococcaceae	Streptococcus	0.51%	1.17%	1.04E-01
	Clostridia	Clostridiales	C. Incertae Sedis XI	Anaerococcus	2.42%	0.50%	**3.67E-04**
				unclassified Bacteria	*4.54%*	*5.27%*	*8.37E-02*
				TOTAL	**96.04%**	**92.44%**	

Table 12 - *Relative abundances and LEfSe analysis results of the 10 most abundant genera and their parent taxa of interest*

The analyses of alpha- (Graphs 14A, 14B, and Table 13) and betadiversity (Graphs 15A, 15B, and Table 13) confirmed that the differences in genera populations was representative of bigger patterns. In fact, the calculated *p*-values are below the 0.05 significance threshold for (almost) all taxonomic levels, indicating that

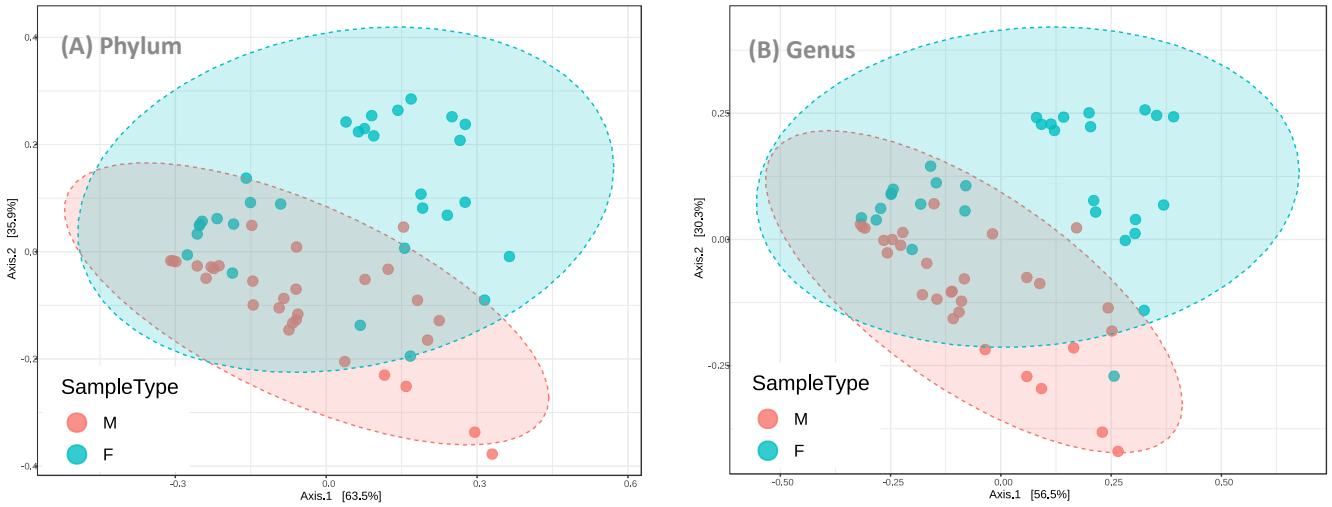
the male and female microbiota differ not only in relative taxa abundances, but also in overall taxa richness, taxa evenness, and community structure.



	alpha		beta
	Shannon	Simpson	
PHYLUM	7.47E-02	6.76E-02	0.001
CLASS	8.77E-03	8.38E-03	0.001
ORDER	3.19E-03	7.65E-03	0.001
FAMILY	3.36E-03	8.01E-03	0.001
GENUS	1.52E-03	6.34E-03	0.001

Table 13. *Statistical diversity values, at the sample level (alphadiversity) and the group level (betadiversity)*

Graph 14. *Visual representation of the distribution across samples of taxa richness (A) and evenness (B) at the phylum and genus levels, depending on the volunteers' sex.*



Graph 15. *Visual representation of the distribution across samples of community composition at the phylum (A) and the genus levels (B), depending on the sampled area.*

With this preliminary study, we demonstrated the applicability of our workflow to larger quantities of samples and hypothesised important differences between the male and female microbiotas. In fact, the latter might explain why no statistical demarcation between the cheek and the forehead could be determined. From a fundamental research point of view, this realisation pushed us to focus on how intrinsic and extrinsic factors might influence the cutaneous microbiome of various body areas (see Chapter III). In parallel (and because the vast majority of our samples come from client-ordered studies), we evaluated several variants to our pipeline in an effort to gain a maximum of effectiveness in conducting large studies while maintaining the results' quality.

As mentioned above, we first tested and validated two new sampling devices from Copan (the Smart eNAt® and the Active Drying System® or ADS®). We then evaluated the efficacy of a novel Copan proprietary device to increase pots-sampling microbial DNA recovery: the NAO® Basket. Finally, we compared the quality of results between DNA extracted with the Qiagen kit and two other commercial kits (from ZymoResearch and Invitrogen)

II. Sample collection and preparation

II.1. Sampling devices

II.1.1. Device presentation

One of the main challenges in personal care today is the regular and reliable specimen collection, especially when dealing with multiple experimental times and/or sampling points. Often, this obliges the patient or volunteer to drop at dedicated points of collection (POCs) at specific times, requiring complicated planning from both the technicians and the subjects. One way to overcome this issue is to develop alternative, safer devices that allow for self-collection. To this end, Copan designed two new devices based on their proven kit, the FLOQswab® + eNAt® (Figure 9), composed of a swab and a sterile tube containing a guanidinium thiocyanate-based preservation medium. Contrarily to many competitors that use woven or twirled cotton or polymers, the Copan swabs' heads are assembled by flocking short nylon strands perpendicular to the head's template using static electricity. Because of the 90° angle and the strands are independent from one another, the release of the specimen from the device's head into the preservation medium is facilitated.

Figure 13. *FLOQswab® and sterile tube containing either 2 or 1 mL of preservation medium (courtesy of Copan).*

Due to the presence of guanidinium thiocyanate, the contact with the conservation medium can be irritating or harmful and using a "simple" tube already filled increases the risk of spilling. Kits such as the FLOQswab® + eNAt® therefore allow sample collection only by a gloved technician in a controlled environment (POC). On the contrary, the new devices completely avoid any contact between the operator (trained technician or patient/volunteer) and the preservation medium: the Smart eNAt® (Figure 10) because the preservation medium is stored in the cap and released only after closing the specimen tube (Figure 11), and the ADS® (Figure 12) because sample preservation is ensured by a silica-based material stored in the cap.

Figure 13. *FLOQswab® ans Smart eNAt® tube, containing 1 mL of preservation medium (courtesy of Copan).*

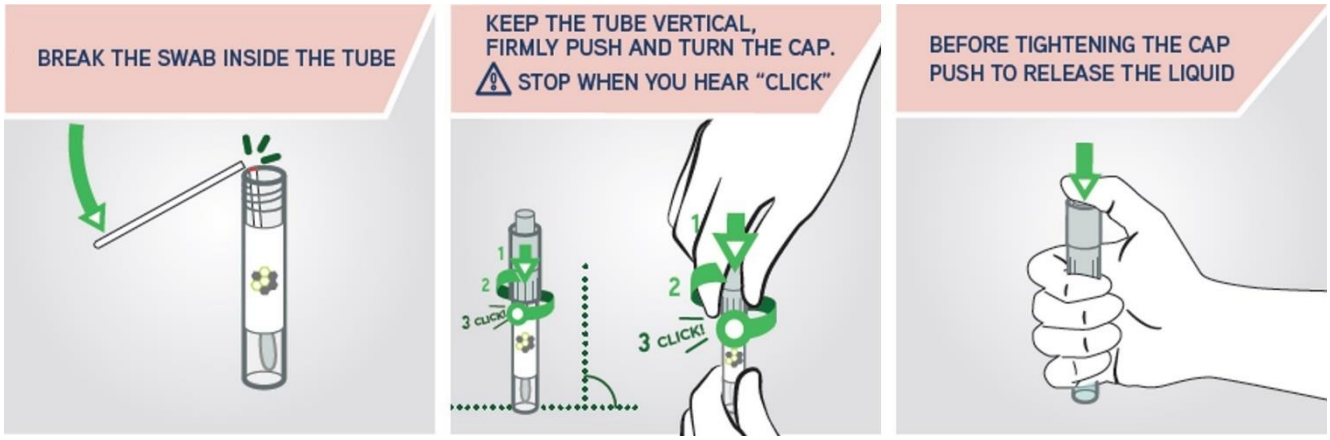
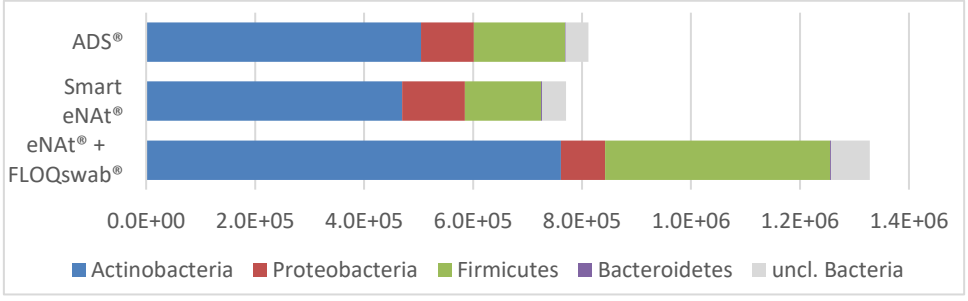


Figure 14. *Preservation medium realease procedure of the Smart eNAt® device (courtesy of Copan).*

Figure 15. *hDNAfree FLOQswab® and Active Drying System® device (courtesy of Copan).*

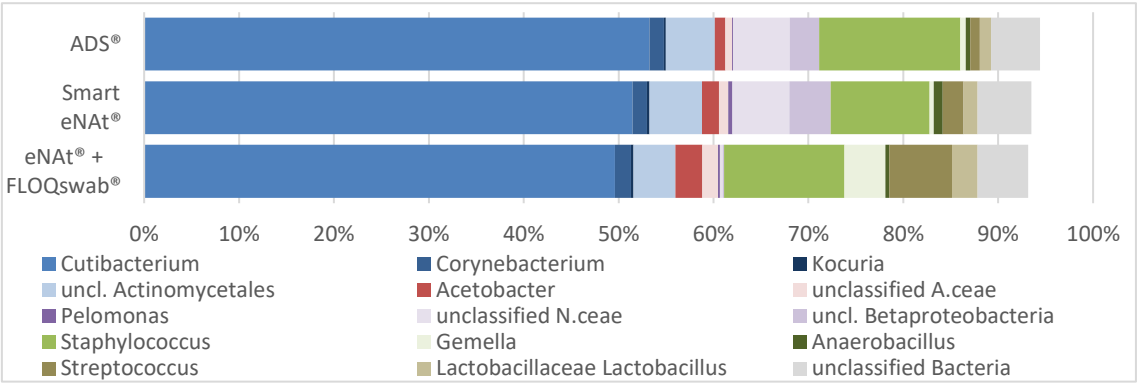
II.1.2. Study design, results, and discussion

To evaluate the efficacy of the two now device compared with the performance of the FLOQswab® + eNAt® usually employed, we collected 36 samples from the right cheeks of four volunteers aged 25-45 years (3 per device per volunteer) over three consecutive days. All samples were collected following the same procedure, then prepared, extracted, amplified, sequenced, and analysed according to our base pipeline. The quality of the run and sequencing results was confirmed with the good run parameters and sequencing statistics numbers (data not shown). At first glance, the original FLOQswab® + eNAt® kit seem to dive better results than the Smart eNAt® and ADS® devices (Graph 16, absolute bacterial abundances).



Graph 16. *Absolute abundences obtained after secondary analysis, by device and detected phyla.*

The overall read numbers remained nevertheless more than satisfying also for the other devices, therefore we proceeded with the relative taxa abundances and LEfSe analysis of the 10 most abundant genera (Graph 17 and Table 14), and the biodiversities (Graphs 18A, 18B, 19A, 19B, and Table 15).

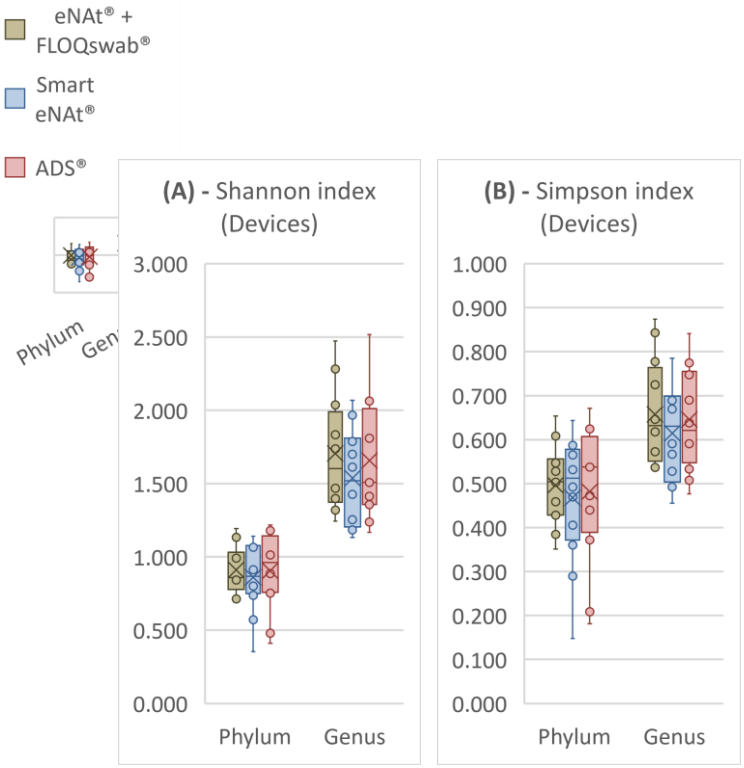


Graph 17. *Relative abundances of the 10 most abundant genera and their parent taxa of interest, depending on the sampling kit.*

PHYLUM	CLASS	ORDER	FAMILY	GENUS	eNAt® + FLOQswab®	Smart eNAt®	ADS®	LEfSe *p*-value
Actino-bacteria	Actino-bacteria	Actino-mycetales	Propionibacteriaceae	Cutibacterium	49.60%	51.48%	53.25%	9.16E-01
			Corynebacteriaceae	Corynebacterium	1.70%	1.49%	1.53%	8.55E-01
			Micrococcaceae	Kocuria	0.28%	0.28%	0.19%	7.68E-01
				uncl. Actinomycetales	*4.41%*	*5.52%*	*5.17%*	*9.21E-01*
Proteo-bacteria	Alpha-proteobacteria	Rhodospirillales	Acetobacteraceae	Acetobacter	2.84%	1.80%	1.13%	2.38E-01
				uncl. A.ceae	*1.65%*	*0.98%*	*0.71%*	*3.13E-01*
	Beta--proteobacteria	Burkholderiales	Comamonadaceae	Pelomonas	0.21%	0.41%	0.09%	4.19E-01
		Neisseriales	Neisseriaceae	*uncl. N.ceae*	*0.30%*	*6.04%*	*5.95%*	*4.59E-01*
				uncl. Betaproteobacteria	*0.12%*	*4.33%*	*3.11%*	*2.30E-01*
Firmicutes	Bacilli	Bacillales	Staphylococcaceae	Staphylococcus	12.70%	10.44%	14.89%	5.97E-01
				Gemella	4.30%	0.41%	0.55%	5.12E-01
			Bacillaceae 1	Anaerobacillus	0.41%	0.96%	0.49%	7.34E-01
		Lactobacillales	Streptococcaceae	Streptococcus	6.61%	2.18%	1.01%	5.32E-01
			Lactobacillaceae	Lactobacillus	2.69%	1.50%	1.20%	3.77E-01
				unclassified Bacteria	*5.35%*	*5.66%*	*5.15%*	*4.97E-01*
				TOTAL	**93.17%**	**93.49%**	**94.41%**	

Table 14 - *Relative abundances and LEfSe analysis results of the 10 most abundant genera and their parent taxa of interest.*

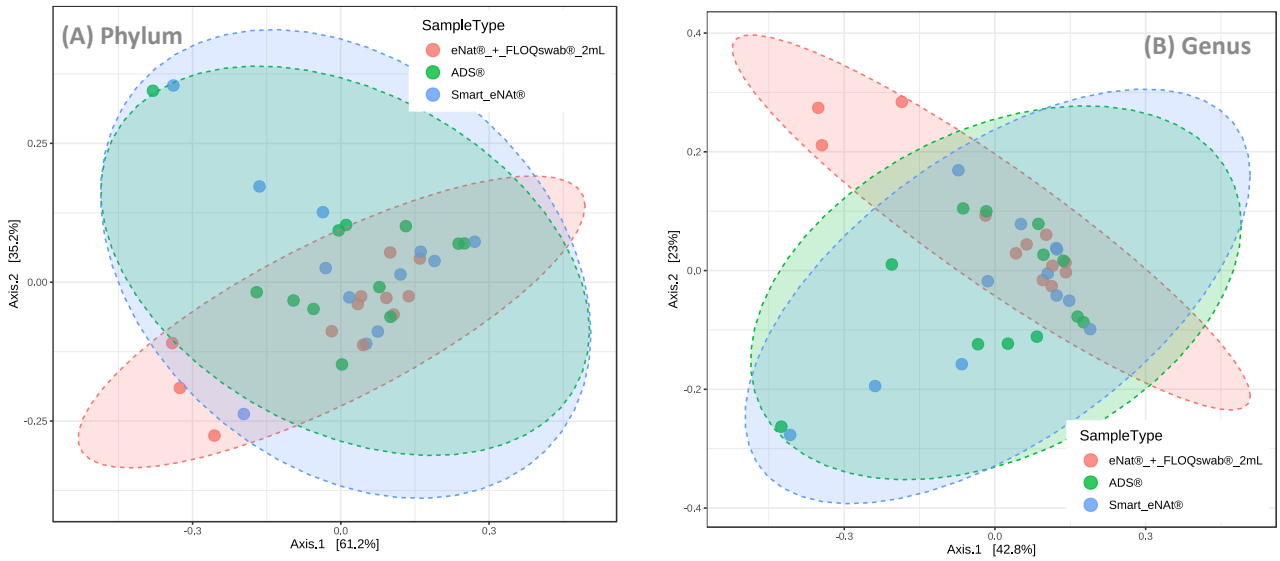
We can readily observe that the relative abundances (Graph 17) do not reflect the noticeable differences in absolute abundances (Graph 16), except maybe concerning the *Betaproteobacteria* (in purple shades). In fact, the LEfSe (Table 14), alpha- (Graphs 18A, 18B, Table 15), and betadiversity analyses (19A, 19B, Table 15) all gave *p*-values well over the 0.05 threshold, confirming i) the lack of statistically relevant differences in microbiota composition between the different kits, and ii) that the new Copan devices (Smart eNAt® and ADS®) are as performant as the original FLOQswab® + eNAt®.



	alpha		beta
	Shannon	Simpson	
PHYLUM	8.43E-01	9.30E-01	0.401
CLASS	6.81E-01	9.16E-01	0.260
ORDER	8.33E-01	9.19E-01	0.216
FAMILY	4.33E-01	7.63E-01	0.407
GENUS	4.91E-01	6.65E-01	0.406

Table 15. *Statistical diversity values, at the sample level (alphadiversity) and the group level (betadiversity).*

Graph 18. *Visual representation of the distribution across samples of taxa richness (A) and evenness (B) at the phylum and genus levels, depending on the sampling kit.*



Graph 19. *Visual representation of the distribution across samples of community composition at the phylum (A) and the genus levels (B), depending on the sampling device.*

In conclusion, the choice of the sampling device should depend on the study design and the maximum storage time. The eNat® medium ensures sample stability for one month at RT and up to 6 months at -20°C so the Smart device is particularly useful when dealing with multiple zones and time points, as it allows safe self-collection by the subject. Similarly, the ADS® device is well adapted to private use, but if sample stability is already proven for 2 weeks at RT, long-term stability tests are still ongoing. Finally, the FLOQswab® + eNAt® initial kit should be preferred for studies requiring POC specimen collection.

II.2. Sample preparation

II.2.1. Study design

As described in the Materials and Methods, the samples were prepared using the NAO® basket specialty device, a perforated basket placed inside a 2 mL microtube (Copan, Figure 13A). Traditionally, the method for immersed swabs consists in vortexing the swab in the preservation medium to detach all collected material, before pressing it against the tube's walls and removing it, and finally extract the microbial DNA from the remaining liquid. In our case, the NAO® basket allows to completely dry the swab's head through centrifugation rounds and thus recover more medium and thus more material (Figure 13B). In fact, increasing the biomass recovered by cutaneous swabbing is also of critical biomedical interest, because chronic wounds microbiome characterisation (diabetic ulcers, bedsores, AD and psoriatic flares, severe acne…) is hampered by the low biomass of typical samples (Verbanic et al. 2019; Gregório, García-Ruiz, and Martínez 2019).

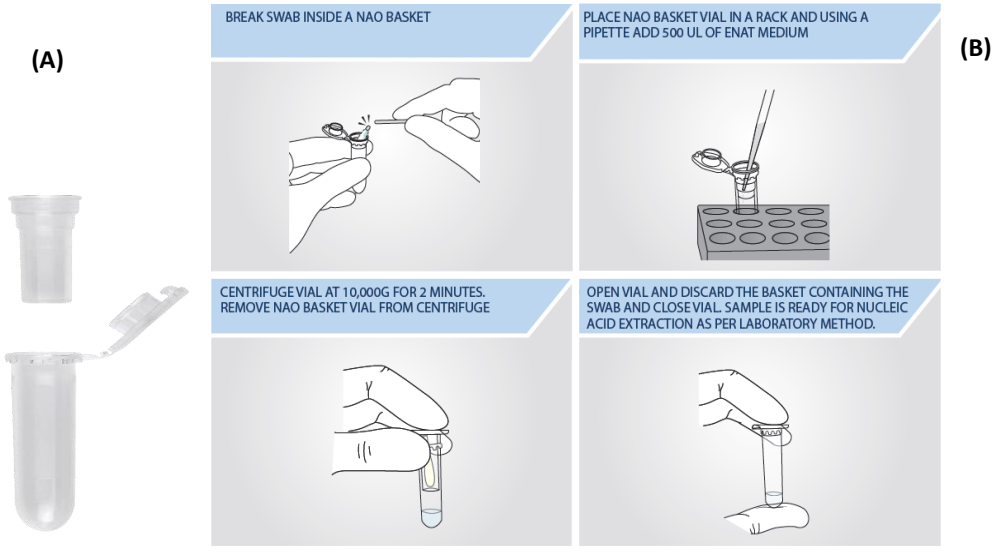
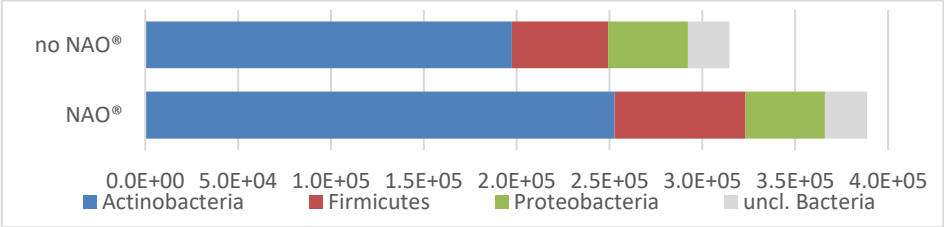


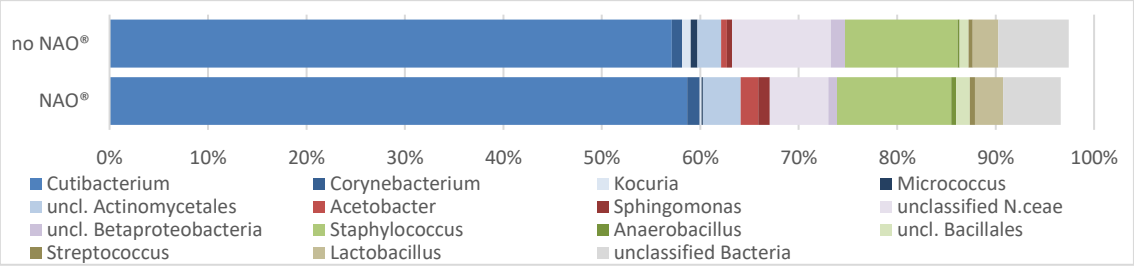
Figure 17. *NAO® Basket (A) and protocol for sample preparation(B) (Copan).*

For this study, we collected 24 samples from the same 4 volunteers as for the devices study, one on each cheek for three consecutive days. For each volunteer, half of the 6 specimens were prepared using the NAO® basket, and the remaining three were prepared using the traditional technique. For each condition, we compared the absolute and relative abundances (Graphs 20, 21, and Table 16), and determine eventual result differences by biodiversities (alpha- and beta-, Graphs 22, 23, and Table 17) and LEfSe (Table 16) analyses.



Graph 20. *Absolute abundences obtained after secondary analysis, by preparation conditions and detected phyla.*

The use of the basket allowed to obtain more reads for the analysed sampled, indicative of better-quality results. Some composition differences can also be observed on the relative abundances' representation (Graph 21), but only few statistical differences could be determined (LEfSe analysis, Table 16) and the only genus difference concerns Acetobacter, a taxon often associated with environmental contamination. In fact, the use of the NAO® basket requires several more steps than the pressing method, and we carried them out on a normal laboratory bench. In the future, the sample preparation step should be performed under a bacterial hood to limit external contaminations.

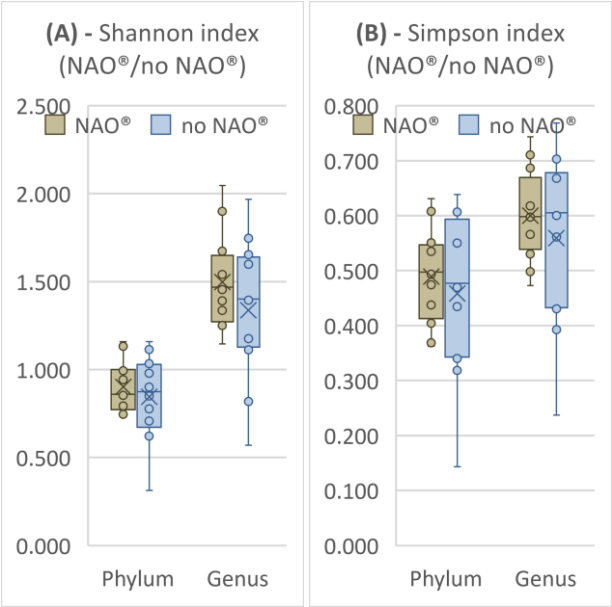


Graph 17. *Relative abundances of the 10 most abundant genera and their parent taxa of interest, by preparation condition and detected phyla.*

PHYLUM	CLASS	ORDER	FAMILY	GENUS	NAO®	no NAO®	LEfSe *p*-value
Actinobacteria	Actinobacteria	Actinomycetales	Propionibacteriaceae	Cutibacterium	58.68%	57.10%	1.00E+00
			Corynebacteriaceae	Corynebacterium	1.25%	1.04%	2.25E-01
			Micrococcaceae	Kocuria	0.21%	0.90%	6.44E-01
				Micrococcus	0.14%	0.67%	3.83E-01
				uncl. Actinomycetales	*3.83%*	*2.40%*	*5.67E-02*
Proteobacteria	Alphaproteobacteria	Rhodospirillales	Acetobacteraceae	Acetobacter	1.79%	0.59%	**1.53E-02**
				Sphingomonas	1.18%	0.51%	2.98E-01
		Neisseriales	Neisseriaceae	*unclassified N.ceae*	*5.95%*	*10.05%*	*7.73E-01*
				uncl. Betaproteobacteria	*0.87%*	*1.45%*	*7.29E-01*
Firmicutes	Bacilli	Bacillales	Staphylococcaceae	Staphylococcus	11.61%	11.41%	5.64E-01
			Bacillaceae 1	Anaerobacillus	0.49%	0.22%	9.41E-02
				uncl. Bacillales	*1.40%*	*0.89%*	***9.37E-03***
		Lactobacillales	Streptococcaceae	Streptococcus	0.53%	0.43%	8.17E-01
			Lactobacillaceae	Lactobacillus	2.85%	2.63%	3.86E-01
				unclassified Bacteria	*5.85%*	*7.15%*	*4.53E-01*
				TOTAL	**96.62%**	**97.43%**	

Table 16 - *Relative abundances and LEfSe analysis results of the 10 most abundant genera and their parent taxa of interest*

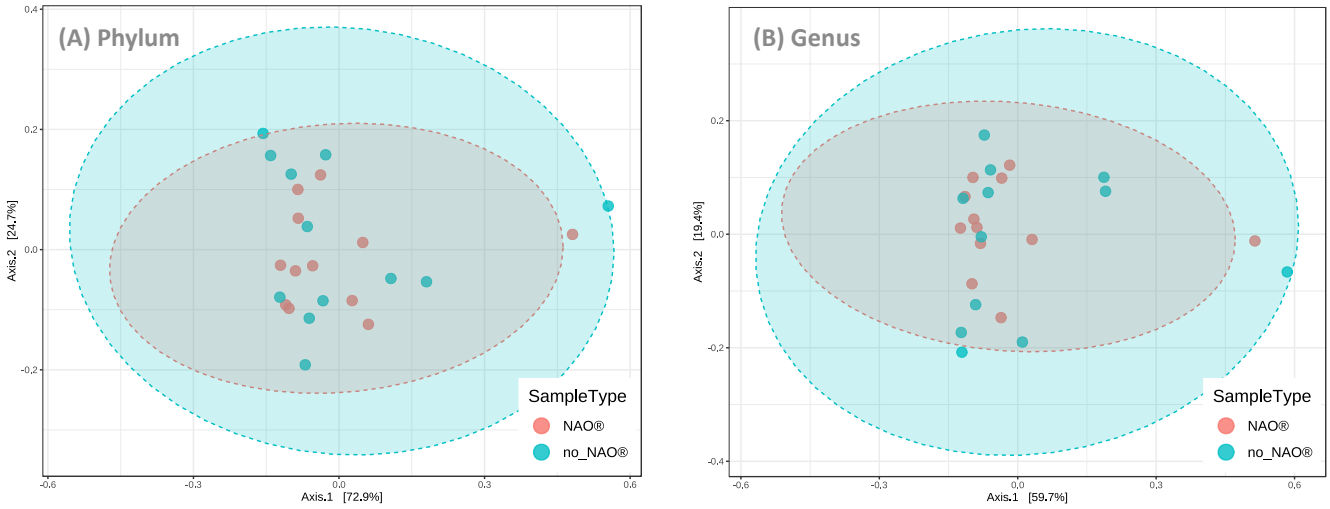
The diversity analyses (Graphs 22, 23) and their *p*-values calculations at each taxonomic level (Table 17) confirm the lack of statistical differences between the two treatments whether in terms of taxa richness (alphadiversity, Shannon index), evenness (alphadiversity, Simpson index), or community structure (betadiversity, PERMANOVA).



	alpha		beta
	Shannon	Simpson	
PHYLUM	7.13E-01	6.30E-01	0.695
CLASS	6.71E-01	7.55E-01	0.590
ORDER	6.30E-01	7.13E-01	0.640
FAMILY	4.10E-01	8.43E-01	0.599
GENUS	4.10E-01	7.55E-01	0.607

Table 17. *Statistical diversity values, at the sample level (alphadiversity) and the group level (betadiversity)*

Graph 22. *Visual representation of the distribution across samples of taxa richness (A) and evenness (B) at the phylum and genus levels, depending on the sampling kit.*



Graph 23. *Visual representation of the distribution across samples of community composition at the phylum (A) and the genus levels (B), depending on the preparation technique.*

In conclusion, there again the decision of using the basket or not depends on the general study design, the technician's expertise, and the available material. On one hand, it is preferable to prepare samples through the NAO® basket to obtain greater quantity of nucleic acids from the samples (Gregório, García-Ruiz, and Martínez 2019)but it can be relatively time-consuming (especially when dealing with volumes > 1 mL). On the other hand, the pressing method should be chosen if there is no access to a laminar flow hood for the sample preparation step, or if the timing must be quickened.

III. DNA extraction – Qiagen *vs.* Invitrogen and ZymoResearch

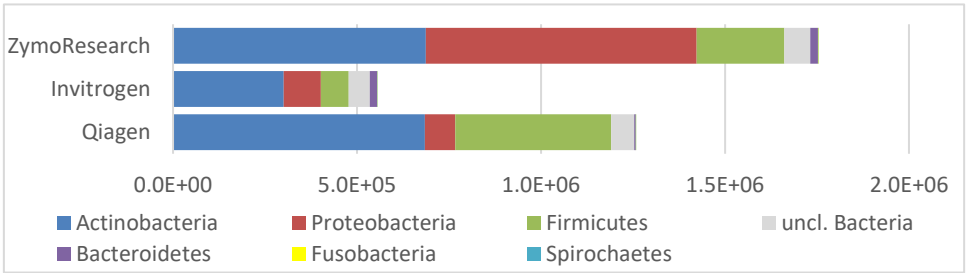
III.1. Study design

As described above, our base pipeline was established using the QIAamp® DNA Microbiome extraction kit (Qiagen) since it is one of the most performant on the market, and one of the rare that include a host DNA removal step in its protocol. However, the whole procedure is longer than those of the competitor brands. At Complife, we thrive to offer tailored testing solutions to our clients, therefore we deemed appropriate to evaluate cheaper and shorter alternative without compromising on results quality. Having flexible prices depending on the experimental approach could in fact allow us to attract clients looking to test lower-margin products on the microbiome. Studies on the human faecal microbiome already demonstrated the reliability of several kits while highlighting the quality of the Qiagen products (Claassen et al. 2013; Thomas-Poulsen et al. 2019), Therefore we felt logical to conduct our own experiment and we compared the QIAamp® DNA Microbiome extraction kit(Qiagen) to its equivalent by ZymoResearch (ZymoBIOMICS™ DNA Miniprep Kit) and Invitrogen (PureLink™ Microbiome DNA Purification Kit).

For this study we collected 8 samples – from both cheeks of the same 4 volunteers as the device and preparation studies – and pooled each volunteer's samples together to give 2 mL of microbiota extract per subject. Each microbial DNA extraction kit was then tested on 500 µL of each of the pooled samples, giving a total of 24 samples which were successively amplified and sequenced together. Result quality was assessed by controlling the run parameters and sequencing statistics, which were satisfying for 21 out of 24 samples: in fact, two samples extracted with the Invitrogen and one with the ZymoResearch kits did not meet our standards (total read number < 20 000, data not shown).

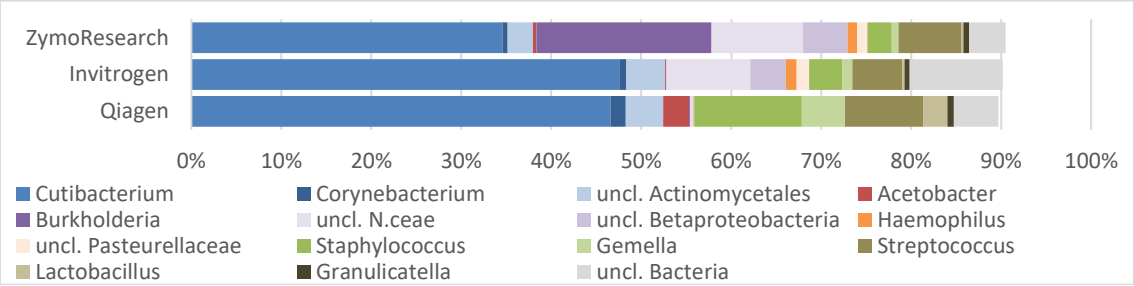
III.2. Results and Discussion

First, the absolute abundances analysis at the genus level (Graph 24) shows that Invitrogen samples had the smaller number of reads and ZymoResearch samples the most, but the latter also display very high amounts of *Proteobacteria* indicative of environmental contamination.



Graph 24. *Absolute abundences obtained after secondary analysis, by extraction kit and detected phyla.*

The relative abundances analyses (Graph 24 and Table 18) confirm the abnormal proportion of *Proteobacteria* for the ZymoResearch kit, while seemingly underestimating the *Alphaproteobacteria* (here, Acetobacter) population. Similarly, samples extracted with the Invitrogen product exhibits unusually high percentages of *Betaproteobacteria* but very low abundances of *Alphaproteobacteria* compared to the Qiagen kit. However, the Invitrogen numbers remain within an acceptable range according to other internal results and the literature, while the ZymoResearch umbers suggests an important external contamination, despite performing the extraction steps under a laminar flow hood. Both Invitrogen and ZymoResearch product also underestimate the *Staphylococci spp.* proportions – as confirmed by the LEfSe analysis – which could be a consequence of the over-representation of *Proteobacteria* (Graph 25 and Table 18).

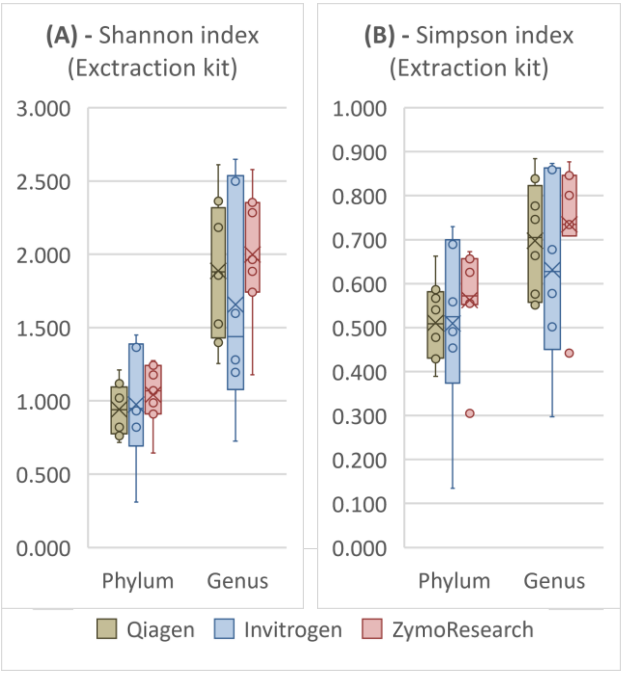


Graph 25. *Relative abundances of the 10 most abundant genera and their parent taxa of interest, by extraction kit.*

PHYLUM	CLASS	ORDER	FAMILY	GENUS	Qiagen	Invitrogen	Zymo Research	LEfSe *p*-value
Actino-bacteria	Actino-bacteria	Actinomycetales	Propionibacteriaceae	Cutibacterium	46.59%	47.57%	34.58%	4.57E-01
			Corynebacteriaceae	Corynebacterium	1.69%	0.78%	0.57%	6.68E-02
				uncl. Actinomycetales	*4.16%*	*4.31%*	*2.82%*	*1.86E-01*
Proteo-bacteria	Alpha-proteobacteria	Rhodospirillales	Acetobacteraceae	Acetobacter	2.87%	0.09%	0.37%	**9.53E-04**
	Beta-proteobacteria	Burkholderiales	Burkholderiaceae	Burkholderia	0.13%	0.03%	19.47%	**1.14E-03**
		Neisseriales	Neisseriaceae	*uncl. N.ceae*	*0.28%*	*9.33%*	*10.18%*	*2.18E-01*
				uncl. Betaproteobacteria	*0.11%*	*3.94%*	*4.92%*	***8.20E-03***
	Gamma-proteobacteria	Pasteurellales	Pasteurellaceae	Haemophilus	0.02%	1.20%	1.07%	4.69E-01
				uncl. Pasteurellaceae	*0.03%*	*1.41%*	*1.16%*	*3.49E-01*
Firmicutes	Bacilli	Bacillales	Staphylococcaceae	Staphylococcus	11.97%	3.68%	2.67%	**1.21E-02**
				Gemella	4.78%	1.12%	0.75%	5.51E-01
		Lactobacillales	Streptococcaceae	Streptococcus	8.70%	5.55%	7.02%	8.95E-01
			Lactobacillaceae	Lactobacillus	2.69%	0.23%	0.23%	**1.98E-02**
			Carnobacteriaceae	Granulicatella	0.74%	0.58%	0.64%	9.89E-01
				uncl. Bacteria	*4.94%*	*10.35%*	*4.03%*	*5.88E-02*
				TOTAL	**89.70%**	**90.19%**	**90.49%**	

Table 18 - *Relative abundances and LEfSe analysis results of the 10 most abundant genera and their parent taxa of interest.*

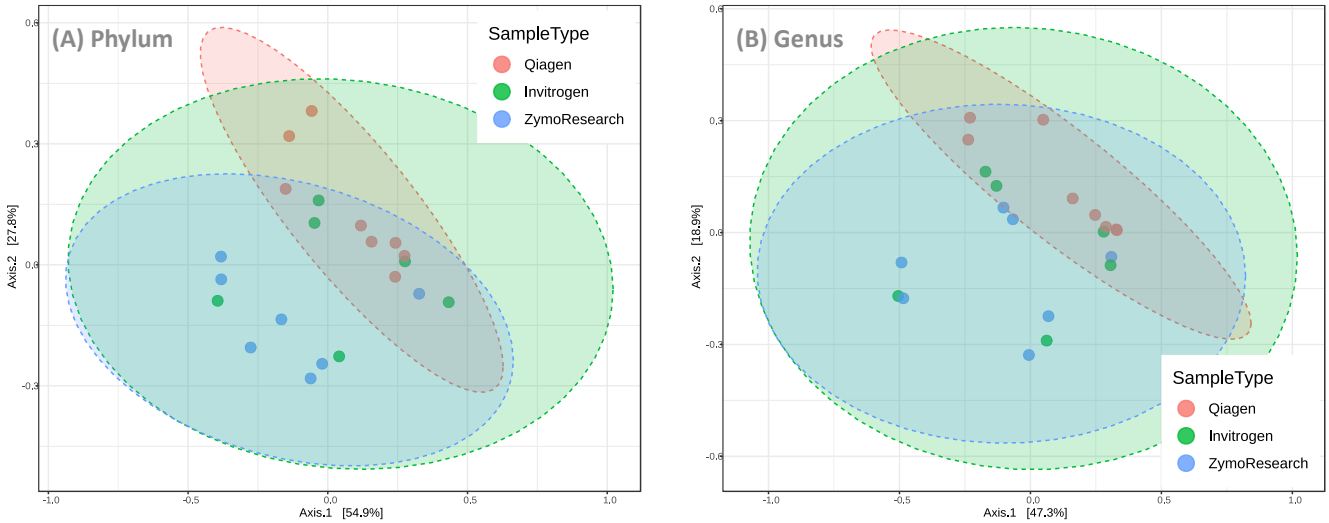
The statistical analyses of the biodiversity confirm that all kits detect similar taxa richness (Shannon index, Graph 25A and Table 19) and taxa preponderance (Simpson index, Graph 25B and Table 19), but different community structures at all taxonomy levels (Graph 26 and Table 19).



	alpha		beta
	Shannon	Simpson	
PHYLUM	6.31E-01	5.52E-01	**0.013**
CLASS	6.06E-01	3.88E-01	**0.006**
ORDER	4.89E-01	6.09E-01	**0.005**
FAMILY	7.22E-01	7.36E-01	**0.015**
GENUS	7.11E-01	6.96E-01	**0.012**

Table 19. *Statistical diversity values, at the sample level (alphadiversity) and the group level (betadiversity)*

Graph 26. *Visual representation of the distribution across samples of taxa richness (A) and evenness (B) at the phylum and genus levels, depending on the extraction kit.*



Graph 27. *Visual representation of the distribution across samples of community composition at the phylum (A) and the genus levels (B), depending on the extraction kit.*

Despite some proportions differences with the Qiagen kit, the Invitrogen kit gave different dispersion but similar distribution results (Graph 26 and Table 19), and most green samples stay within the range of the Qiagen extract composition (Graph 27 and Table 19). Because of these similarities, we deemed the Invitrogen kit a good alternative for quicker and cheaper microbial DNA extraction from skin swabs, but the ZymoResearch kit did not exhibit the same qualities and we would advise against it.

IV. Conclusions and outlooks

The role of the microbiome in maintaining the cutaneous health has gained tremendous interest in the cosmetic field as the analysing techniques got more accessible. Complife decided in November 2019 to join

the fray and implement its own service for analysing the effect – or lack thereof – of cosmetic products on the skin microbiome. Starting from scratch but thanks to the support of UPO, we were able in only a few months to establish a complete pipeline based on the 16S rDNA analysis, from specimen collection to bioinformatic analysis of the sequencing results.

We first demonstrated the reliability of the whole workflow by comparing our data with the literature and by controlling that samples collected and treated at different times gave reproducible and repeatable results. We also validated the suitability of two new devices from our partner Copan for at-home sample collection, allowing in the future to relieve the POCs from the planning pressure linked to studies that involve multiple time and collection points. We proceeded with the optimisation of the sample preparation step and demonstrated the efficacy of the NAO® basket in recovering more and better-quality nucleic acids. Despite the added manipulation time, we believe that such systems should always be employed when dealing with low biomass samples such as skin microbiota specimens, while richer samples may be prepared without (if necessary). Finally, we continued our trials on process simplification by testing two alternative microbial DNA extraction kits. As expected, the one we initially chose from Qiagen gave better results than the others. Nevertheless, we were able to confirm the applicability of the Invitrogen product (when cost and time gains are crucial), while we do not consider the ZymoResearch kit suitable for cutaneous microbiome applications.

However, limiting our approach to skin microbiota studies only to 16S rRNA gene analysis would be a mistake, as many other microorganisms (fungi, viruses, archaea, mites…) actively participate to the ecosystem symbiosis (Flowers and Grice 2020; Lunjani, Hlela, and O'Mahony 2019; Paulino et al. 2006; Iebba et al. 2016). Because of the mainly commercial purpose of the laboratory, the whole-genome sequencing approach remains out of the equation, but other techniques have lower financial impacts and would still help broaden our view of the healthy cutaneous microbiota.

In fact, we recently launched two new projects aiming at diversifying our expertise, starting with adapting our current pipeline to allow the investigation of the fungal component. To this end, we tested several extraction kits, and the amplification step targeted the ITS-1 gene of the fungal genome. The results will be interpretated depending on the extraction kit, and the overall mycobiome composition compared with the existing literature. Second, several clients expressed interest in observing the behaviour of specific fungal and bacterial species upon cosmetic application, but bot the 16S and ITS-1 amplicon approach remains limited for analyses at the species level. To overcome this drawback, we started elaborated a ddPCR protocol for the amplification of species of interest from specifically designed primers.

Finally, we decided to try and develop our own *in vitro* skin microbiome model based on 3D reconstructed epidermis, which should allow to test the effect of isolated cosmetic actives without the need to formulate them beforehand. In the long term, such a technique could even avoid resorting to *in vivo* trials and the associated clinical and ethical issues.

CHAPTER II – Influence of endo- and exogenous factors on the skin microbiome

I. Study design

Over the years, most studies focused either on whole-body characterization or on common skin diseases (atopic dermatitis, psoriasis, acne) (Byrd, Belkaid, and Segre 2018; Grice and Segre 2011; Cogen, Nizet, and Gallo 2008; Peterson et al. 2009), and it can be challenging to find detailed studies rendering the composition of a normal cutaneous microbiota or the indicators for altering conditions (Lloyd-Price, Abu-Ali, and Huttenhower 2016). In fact, the high intra- and interpersonal variability does not allow to establish the general composition of the basal microbiota of the face, and this variability is further reinforced by the important influence of endo- and exogenous factors (Cundell 2018; Samaras and Hoptroff 2020; Baldwin et al. 2017). Additionally, most studies focusing on the facial cutaneous microbiota have been conducted in eastern Asia and information is scanty for the western populations (Oh et al. 2016).

Thanks to its clients, Complife collected thousands of specimens in just three years and thus could leverage data from small and larger studies on healthy volunteers to study specific patients cohorts, since very large sample numbers allow the use of statistical techniques (Kong et al. 2017; Li et al. 2012). Aware that both genetics and environmental factors are key players in shaping and maintaining the microbiota (Byrd, Belkaid, and Segre 2018; Lloyd-Price, Abu-Ali, and Huttenhower 2016; Lunjani, Hlela, and O'Mahony 2019; Arweiler and Netuschil 2016; Boxberger et al. 2021), the volunteers were asked to fill in a questionnaire detailing their lifestyle (family, pets, work, living environment, health…) and habits (beauty/sleep/food routines, sports, hobbies…) to be able later to evaluate microbiome variations based on chosen variables.
Routinely, the *in vivo* studies at Complife include a two-weeks wash-out period where the volunteers are asked not to use any product on the future tested area. This ensures a "clean sheet" at the beginning of the study and allowed us to use the T0 samples for microbiome characterisation. We retained the specimens from the untreated areas as well, but not those from the placebo areas as the microbiota might be modified even by "neutral" products.

This got us 980 suitable samples at the end of 2022, where each sample was treated as coming from a different individual even when the same volunteer was sampled more than one time. In fact, it has been demonstrated that the same locations are more similar between different subjects than to other locations on the same person, but also that the microbiota can suffer from temporal variations within the same area of the same subject (Grice et al. 2009). Furthermore, microbiome researchers often prefer to rely on high-heterogeneity pools as less accurate data about a large number of samples is much more informative than more accurate

data about small number of samples, especially when trying to uncover general trends in the host physiology/microbiota and host environment/microbiota relationships (Hamady and Knight 2009).
Out of these 980 samples (745 from women vs. 235 from men), 472 were collected on the cheeks (either left or right, 371 *vs.* 101), 50 on the forehead (35 *vs.* 15), and 458 from other areas of the body (armpits, manubrium, groin, thigh; 339 *vs.* 119). In accordance with our proven protocol, the volunteers avoided washing the tested area 8-12 hours before collection (Kong et al. 2017) and all specimens were treated following the same, unmodified procedures used in the pilot and preliminary studies (See Materials and Methods and Ch. I, § I.2.).

Because we already suspected important facial microbiota variations depending on the sex, we naturally focused on rationalising and expanding this study first from the statistical point of view (Ch. II, § II.1.) and then geographically to see if all body sites show (the same?) remarkable differences (Ch. II, § II.2.). In fact, because areas exposed to the environment have more variable microbiotas (Moskovicz, Gross, and Mizrahi 2020) and because different micro-environments (humidity and salt levels, pH, nutrient availability, etc.) modulate the structure and composition of the microbiota (Costello et al. 2009; Oh et al. 2014), the same factor might not have the same consequences on different areas (if it has any).
After exploring the differences between the sexes, we were curious about other intrinsic and extrinsic factors (easily accessible from our questionnaire's database) and decided on the subject's age, the season in which the sample was collected, and the sampling centre – since our POCs are located in diversly urbanised areas. Because sequences in 16S rDNA metagenomic studies are generally too short to achieve accurate species affiliation (Cosseau et al. 2016), the analysis was limited to the genus level and only three different phyla (Firmicutes, Proteobacteria, and Actinobacteria) were detected across all variable/body site combinations.

II. Microbiome differences depending on the sex and the age

II.1. The cheek microbiome and the sex

From the almost 500 samples collected from the cheeks of men and women, we matched one-to-one 48 women and 48 men based on age, season, and living area (to limit the influence of these factors). The subjects were aged 25-71 years (A=25-35, B=36-45, C=46-55, D=56-65, E=66-75 years old), all residing in central northern Italy, Caucasian, presenting a healthy facial skin, and not following any antibiotic therapy; because about 85% of our volunteers do not smoke, the smoking status was not considered. All studies carried out at Complife comply with the international ethical rules and the Helsinki convention, therefore all volunteers give their informed consent upon participating to said studies. All samples were collected by trained technicians from Complife Italia Spa (Garbagnate Milanese, Italy).

To characterise the differences between the male and female facial microbiomes, we first profiled the community through Principal Component Analysis (using all the phyla and species presenting a relative abundance > 0.2%, Graphs 28) while the two-way ANOVA method was used to discriminate the effects of the two factors ("sex" and "age") and of their interaction ("sex × age") (Table 19). These analyses were performed using R (v. 3.5.1) R Core Team (Team 2020) using FactoMineR and Factoextra packages, and differences were considered significant for p-values < 0.05. We also observed the microbiotas of both groups based on alphadiversity (Shannon and Simpson indices), betadiversity (PERMANOVA), and LEfSe analyses (§ II.1.2 – II.1.4), similar to the approaches already presented in Chapter I.

II.1.1. Community profiling

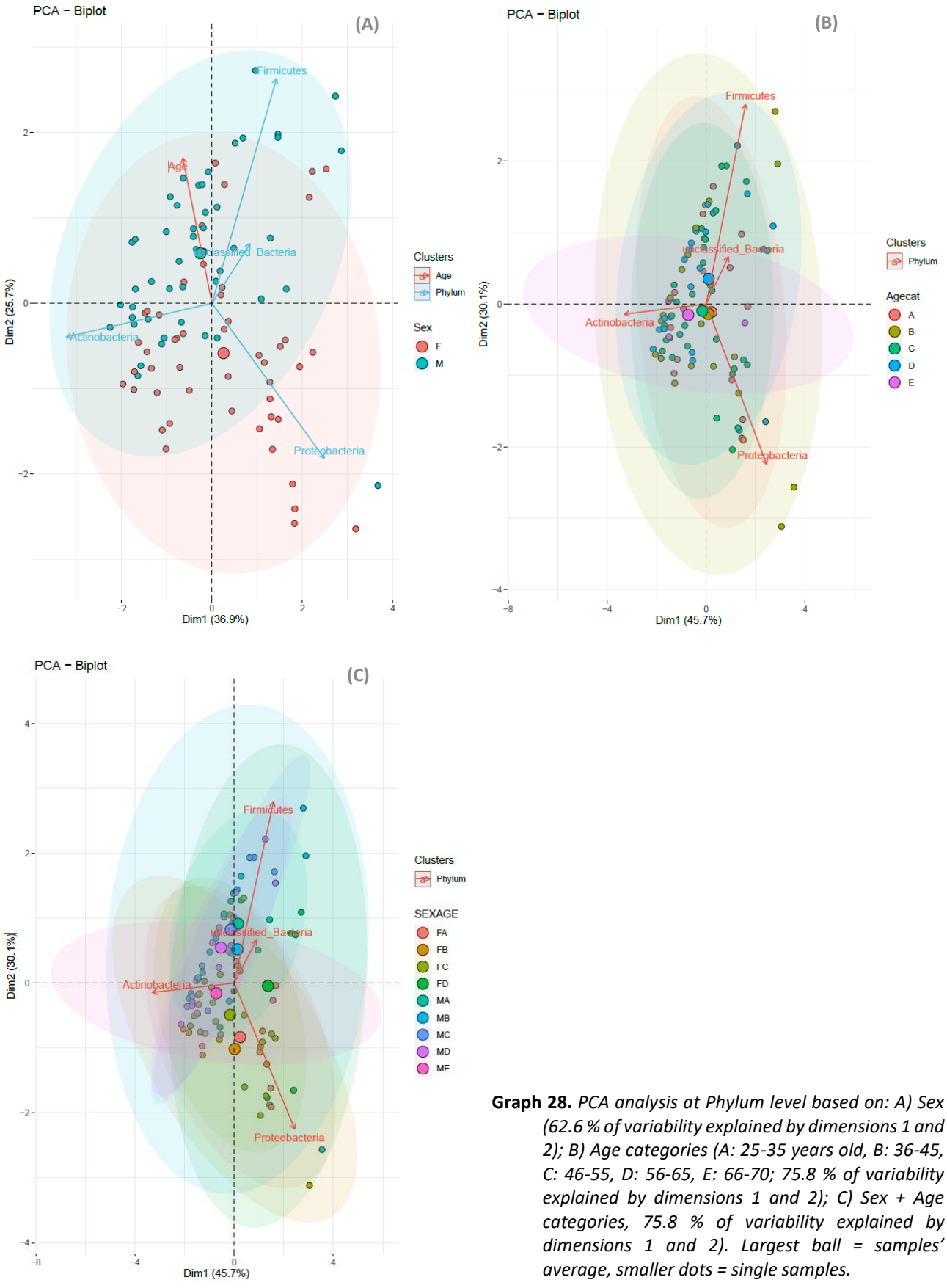
The multivariate analysis conducted at the phylum level confirmed that the skin microbiota of the face of males and females was significantly different, and that two particular phyla strongly modulated these variations: Firmicutes influenced mainly the male skin microbiota, while Proteobacteria influenced the female one (Figure 1A). On the contrary, when the samples were labelled by age category or considering the combination of gender and age, the results appeared less separated according to dimensions 1 and 2. These observations were corroborated by the two-way ANOVA (Table 19) and supported the sex factor as a significant influence on both *Firmicutes* and *Proteobacteria*, while the age factor was never significant

.

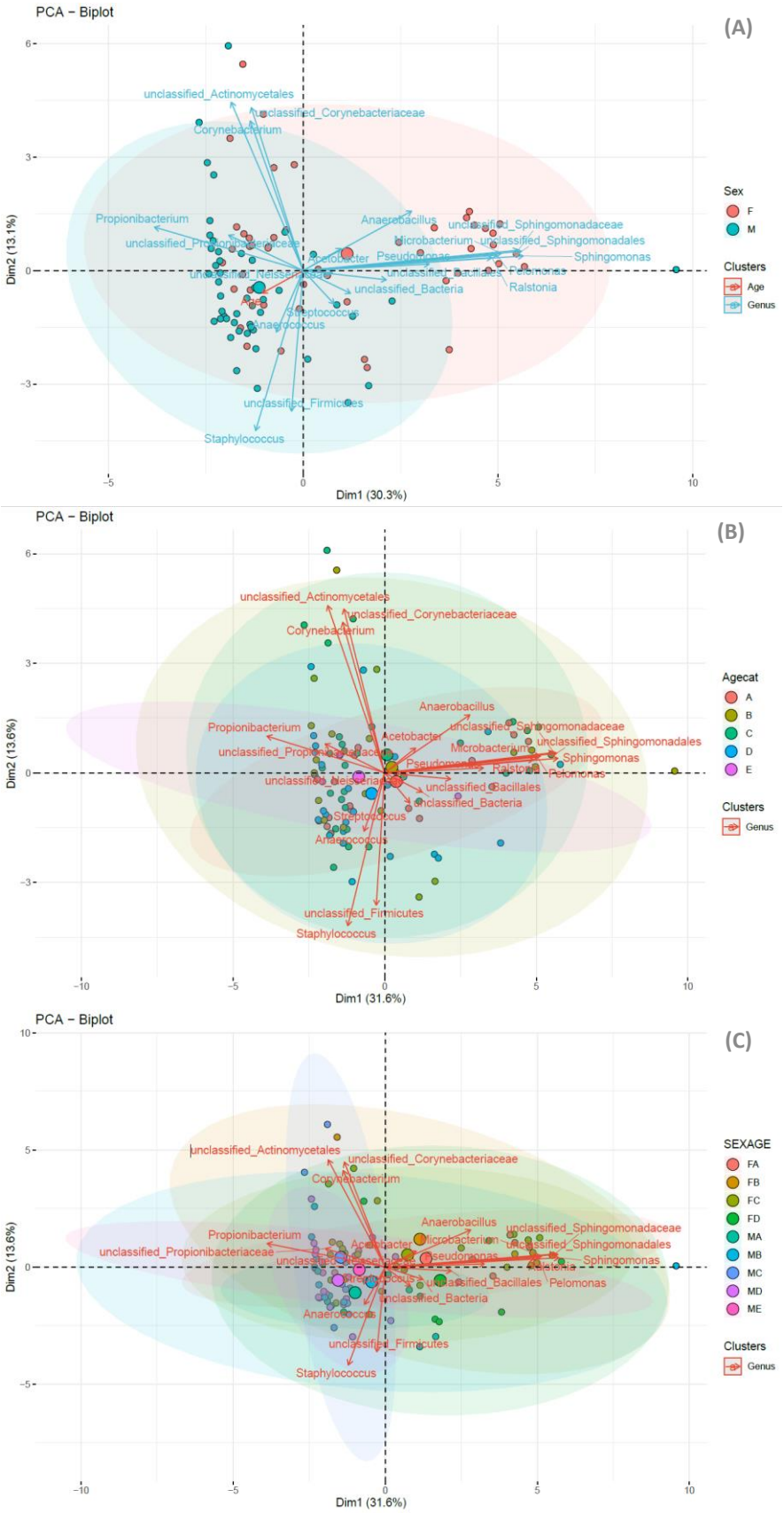
	Actinobacteria	Proteobacteria	Firmicutes	uncl. Bacteria
Sex	N.S.	***	***	N.S.
Age	N.S.	N.S.	N.S.	N.S.
Sex *x* Age	*	N.S.	*	N.S.

Table 19. *two-way ANOVA results, by phyla and considering the two factors "Sex", "Age" and their interaction "Sex x Age". N.S.= not significant; *= significant at P<0.05; **= significant at P<0.01; ***= significant at P<0.00*

Given the results obtained at the phylum level, the same multivariate analysis was conducted also at the genus level (Graph 29 and Table 20). There again, the role of the sex in shaping microbiota is evident while the age does not seem to have any impact (at least in this study setting).



Graph 28. *PCA analysis at Phylum level based on: A) Sex (62.6 % of variability explained by dimensions 1 and 2); B) Age categories (A: 25-35 years old, B: 36-45, C: 46-55, D: 56-65, E: 66-70; 75.8 % of variability explained by dimensions 1 and 2); C) Sex + Age categories, 75.8 % of variability explained by dimensions 1 and 2). Largest ball = samples' average, smaller dots = single samples.*



Graph 29. *PCA analysis at Genus level based on: A) Sex (39.8 % of variability explained by dimensions 1 and 2); B) Age categories (See Graph 28; 41.3 % of variability explained by dimensions 1 and 2); C) Sex + Age categories, 41.3 % of variability explained by dimensions 1 and 2). Largest ball = samples' average, smaller dots = single samples.*

	Sex	Age	Sex *x* Age
Acetobacter	**	N.S.	*
Anaerobacillus	*	N.S.	N.S.
Anaerococcus	**	N.S.	N.S.
Corynebacterium	N.S.	N.S.	N.S.
Microbacterium	***	N.S.	N.S.
Pelomonas	**	N.S.	N.S.
Cutibacterium	N.S.	N.S.	**
Pseudomonas	***	**	**
Ralstonia	**	N.S.	N.S.
Sphingomonas	***	N.S.	N.S.
Staphylococcus	***	N.S.	*
Streptococcus	N.S.	N.S.	N.S.
uncl. Actinomycetales	N.S.	N.S.	N.S.
uncl. Bacillales	**	N.S.	N.S.
uncl. Bacteria	N.S.	N.S.	N.S.
uncl. Corynebacteriaceae	N.S.	N.S.	N.S.
uncl. Firmicutes	**	N.S.	*
uncl. Neisseriaceae	*	N.S.	N.S.
uncl. Propionibacteriaceae	N.S.	N.S.	N.S.
uncl. Sphingomonadaceae	***	N.S.	N.S.
uncl. Sphingomonadales	***	N.S.	N.S.

Table 20. *Two-way ANOVA results, by genus and considering the two factors "Sex", "Age" and their interaction "Sex x Age". N.S.= not significant; *= significant at P<0.05; **= significant at P<0.01; ***= significant at P<0.001.*

II.1.1.1. Alpha-diversity

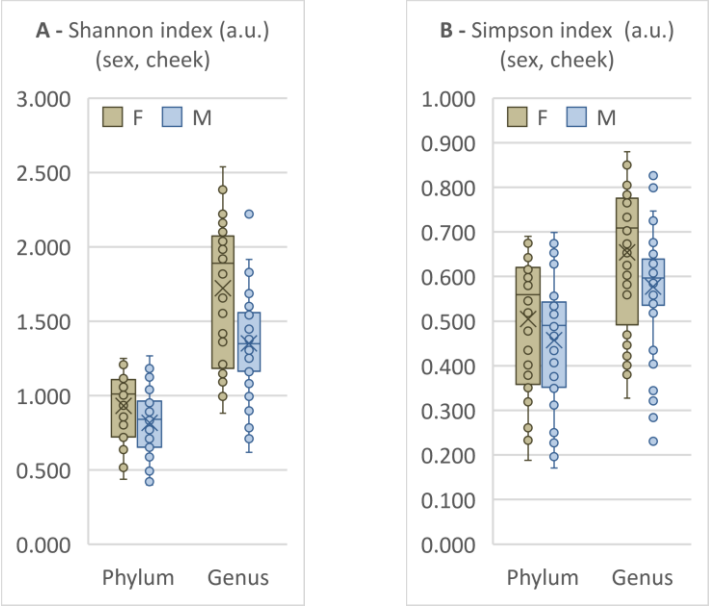
Similar to our pilot and preliminary studies, richness within the individual samples was assessed through Shannon index calculations (H' = ln(total number of taxa, H' ≥0), while evenness of taxa distribution was assessed using the Simpson index (distribution of abundances, 0 ≤ D ≤ 1). We chose to implement the calculations using the non-parametric tests Kruskal-Wallis and Mann-Whitney, because the data is not expected to follow a normal distribution, making it impossible to use the τ test (Grogan et al. 2019). The boxplots in Graph 30 illustrate the diversity distribution in the two groups at the phylum and genus levels and the statistical values are presented in Table 21, with a difference threshold maintained at 0.05.

				Median absolute values		Total spread	
		p-value	MW/KW	F	M	F	M
Shannon	Phylum	7.67E-03	1514	1.009	0.839	0.573	0.442
	Genus	2.28E-04	1647	1.888	1.349	1.008	0.731
Simpson	Phylum	2.29E-02	1462	0.560	0.491	0.501	0.528
	Genus	3.63E-03	1546	0.709	0.597	0.553	0.595

Table 21. *Values of interest at the phylum and genus levels, calculated from Shannon and Simpson indexes.*

The Shannon index absolute values remained generally quite low (particularly at the phylum level, Graph 30A and Table 21), indicating an overall low taxa number. At the phylum level, the men and women's groups display neighbouring minimum and maximum values, with a comparable spread and similar dispersion and distribution, suggesting that a similar range of taxa is represented in each group. However, the significant results of the statistics calculations ($p < 0.05$) demonstrate that fundamental membership differences exist

between the two cohorts. At the genus level, the ever-lower *p*-value further emphasizes these statistical differences between the two sexes. The lower median in men, accompanied by lower minimum and maximum values, highlight the overall higher biodiversity in women. Finally, despite both having similar dispersions (see spread), the higher distribution in women indicates higher microbiota interpersonal variability in this group.



Graph 30. *Graphic representation of the α-diversity at the phylum and genus taxonomic levels, calculated using the Shannon (A) and Simpson (B) indice and depending on the sex. a.u.=arbitrary unit.*

Considering the Simpson index, here again the *p*-values are considerably lower than 0.05 at any taxonomic level (data not shown), therefore further supporting the theory of radical structure differences between male and female facial microbiomes. Following the same trends as the H' results, the female microbiome is more diverse and distributed (higher medians and longer boxes) than the male's, despite both displaying similar dispersions (spread, Table 21). Because the women's medians are closer to 1 and are skewed right at the lower taxonomic level observed, it suggests that the taxa found in the female microbiota are more diverse from one volunteer to another compared to the male microbiota. Overall, our data are in agreement with the literature as they consistently displayed a reduced alpha-diversity at each taxonomic level independently of the chosen indexes (Ying et al. 2015; Giacomoni, Mammone, and Teri 2009; Noah Fierer et al. 2008).

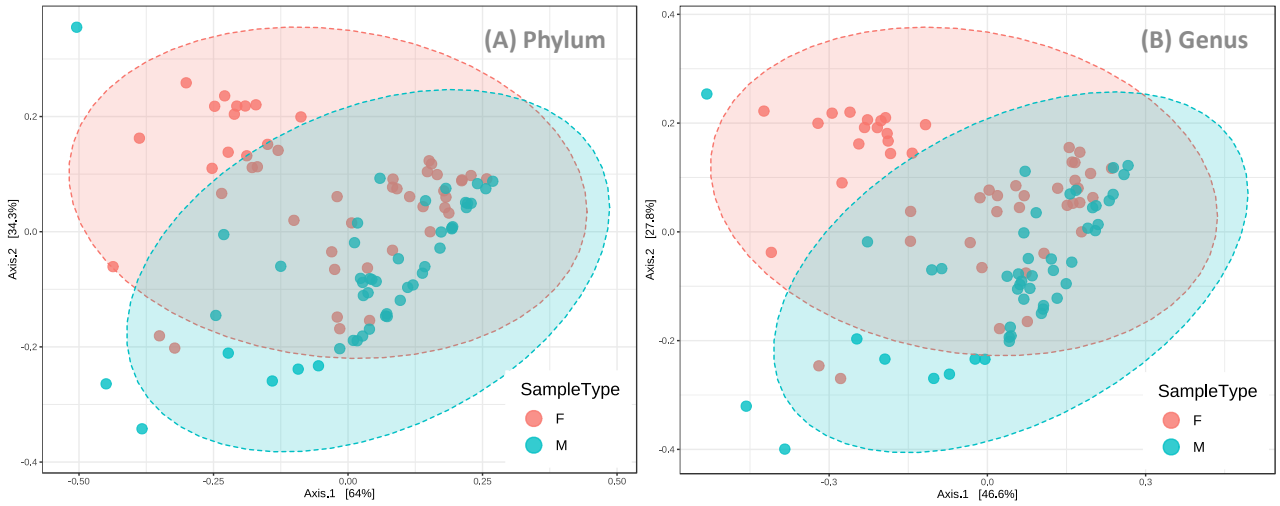
In fact, the biodiversity of a given environment is highly dependent on nutrient availability and variety, so the traditionally high facial sebum levels and the consequent anoxic environment limits the access to such resources, while naturally excluding the presence of aerobic members which in turn reinforces the monopoly of anaerobic bacteria such as *Cutibacterium* spp. (Zheng et al. 2021; Wallen-Russell and Wallen-Russell 2020). Because men traditionally present higher numbers of sebaceous and sweat glands, the increased release of free fatty acids by the first and of lactic acid by the second are thought to participate to the low biodiversity in male samples (Somboonna et al. 2017; Wallen-Russell and Wallen-Russell 2020). On the contrary, the higher

numbers in women are generally attributed to their reduced sweat production and thinner skin (Ying et al. 2015), but better skin hydration and more substantial use of cosmetic products are also believed to increase the cutaneous biodiversities by providing more varied substrates (Grice et al. 2009; Wallen-Russell and Wallen-Russell 2020; Hwang et al. 2021; Pinto et al. 2021; Dimitriu et al. 2019). Additionally, the recurrent washing and regular chemical and mechanical exfoliation of the skin accelerate the renewal of the skin layers, thus continuously changing the population of transient species – which would also explain the higher interpersonal variability in women compared to men.

II.1.1.2. Beta-diversity

The difference in community structure based only on the sex was evaluated by PERMANOVA analysis, and the results are presented in Graph 31 as PCoA plots with ellipses indicating confidence intervals of 95%. If the partly overlapping (but not superimposed) ellipses do not particularly highlight the group differences, all p-values (calculated at each taxonomic levels, data not shown) were below 0.001 and confirmed the structural differences already suggested by the α-diversity (Figure 30) and multivariate (Figures 28 and 29) analysis results.

The alpha-diversity values already highlighted important contrasts between the male and female microbiota – with women hosting a greater bacterial diversity while the male's microbiota seem dominated by a few members – and the beta-diversity results confirm this trend by implying that each sex hosts different members of the bacterial community.

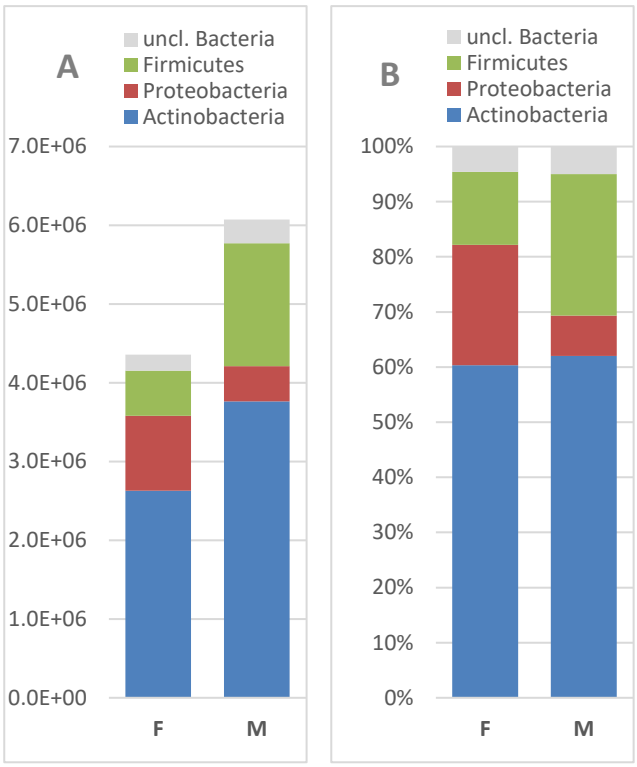


Graph 31. *Graphic representation of β-diversity at the phylum and genus taxonomic levels, depending on the sex.*

II.1.2. Community composition

II.1.2.1. Taxa abundance

To try and characterise these composition differences, we performed taxa abundance and LEfSe analyses, and the MicrobiomeAnalyst tool detected 3 phyla, 6 classes, 13 orders, 20 families, 22 genera, and 71 species. The absolute abundances data (Graph 32A and Table 22) shows higher read numbers for the men's groups, with a total of 6,073,226 reads for the male group and 4,356,001 for the female group.



Beyond the total number of reads, Graph 32A suggests that the microbiota divergences concern each phylum, with women displaying more *Proteobacteria* against both *Firmicutes* and *Actinobacteria* for men. However, where this is substantiated by the relative abundances (Graph 32B) and LEfSe analysis (Table 22) for *Firmicutes* and Proteobacteria, *Actinobacteria* is present in similar percentages and importance in both sexes, despite the higher read numbers in male subjects.

Graph 32. *Absolute (A) and relative (B) abundances as calculated by MicrobiomeAnalyst, by phylum and by sex.*

	Total read numbers		Relative abundances		p-values	FDR	LDA score
	F	M	F	M			
Actinobacteria	2628307	3765433	60.34%	62.00%	2.47E-01	3.29E-01	-5.26
Proteobacteria	951602	446337	21.85%	7.35%	7.40E-09	2.96E-08	5.89
Firmicutes	575608	1559421	13.21%	25.68%	4.62E-05	9.24E-05	-5.75
uncl. Bacteria	200484	302035	4.60%	4.97%	9.65E-01	9.65E-01	-4.35
TOTAL	4356001	6073226					

Table 22. *Results of taxa abundance and LefSe analyses, by sex at the phylum level.*

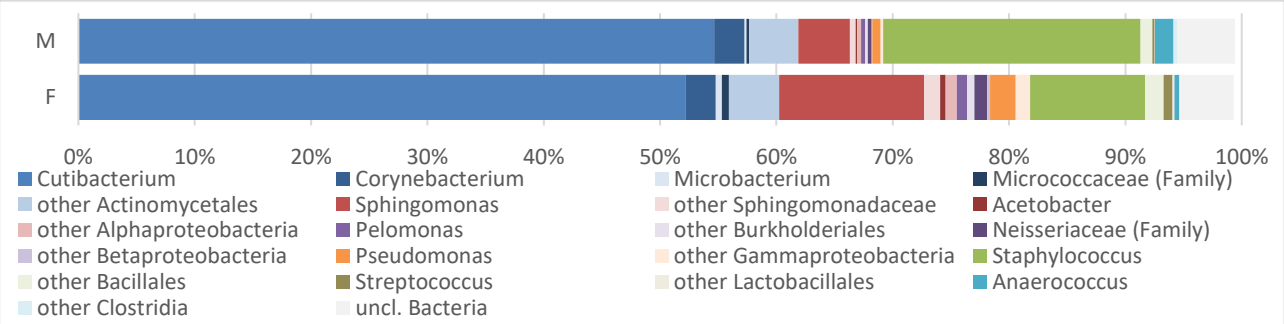
The focus was then put on the 10 most abundant genera, as, together, they already represent over 80 % of the microbiota's bacterial component (Table 23 and Graph 33). In the interest of clarity, the close parent taxa with % <0.45 for both groups were removed from the graphical representation (proportion of *Acetobacter*, 10th most abundant genera). Among these 10 taxa, three genera belonged to the Actinobacteria phylum (*Cutibacterium*, *Corynebacterium*, and *Microbacterium*); four were *Proteobacteria* – two *Alphaproteobacteria* (*Sphingomonas* and *Acetobacter*), one *Betaproteobacteria* (*Pelomonas*), and one *Gammaproteobacteria*

(*Pseudomonas*); three were *Firmicutes* – *Staphylococcus* (*Bacillales* order), *Streptococcus* (*Lactobacillales* order), and *Anaerococcus* (*Clostridia* class).

PHYLUM	CLASS	ORDER	FAMILY	GENUS	F	M
Actinobacteria	Actinobacteria	Actinomycetales	Propionibacteriaceae	Cutibacterium	52.18%	54.65%
			Corynebacteriaceae	Corynebacterium	2.60%	2.61%
			Microbacteriaceae	Microbacterium	0.54%	0.16%
				Micrococcaceae (Family)	0.61%	0.23%
				other Actinomycetales	4.30%	4.25%
Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Sphingomonas	12.48%	4.41%
				other Sphingomonadaceae	1.38%	0.50%
		Rhodospirillales	Acetobacteraceae	Acetobacter	0.45%	0.14%
				other Alphaproteobacteria	0.97%	0.33%
	Betaproteobacteria	Burkholderiales	Comamonadaceae	Pelomonas	0.88%	0.35%
				other Burkholderiales	0.62%	0.23%
		Neisseriales		Neisseriaceae (Family)	1.11%	0.33%
				other Betaproteobacteria	0.25%	0.06%
	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	2.20%	0.71%
				other Gammaproteobacteria	1.24%	0.23%
Firmicutes	Bacilli	Bacillales	Staphylococcaceae	Staphylococcus	9.88%	22.12%
				other Bacillales	1.58%	1.00%
		Lactobacillales	Streptococcaceae	Streptococcus	0.77%	0.21%
				other Lactobacillales	0.19%	0.03%
	Clostridia	Clostridiales	Clostridia Incertae Sedis XI	Anaerococcus	0.40%	1.59%
				other Clostridia	0.08%	0.30%
				uncl. Bacteria	4.60%	4.97%
				TOTAL	99.31%	99.41%

Table 23. *Relative abundance of the most abundant genera and of their most representative parent taxa.*

The relative taxa abundance analysis is coherent with the results gotten with the low Shannon alphadiversity values, since they show that only four genera account for more than 75% of the bacterial flora in both sexes: *Cutibacterium* and *Corynebacterium* (*Actinobacteria*), *Sphingomonas* (*Proteobacteria*), and *Staphylococcus* (*Firmicutes*). They also align with the literature, with *Cutibacterium* spp. reportedly accounting for about 50% of all microbial flora for both sexes (Grice et al. 2009), and Staphylococcus spp. next-off overall (Lee et al. 2021). Our results however demonstrate that, if the latter is indeed second for men, it is trumped from women by *Sphingomonas*, an *Alphaproteobacteria* which has rarely been described as a common member of the basal microbiome of the skin (Cundell 2018).



Graph 33. *Bar graph of the most abundant genera and their most representative parent taxa.*

Actinobacteria members generally thrive in the oily environment of the sebaceous areas (such as the face, back, and chest) (Grice et al. 2009), as evidenced by the high percentages of *Cutibacteria* and *Corynebacteria* spp. (the fourth most abundant genus). The LEfSe analysis (Table 24 and Graph 34) confirms that these two taxa are not affected by the sebum level variations, but it might impact the proportions of other aerobic or facultative anaerobic bacteria (here: *Staphylococci*, *Sphingomonas*, *Pseudomonas*, *Anaerococci* and *Microbacteria* spp.).

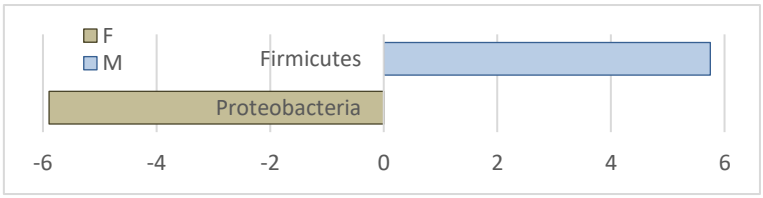
For example, the *Anaerococcus* genus has been described in the cutaneous microbiota and associated to bacteraemia (Cobo and Navarro-Marí 2020), but we expect here that its superior abundance in men is related to its anaerobic properties (J. C. Lagier et al. 2012) rather than to a diffused diseased state.
In the case of *Staphylococcus*, its higher fraction in the male microbiota should on the contrary be attributed to the greater sweat secretion in men, as it produces preferred nutrients that counteract the oily environment (Luebberding, Krueger, and Kerscher 2013). Similarly, the larger proportion of *Microbacterium* in women may hand be attributed to their lower sweat production and thus salt levels, as its growth is reportedly inhibited by high concentrations (Mounier et al. 2007). But it might also be explained by the presence of heavy metals in make-up products as well, as their presence in certain formulations is currently of utmost concern in the cosmetic industry (Corretto et al. 2020).
Although *Lactobacillus* members were expected in its place (unpublished results, (Zheng et al. 2021; Kong et al. 2012)), the *Streptococcus* genus was the only *Firmicute* showing lower proportions in men than in women: these results suggest that members of the *Lactobacillales* order adopt the same behaviour independently of their genera, remaining in line with other reports (Zheng et al. 2021; Leung et al. 2018).

The case of the *Proteobacteria* component was a bit more complex as their commensal role in the cutaneous skin microbiota is scarcely documented. Most of this phylum's members are in fact commonly accepted as environmental bacteria and often reduced to contaminants, but their consistent presence in our analyses may confirm that the frequent depletion and repopulation of the microbiota in women leads to exposome-driven bacteria recolonization in the more exposed skin areas.
First, while *Sphingomonas* (along with *Acetobacter* and most members of the *Alphaproteobacteria* class) is traditionally linked to plant colonization (D. C. White, Suttont, and Ringelberg 1996), only recent works identified it as a typical skin inhabitant (Hwang et al. 2021; Robert et al. 2022). Because it may play a role in maintaining the microbiome's balance in the presence of pollutants (Leys et al. 2004), we believe this aspect should be further investigated as a possible marker of rural vs. urban environments.
Similarly, the *Pelomonas* genus was reported just a short time ago, but as a core commensal of the dermal compartment rather than of the skin surface like in our case (Bay et al. 2020), and we believe it travelled with the keratinocytes during their migration towards the outer-most epidermal layers.

Finally, the *Pseudomonas* members are widely reported as common members of the normal adult gut microbiota – despite often being reduced to its pathogenic members (Laughlin et al. 2000) – but have also been described in and on the skin in the past years (J. H. Kim et al. 2021; Fujii et al. 2014; Bay et al. 2020). Here again, we believe it was transported from the deeper cutaneous compartments during the natural skin regeneration processes.

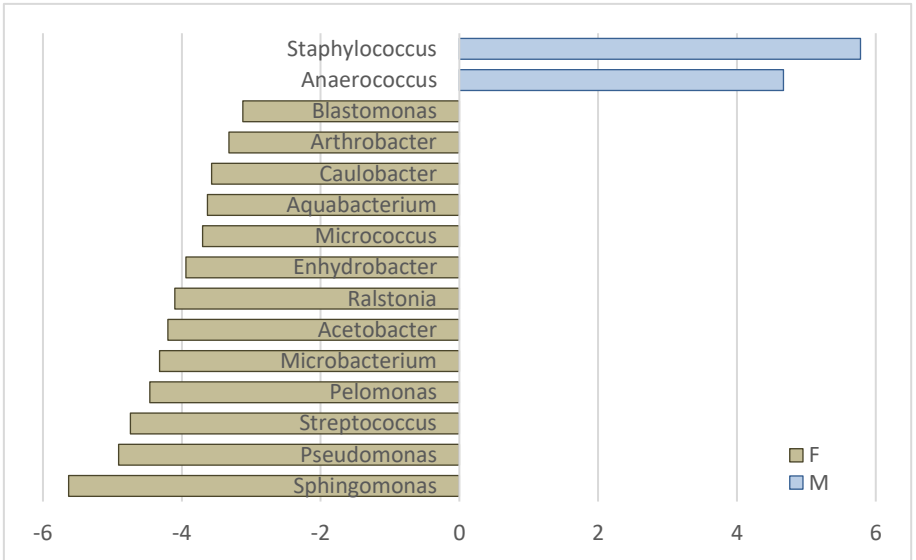
II.1.2.2. LefSe analysis of signature taxa

Beyond the observation of the relative taxa abundances differences, we performed a LEfSe analysis at the phylum (Table 22 and Graph 34) and genus levels (Table 24 and Graph 35) to identify the statistically different members (|LDA score|> 2.0, p-value <0.05) and to estimate the effect size of each highlighted taxon (J. H. Kim et al. 2021). The similar representation of *Actinobacteria* in both sexes is confirmed by the high p-value (p = 0.247) and confirms the removal of bias linked to read numbers. Likewise, the absolute abundances differences for *Proteobacteria* and *Firmicutes* (p = 7.40E-09 and p = 4.62E-05, respectively) confirm their involvement in the variations of our study groups' community composition.



Graph 34. *LDA scores at the phylum level, given by the LefSe analysis and shown for p<0.05.*

The same statistical analysis was performed at each lower taxonomic level (class, order, family; data not shown) down to the genus level (Graph 35) and the results overall followed the same trend – except from the order level down, where *Bacillales* and *Lactobacillales* stop adopting the same behaviour.



Graph 35. *LDA scores at the genus level, given by the LefSe analysis and shown for p<0.05.*

The genus-level LEfSe analysis determined that 15 out of the total 22 identified genera have significantly different importance from one group to the other, with p-values well below the 0.05 threshold (when applicable, Graph 35 and Table 24). More importantly, almost each of the 10 most abundant genera showed sex-specific variations (Table 24, in black), and other members of the same family frequently accompanied the *Proteobacteria* entities (*Blastomonas* and *Sphingomonas*, *Aquabacterium* and *Pelomonas*, and *Enhydrobacter* and *Pseudomonas*). Similarly, at least one member of the same order is represented along four of our genera of interest: *Ralstonia*, *Pelomonas*, and *Aquabacterium* belong to the *Burkholderiales* order, and *Cutibacterium*, *Corynebacterium*, and *Microbacterium* are *Actinomycetales*. Interestingly, two *Micrococcaceae* were also identified in the *Actinomycetales* order (*Arthrobacter* and *Micrococcus*) despite none being highlighted as a major actor, confirming the relevancy of the family's presence in data analysis.

To confirm the outcomes of the LEfSe analysis, a heat tree analysis was carried out at the genus level and evidenced the same taxa, categorizing them in the same order with similar *p*-values (data not shown). *Staphylococcus* and *Anaerococcus* were confirmed as more prevalent in men than in women, while the other taxa consistently showed higher proportions in women. The graphical representation (data not shown) also highlighted the fact that the disparities between males and females were caused by the female group's higher bacterial diversity.

			Median read number		
	***p*-values**	**FDR-corrected**	**F**	**M**	**LDA score**
Microbacterium [A]	3.63E-09	5.72E-08	55004	13319	-4.32
Ralstonia [β‡]	4.67E-09	5.72E-08	36469	11060	-4.1
Arthrobacter [A*]	7.53E-09	7.38E-08	5514.1	1367.8	-3.32
Sphingomonas [α•˚]	1.35E-08	9.34E-08	1239200	383550	-5.63
Blastomonas [α•˚]	2.41E-08	1.07E-07	3575.1	947.4	-3.12
Pelomonas [β‡†]	4.35E-08	1.78E-07	92260	34183	-4.46
Staphylococcus [FB]	7.02E-07	2.29E-06	1088700	2297500	5.78
Caulobacter [α]	1.87E-05	4.16E-05	9776.9	2312.5	-3.57
Micrococcus [A*]	5.88E-05	1.25E-04	13784	3772.4	-3.7
Pseudomonas [γp]	8.32E-05	1.70E-04	207110	43019	-4.91
Aquabacterium [β‡†]	1.04E-04	1.91E-04	14808	6208.9	-3.63
Enhydrobacter [γp]	1.05E-04	1.91E-04	20008	2465.2	-3.94
Streptococcus [FL]	2.03E-04	3.55E-04	132430	23639	-4.74
Acetobacter [α]	2.91E-03	4.60E-03	48747	17091	-4.2
Anaerococcus [FC]	3.54E-03	5.26E-03	52957	146280	4.67
Cutibacterium [A]	1.68E-01	2.17E-01	5004700	5385600	5.28
Corynebacterium [A]	5.73E-01	6.53E-01	301120	349850	4.39

Table 24. *Results given by the LefSe analysis at the genus level. [A] Actinobacteria (phylum): * Micrococcaceae (family); [α] Alphaproteobacteria (class): • Rhodospirillales (order), ˚ Sphingomonadaceae (family); [β] Betaproteobacteria (class): ‡ Burkholderiales (order), † Comamonadaceae (family); [γ] Gammaproteobacteria (class): [p] Pseudomonadales (order); [F] Firmicutes: [B] Bacillales (order), [L] Lactobacillales (order), [C] Clostridiales (order).*

Overall, the identification of only three phyla is in line with published results (Grice and Segre 2011; Ederveen et al. 2020), but while we could demonstrate their statistical relevance in our cohort, the scientific community disagrees on whether men and women host similar cutaneous microbiotas. Indeed, some works noted some variations on the hands and across the whole body as well (Noah Fierer et al. 2008; Ying et al. 2015), but others contradict these conclusions, locally and globally (Si et al. 2015; Oh et al. 2012; Leung et al. 2018).

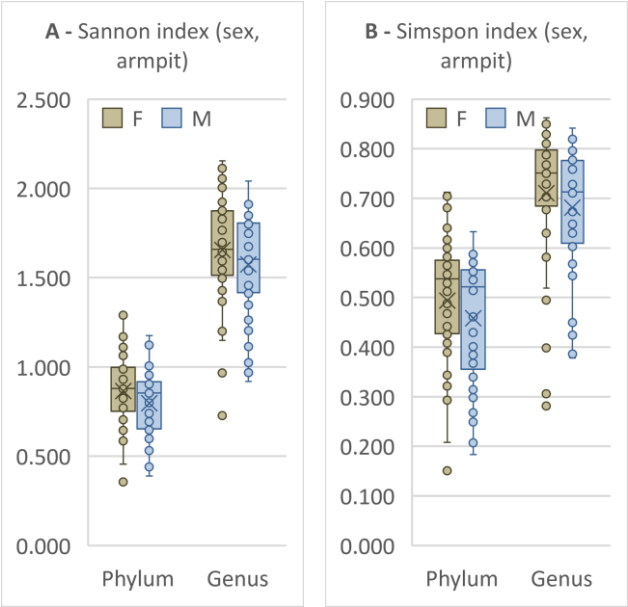
Generally, *Corynebacteria* and *Micrococci* spp. are considered ordinary minor members of the microbiota (Cundell 2018), but our results strongly suggest that the other low-abundance members should be equally investigated. The research presented here indeed concluded that *Lactobacilli* spp. may be substituted by *Streptococci* spp. in the facial microbiome, and that *Proteobacteria* and *Clostridia* taxa warrant further investigations. Finally, LefSe results at the family level (data not shown) evidenced the two families *Micrococcaceae* and *Neisseriaceae* (data not shown) as mathematically more predominant in women, but no descendants were recognised by the MicrobiomeAnalyst tool. While it might be a consequence of the traditionally low resolution of 16S rRNA metagenomic studies, we believe in this case that a lack of reference genomes is at fault.

II.1.3. Influence of the sex on other districts

Putting the focus on the cheek, we could observe that the differences in community structure between males and females reflect the physiological and anatomical distinctions between the two sexes (J. H. Kim et al. 2021; Skowron et al. 2021). But are all geographical niches similarly affected? Different skin areas indeed have different exposomes, and the zones more often in contact with the external environment (hands, face) even demonstrated increased fluctuations compared to more sheltered areas (armpit, navel) (Moskovicz, Gross, and Mizrahi 2020). Moreover, the microbiota composition is highly dependent on local microenvironment: *Proteobacteria* were reported to inhabit principally dry sites, while moist sites can be dominated by several bacterial types (either *Corynebacteria* spp., *Staphylococci* spp., or *Proteobacteria*), and sebaceous areas are generally mostly composed of *Cutibacteria* spp. (Byrd, Belkaid, and Segre 2018; Grice and Segre 2011; Grice et al. 2009). Systematically surveying multiple skin sites is therefore instrumental to determine the ubiquity of taxa across various body habitats, and a central component for studying the microbial community diversity in "normal", healthy individuals to then understand how each body region support alternative microbial communities (Li et al. 2012) (Grice et al. 2009) (Oh et al. 2014).

II.1.3.1. Armpit

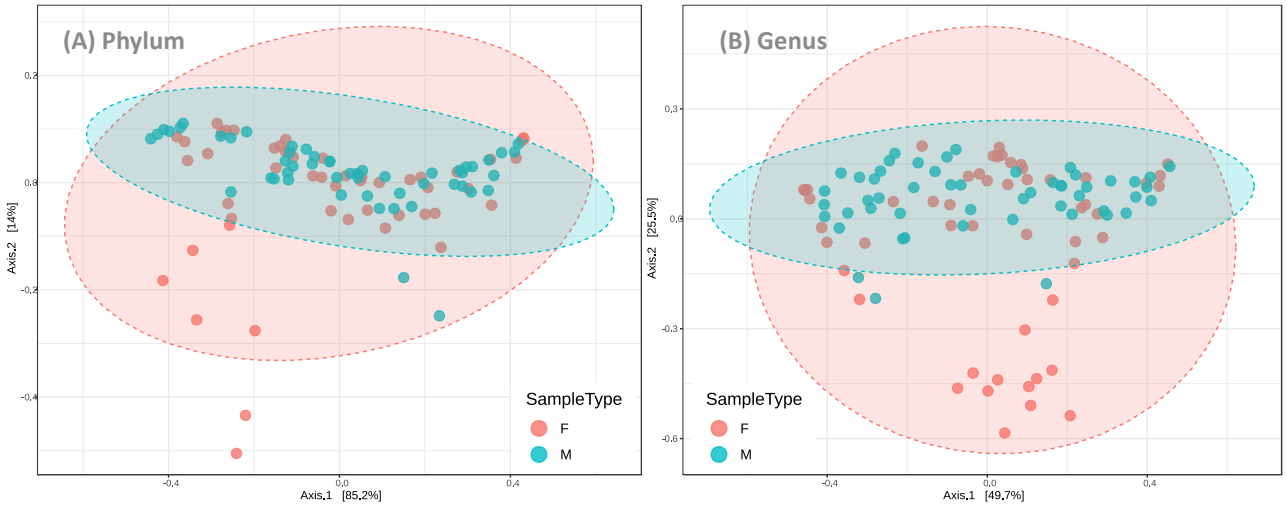
From our samples' library, we were able to match 52 samples one-to-one – following the same approach as we did for the cheek – and thus to obtain statistically sound results (Table 25 and 26, and Graphs 36, 37, 38).
The alpha- and betadiversity calculations revealed much less differences than when studying the facial microbiome: here, only a few taxonomic levels show statistically relevant numbers. The high *p*-values of the Shannon index (Table 25 and Graph 36A) imply that both sexes host the same taxa richness, while the Simpson index values suggest some differences in genus prevalence (Graph 36B and Table 25). The betadiversity results seem to confirm this hypothesis, as *p* is below the 0.05 threshold for both the family and genus levels and thus indicates structural community differences.



	alpha		beta
	Shannon	Simpson	
PHYLUM	7.65E-02	1.50E-01	0.208
CLASS	1.26E-01	9.67E-02	0.089
ORDER	1.55E-01	1.13E-01	0.089
FAMILY	3.78E-01	2.73E-01	**0.012**
GENUS	6.16E-02	**5.00E-02**	**0.005**

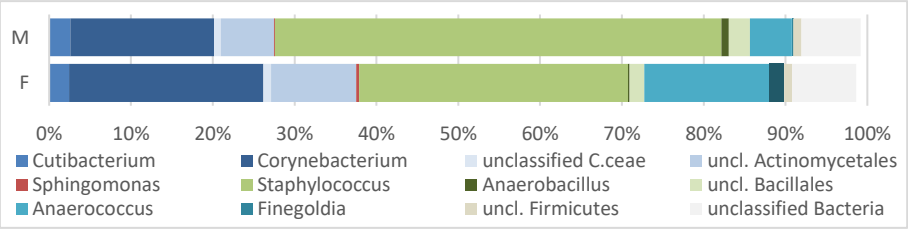
Table 25. *Statistical diversity values, at the sample level (alphadiversity) and the group level (betadiversity).*

Graph 36. *Graphic representation of the α-diversity at the phylum and genus taxonomic levels, calculated using the Shannon (A) and Simpson (B) indices and depending on the sex. a.u.=arbitrary unit.*



Graph 37. *Graphic representation of β-diversity at the phylum and genus taxonomic levels, on the armpit and depending on the sex.*

The taxa abundance analysis (Graph 38 and Table 26) shows a completely different microbiota structure in the armpits compared to the face: *Cutibacteria* spp. hold much less ground and *Proteobacteria* almost completely disappeared, while the *Staphylococcus* genus is ever more prevalent, and *Corynebacterium* and *Clostridia* members show considerably higher proportions. These obvious differences between the two niches highlight the importance of analysing the different body areas separately.



Graph 38. *Bar graph of the seven identified genera and their most abundant parent taxa, on the armpit and based on the sex.*

For a long time, *Corynebacterium* was suspected of being a significant constituent of the normal flora, but its role has been underappreciated until the advent of NGS techniques, as they are extremely fastidious and slow-growing organisms (McGinley et al. 1985; Roth and James 1988; Grice and Segre 2011). As we can see here, *Corynebacteria* spp. definitely constitute a large proportion of the bacterial branch of the microbiota and although they tolerate anaerobic environments, they seem more adapted to the armpit area (Roth and James 1988): similar to the Staphylococci, they thrive in salt-rich and humid environments (Luebberding, Krueger, and Kerscher 2013). *Cutibacteria* spp. are reportedly depleted in moist skin sites, and their low proportions is associated with lower pHs that favours the growth of opportunistic *Staphylococci* (Larson et al. 2022). The *Anaerococcus'* genus members are usually better suited to anaerobic environments, but their higher percentage here could suggest that, on the face, the monopoly of *Cutibacteria* spp. prevents the growth of other bacteria.

PHYLUM	CLASS	ORDER	FAMILY	GENUS	F	M
Actinobacteria	Actinobacteria	Actinomycetales	Propionibacteriaceae	Cutibacterium	2.42%	2.61%
			Corynebacteriaceae	Corynebacterium	23.78%	17.57%
				uncl. C.ceae	*0.94%*	*0.82%*
				uncl. Actinomycetales	*10.39%*	*6.47%*
Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Sphingomonas	0.37%	0.13%
Firmicutes	Bacilli	Bacillales	Staphylococcaceae	Staphylococcus	32.87%	54.55%
			Bacillaceae 1	Anaerobacillus	0.20%	0.92%
				uncl. Bacillales	*1.77%*	*2.56%*
	Clostridia	Clostridiales	C. Incertae Sedis XI	Anaerococcus	15.30%	5.13%
				Finegoldia	1.81%	0.17%
				uncl. Firmicutes	*0.98%*	*1.02%*
				unclassified Bacteria	*7.83%*	*7.21%*
				TOTAL	**98.64%**	**99.17%**

Table 26. *Relative abundance results of the 7 identified genera and of the parent taxa of interest (Σ>1%).*

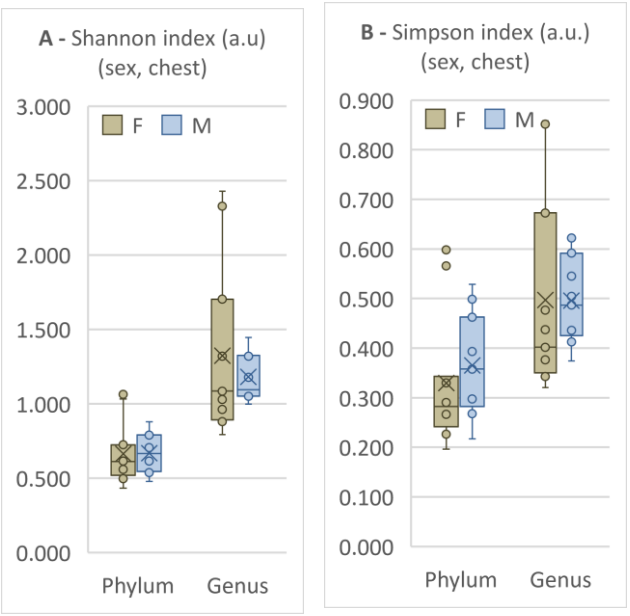
In accordance with the general lack of significant differences in biodiversities (whether at the taxa or community level), the LEfSe analysis calculated only borderline *p*-values (Table 27). Interestingly, those two values regard the two most different genera from the facial microbiome study: women show higher levels of *Sphingomonas*, while men host more *Staphylococcus*, suggesting that the fundamental physiological differences between men and women have the same impact over all areas of the skin microbiota.

PHYLUM	CLASS	ORDER	FAMILY	GENUS	LEfSe *p*-value	FDR-corrected	LDA score
Actinobacteria	Actinobacteria	Actinomycetales	Propionibacteriaceae	Cutibacterium	7.70E-02	2.48E-01	-5.56
			Corynebacteriaceae	Corynebacterium	3.33E-01	5.02E-01	4.97
				unclassified C.ceae	*1.51E-01*	*3.39E-01*	*4.56*
				uncl. Actinomycetales	*4.63E-01*	*5.95E-01*	*5.31*
Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Sphingomonas	5.25E-02	2.48E-01	-5.08
Firmicutes	Bacilli	Bacillales	Staphylococcaceae	Staphylococcus	5.60E-02	2.48E-01	5.62
			Bacillaceae 1	Anaerobacillus	6.91E-01	8.29E-01	4.02
				uncl. Bacillales	*1.22E-01*	*3.13E-01*	*4.57*
	Clostridia	Clostridiales	C. Incertae Sedis XI	Anaerococcus	8.91E-01	8.91E-01	-4.94
				Finegoldia	2.56E-01	5.02E-01	-4.91
				uncl. Firmicutes	*2.95E-01*	*5.02E-01*	*3.92*
				unclassified Bacteria	*3.63E-01*	*5.02E-01*	*-4.87*

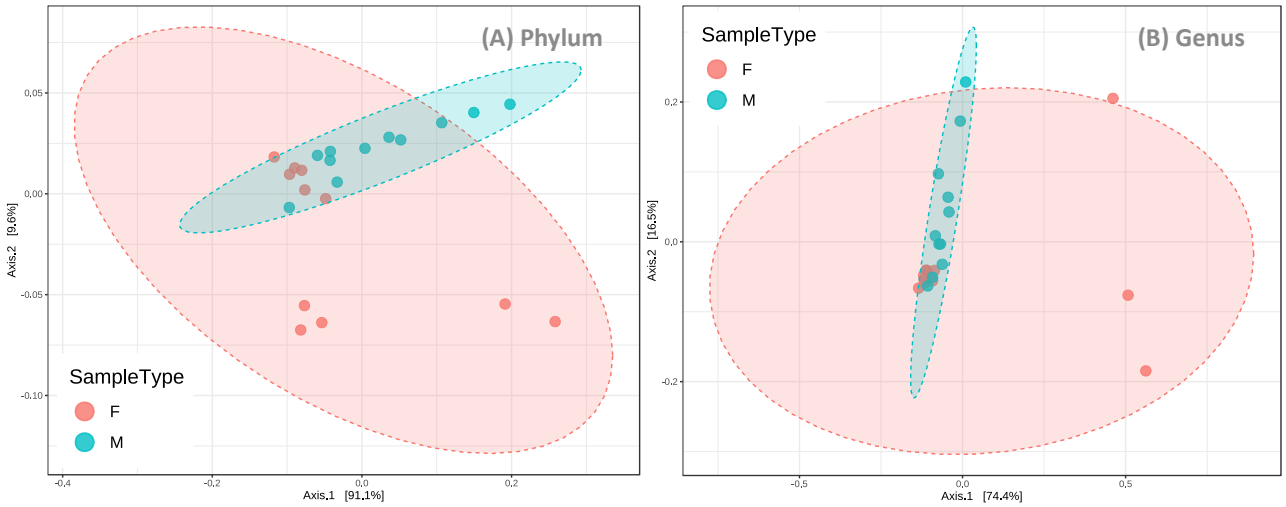
Table 27. *LEfSe analysis results of the 7 identified genera and of their parent taxa of interest (Σ>1%).*

II.1.3.2. Chest

Because the chest is one of the lesser-sampled niches, we could match by age/sampling season/living area only 22 samples in total. Therefore, the following results should be understood as trends rather than statistically accurate data. In fact, this very small cohort might explain why the alpha- and biodiversities analyses did not highlight any difference between the two groups (data not shown), when some are visible on the associated Graphs 39 and 40, and the LEfSe analysis identified some statistically relevant differences (Graph 41).



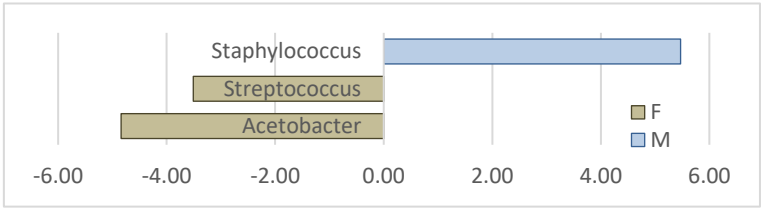
Graph 39. *Graphic representation of the alphadiversity at the phylum and genus taxonomic levels, calculated using the Shannon (A) and Simpson (B) indices and depending on the sex. a.u.=arbitrary unit.*



Graph 40. *Graphic representation of β-diversity at the phylum and genus taxonomic levels, on the upper chest and depending on the sex.*

Nevertheless, the result overall underline the same taxa and trends as previously observed on the face and the armpits. The *Staphylococcus* genus has a major abundance in men, while women show higher percentages of *Streptococci* spp. and *Alphaproteobacteria* (here, *Acetobacter* instead of *Sphingomonas*; data not shown). As expected from the sebaceous nature of the manubrium area (Grice and Segre 2011), the general taxa

distribution quite resembled the facial microbiome but not the armpit's at all – notoriously moist and marginally oily (data not shown).



Graph 41. *LDA scores at the genus level, given by the LefSe analysis and shown for p<0.05.*

Despite not being able to continue the comparison with the thigh and groin areas for lack of men samples, we were able to confirm the major variations in microbiota composition depending on the skin area and their microenvironment: the sebaceous face and chest against the moist armpit (Grice and Segre 2011; Roth and James 1988). We also witnessed that more exposed areas show greater bacterial diversity (J. H. Kim et al. 2021), probably through environmental contamination. But most importantly, we pinpointed sex-specific patterns across several, unrelated body sites.

The male microbiota is typically less diverse than the female's, and while it may be linked to the generally higher sebum levels in men (Skowron et al. 2021), the more frequent use of moisturizer by women – and the consequent major water retention – could be a biodiversity driving force (Y. Belkaid and Segre 2014). In the future, these results should allow to better understand if the men's and women's microbiomes behave similarly under comparable circumstances.

II.2. Influence of the age on the female microbiome

Over the years, the skin surface's physiology is altered by the normal cellular senescence mechanisms (minor sebum and sweat production, immune function degradation) that strongly impact lipid composition, sebum secretion, and pH (Ratanapokasatit et al. 2022). In fat, the hormonal changes over one's lifetime and their physiological repercussions (particularly in women) are also well documented, and their influence on the microbiota is documented since a few years as well (Ying et al. 2015; H. J. Kim et al. 2019). In fact, the lack of significance of the "age" factor in our multivariate analysis (§ II.2) was quite unexpected, as recent works demonstrated significant correlations between the subjects' age and the facial microbiota variations in women (Howard et al. 2022).

To elucidate these discrepancies, we modified our initial approach and decided on three age groups to allow for larger sample numbers: <30 years old to figure the post adolescent and early adult years, 30-50 years old to represent the average mature adult, and >50 to model the more senior individuals. Unfortunately, almost all our male facial samples were collected in volunteers from the 30-50 range, and we could not compare the effect of the age between the two sexes.

II.2.1. Facial microbiome

The one-to-one sample correspondence by season and living location resulted in only 22 matches when considering all three categories. To increase the result accuracy, we decided to dive the study into two sub-groups, which gave us 86 matches for the <30 *vs.* 30-50 comparison and 39 for the 30-50 *vs.* >50 observation.

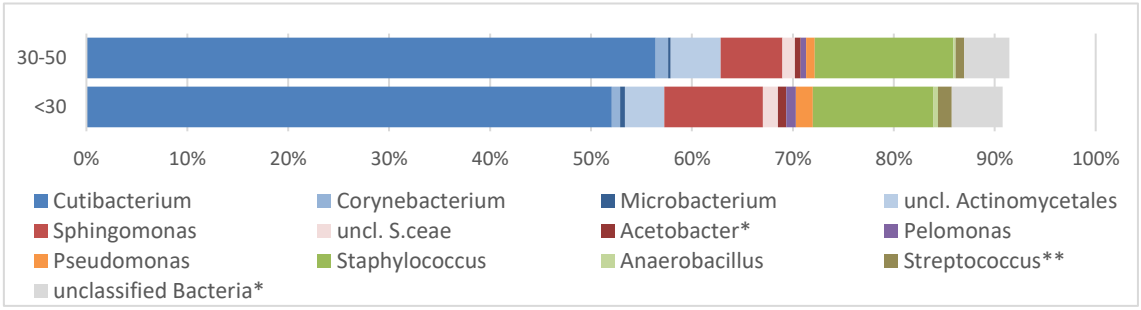
II.2.1.1. < 30 vs. 30-50 years old

The alphadiversity calculations did not detect consistent differences in taxa richness (Shannon index) and evenness (Simpson index) although the PERMANOVA approach (betadiversity) demonstrates statistical microbiome composition variations from one group to the other (Table 28). Interestingly, we can observe a trend in the absolute alphadiversity values where the younger subjects displaying a slightly higher taxa richness, which contradicts many reported results (Jugé et al. 2018; Shibagaki et al. 2017; J. H. Kim et al. 2021; Gratton et al. 2022).

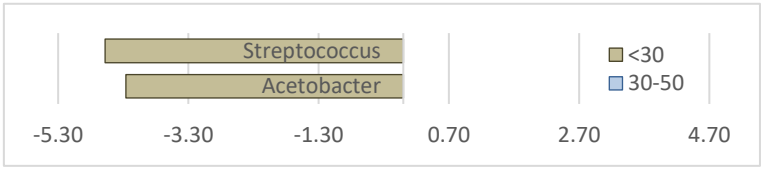
	alpha		beta
	Shannon	Simpson	
PHYLUM	1.15E-01	1.04E-01	**0.034**
CLASS	**3.61E-02**	**3.97E-02**	**0.044**
ORDER	**2.33E-02**	**2.91E-02**	**0.035**
FAMILY	**4.15E-02**	9.66E-02	**0.031**
GENUS	5.78E-02	1.17E-01	**0.028**

Table 28. *Statistical diversity values, at the sample level (alphadiversity) and the group level (betadiversity).*

The taxa abundance analysis (Graph 42 and Annex 4) suggests differences in *Cutibacterium* and *Sphingomonas* genera, but the LEfSe analysis (Graph 43 and Annex 4) revealed that the relevant variations ($p < 0.05$) concerned only the *Streptococcus* genera with borderline values for *Acetobacter*.



Graph 42. *Bar graph of the 10 most abundant genera genera and their relevant parent taxa, on the cheek and based on the age. ** both original p and corrected value <0.05; * only original p <0.05.*

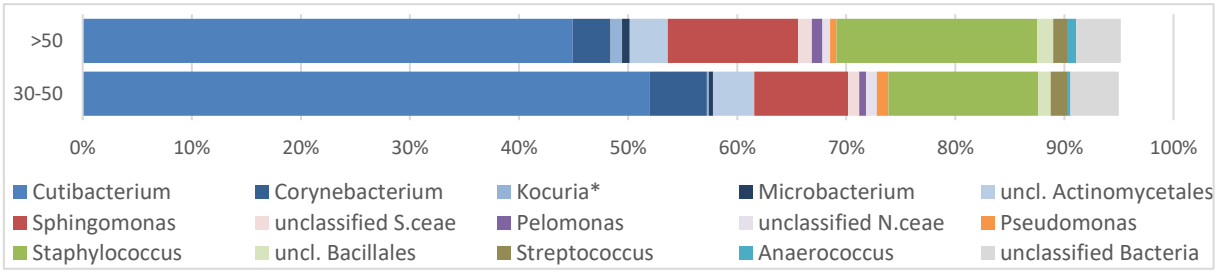


Graph 43. *Relevant LDA scores, given by the LefSe analysis.*

It is commonly accepted that *Streptococcus*, *Betaproteobacteria*, and *Gammaproteobacteria* dominate the microbial communities of children (Oh et al. 2012), but in young adults the composition shifts towards lipophilic microbes (*Corynebacterium*, *Cutibacterium acnes*, *Malassezia* fungi) (Park et al. 2022; Jo et al. 2016; Oh et al. 2012; Y. Belkaid and Segre 2014) as the hormones – driving the physical and sexual developments – directly promote structural and functional changes in the skin (particularly sebum and apocrine sweat production) (Gratton et al. 2022; Park et al. 2022). So far, most of our observation align with the literature, but we could not draw conclusions on the *Corynebacterium* variations, known to increase over time (Dimitriu et al. 2019; Alkema et al. 2021; Shibagaki et al. 2017; Jugé et al. 2018). Furthermore, while skin ageing is traditionally associated with lower *Cutibacterium* levels (Jugé et al. 2018; Howard et al. 2022; Shibagaki et al. 2017), our and others' results suggest otherwise (Alkema et al. 2021).

II.2.1.2. 30-50 vs. > 50 years old

No biodiversity analysis (alpha and beta, data not shown) could identify any significant difference between the two groups, on one hand confirming the stabilisation of the microbiota structure after the end of the puberty (Yasmine Belkaid and Hand 2014; Chen, Knight, and Gallo 2023) but ever more in contradiction with the latest descriptions of the western European populations' facial microbiome (Russo et al. 2023; Feuillie et al. 2018; Jugé et al. 2018; Alkema et al. 2021). Some variations in taxa preponderance can nevertheless be observed on Graph 44 despite irrelevant LEfSe results (Annex 5): the decrease in *Cutibacteria* spp. and increase of *Firmicutes* over time (particularly after menopause) is well documented (Somboonna et al. 2017; Alkema et al. 2021; Howard et al. 2022; H. J. Kim et al. 2019; Shibagaki et al. 2017; Jugé et al. 2018; Russo et al. 2023), but we still failed to observe higher percentages of *Corynebacteria* spp. (Dimitriu et al. 2019; Shibagaki et al. 2017; Russo et al. 2023).



Graph 44. *Bar graph of the 10 most abundant genera genera and their relevant parent taxa, on the cheek and based on the age. ** both original p and corrected value <0.05; * only original p <0.05.*

These underwhelming results reminded us of the difficulty to conduct large-scale microbiome studies and the critical need for precise study design. In fact, Large sample numbers cannot always account for the high degree of heterogeneity of the skin microbiome, even when matched for body site and sexual maturity (Kong et al. 2017). Beyond detailed subject screening (demographic data, medical history, habits…), the tremendous interindividual differences cutaneous microbiota composition are amplified by its susceptibility to many endo-

end exogenous factors (Kong et al. 2017), and areas as exposed as the face are much more likely to host highly variable microorganisms communities (Fredricks 2001; Grice and Segre 2011).

II.2.2. Other body districts

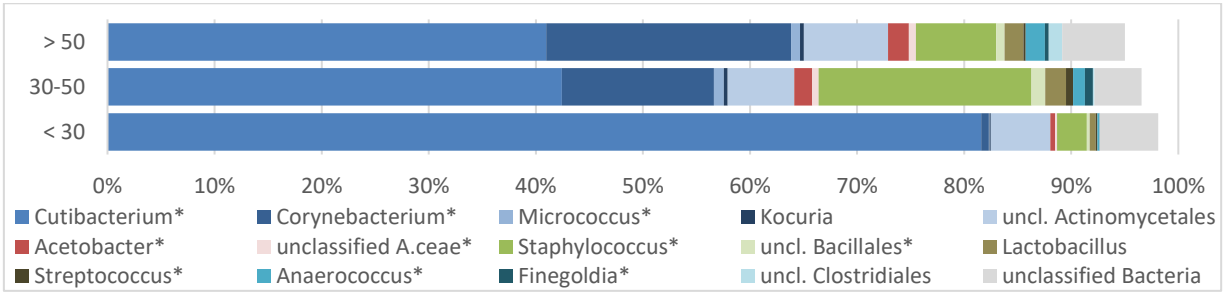
The same analyses were performed on the same age groups together for the upper chest and armpits areas, giving 6 and 11 matches (by season and leaving area) each. Although these numbers are low to obtain accurate, statistically sound results, we were able to determine variations in line with the literature for both niches (Table 29, 30, Graph 45, and Annexes 6 and 7).

	alpha		beta
	Shannon	Simpson	
PHYLUM	3.38E-03	3.22E-03	0.023
CLASS	3.38E-03	3.22E-03	0.015
ORDER	3.22E-03	2.94E-03	0.013
FAMILY	4.32E-03	4.58E-03	0.018
GENUS	4.32E-03	4.58E-03	0.020

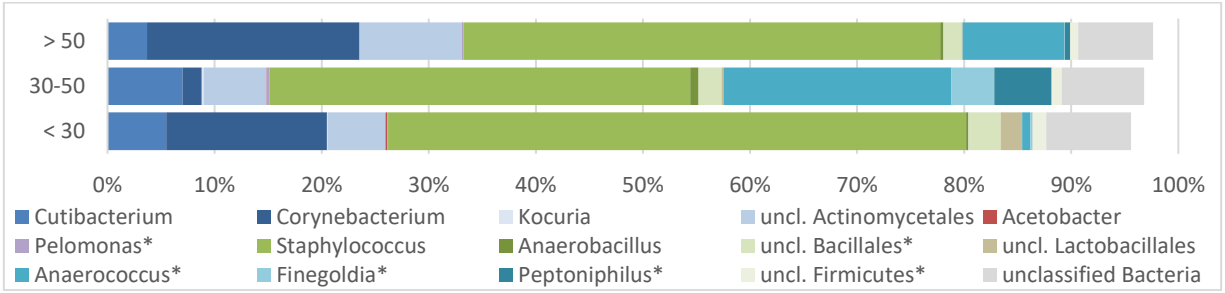
Table 29. *Statistical diversity values, at the sample (alphadiversity) and the group level (betadiversity), for the chest area depending on the age.*

	alpha		beta
	Shannon	Simpson	
PHYLUM	4.41E-02	2.20E-02	0.053
CLASS	3.78E-03	1.33E-03	0.007
ORDER	4.45E-03	1.07E-03	0.008
FAMILY	6.55E-02	1.50E-01	0.006
GENUS	4.73E-02	6.85E-02	0.006

Table 30. *Statistical diversity values, at the sample (alphadiversity) and the group level (betadiversity), for the armpits depending on the age.*



Graph 45. *Bar graph of the 10 most abundant genera genera and the 5 most abundant parent taxa, on the chest and based on the age. ** both original p and corrected value <0.05; * only original p <0.05.*



Graph 46. *Bar graph of the 10 most abundant genera genera and the 5 most abundant parent taxa, on the armpit and based on the age. ** both original p and corrected value <0.05; * only original p <0.05.*

In the case of the chest, the correlation with the literature is evident, as the percentages of *Cutibacterium* are visibly lower and those of *Corynebacterium* (and of most *Firmicutes*) are higher. Traditionally, the increased abundance of *Corynebacterial* taxa is associated to the an age-related decrease in the sebocyte gland area and an increase in the natural moisturizing factors (NMF), skin lipids, and antimicrobial peptides (AMPs), that hinder the growth of *Cutibacteria* spp. (and usually Lactobacillus members) in sebaceous areas (Dimitriu et al. 2019;

Russo et al. 2023; Howard et al. 2022). Moreover, NMFs both absorb water and can promote bacterial proliferation and adherence to the skin, explaining the common higher biodiversity in older subjects (Boireau-Adamezyk, Baillet-Guffroy, and Stamatas 2021; Feuillie et al. 2018; Grice and Segre 2011).

Regarding the armpit samples, we can readily observe an increase in the *Clostridia* taxa proportion with the increasing age while the *Staphylococcus* percentages are quite constant, aligning but not totally corresponding to existing reports (Somboonna et al. 2017; Alkema et al. 2021; Howard et al. 2022; H. J. Kim et al. 2019; Shibagaki et al. 2017; Jugé et al. 2018; Russo et al. 2023). Likewise, The *Actinobacteria* members have inconsistent behaviours: *Corynebacteria* lowers after microbiota maturation before going up again and *Cutibacterium* adopts the inverse comportment, when both should show the contrary (Dimitriu et al. 2019). Once again, this highlights the importance of the microenvironment and how different factors may not have the same impact, also because of the various growth conditions.

Preliminary results on the groin area (moist) supported the traditional increase of *Corynebacterium* and *Staphylococcus* (and *Cutibacterium* decrease) – contrarily to the armpit results – but the middle-age groups showed a higher diversity than the > 50 group. On the contrary, exploratory data from the upper thigh zone (dry) show similar biodiversities and compositions.

The inconsistency of the age impact across different areas strengthened our will to study microbiome-influencing factors depending on the sampled area and not only according to its general characteristics (geographical proximity, microenvironment type: sebaceous, dry, moist). Parallelly, the unexpected results obtained on the cheek area confirms the increased microbiota variability of high-exposure areas, and we decided to explore fluctuations linked to the sampling season – and thus the weather and temperature. In fact, we believe fundamental to understand or exclude the maximum of factors that might drive the cutaneous microbiota structure in order to achieve efficient study design and cohort assembly.

III. Exogenous factors

Because the epidermis constitutes the first line of defence against environmental stressors, its microbiome is necessarily subjected to meteorological factors, solar radiation, pollution, tobacco smoke, … (Passeron et al. 2021). All are suspected to contribute to skin microbiota variations (Dimitriu et al. 2019; Townsend and Kalan 2023), and transient organisms have even become established as resident flora when individuals were consistently exposed (Price 1938; Roth and James 1988; Nielsen and Jiang 2019).

Despite having access to about 1000 samples from various body sites, organise then into representative subsets that allow accurate and statistical comparisons revealed more complex than anticipated. For evaluating the effect of extrinsic element on the skin microbiota structure, we decided to ignore the age factor – since the results in our panel were overall inconclusive, particularly on the face – and focus on arranging our subsets of samples first by sex, and then either by living area (for the seasonality influence) or by season (for the living area effect).

III.1. Seasonality

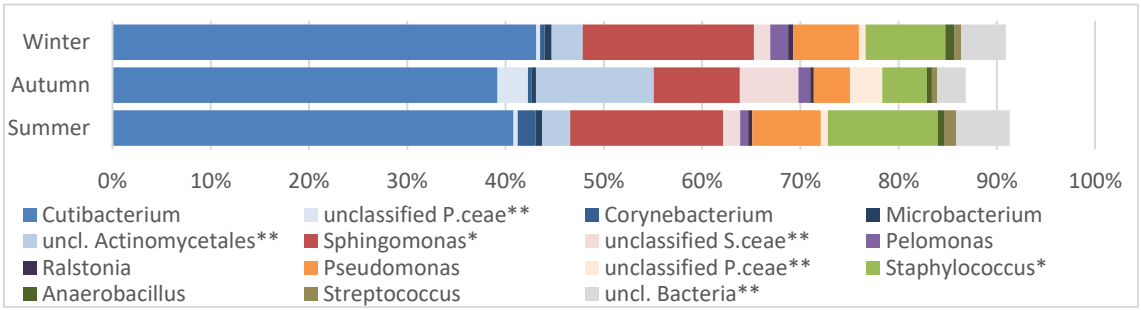
The temporal variability of the skin microbiome is known to be dependent on the site sampled, as more exposed areas (such as the face) commonly display more inconsistent compositions over time (Grice and Segre 2011). In fact, in areas constantly exposed to the external environment, the seasonal variations in ambient temperature and humidity may alter the density of bacterial colonisation and alter the relative ratios of organisms, as their combined action knowingly causes changes in the normal flora distribution (Boxberger et al. 2021; J. H. Kim et al. 2021; Duncan, McBride, and Knox 1969). In fact, we expect to find higher proportions of halo- and hydrophilic bacteria in the summer samples, while the winter ones should demonstrate lower biodiversity and higher percentages of lipophilic organisms, and the spring and autumn samples might have similar composition as the weathers are comparable between these two seasons.

III.1.1. Women

III.1.1.1. Face

First, we could match 45 female cheek samples collected in our Pavia, Italy POC over three seasons (Summer, Autumn, and Winter) across all ages. Of note, the Autumn and Winter samples were collected only in younger and middle-aged subjects (all < 50 years old), while the summer samples were collected for all ages over 18. Considering the results obtained in paragraph II.2.1.1., any particularity related to the *Streptococcus* and *Acetobacter* genera should be carefully interpretated.

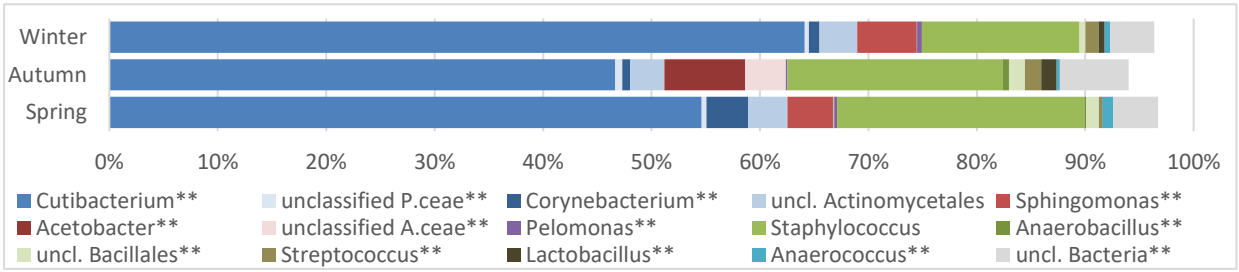
The alphadiversity analyses (data not shown) indicated that all groups had similar taxa richness and evenness (Shannon and Simpson indices, respectively: all *p*-values > 0.05) but the beta diversity revealed some structure differences, although only at the family ($p = 0.023$) and genus ($p < 0.001$) levels. While many genus abundances varied across the groups (Graph 47), the LEfSe analysis showed relevance only for *Staphylococcus* and *Sphingomonas*, while *Pseudomonas* had borderline values (Annex 8).



Graph 47. *Bar graph of the 10 most abundant genera genera and the 5 most abundant parent taxa, on the cheek and based on the season (women, Pavia POC). ** both original p and corrected value <0.05; * only original p <0.05.*

Since the *Acetobacter* and *Streptococcus* genera did not reveal statistical differences, it apparently confirms the lack of relevance of the age factor in this approach. Interestingly, the summer and winter microbiomes seem to resemble each other more than each to the autumnal microbiome. Other than melanocytes stimulation and potential DNA damage, solar radiations are known to promote oxidative stress and inflammation, eventually leading to decreased immunity against microbial challenges, and barrier function and microbiome alteration (Passeron et al. 2021). In addition, the size of the bacteria does not allow efficient photoprotection against solar radiation, which makes them particularly vulnerable to acute exposition since their genetic material represents a significant portion of their total volume (Harel et al. 2022; Garcia-Pichel 1994; Jeffrey et al. 1996). We would therefore expect that the summer microbiome would exhibit proofs of this action, but it rather seems to have impacted the autumn samples. In fact, while one could speculate that most members of the microbiome have evolved with their host/niche and so seasonal changes are generally easily tolerated (Harel et al. 2022), these preliminary results could indicate a delayed effect of the summer solar exposition on the exposed skin microbiota.

We could assemble a second cohort from another one of our POC (Biella, Italy), gathering 63 cheek samples collected in Winter, Autumn, and Spring (Graph 48 and Annex 9). There again, we could match the samples only by sampling locations and not by age, as the autumn and winter specimens were sampled from volunteers younger than 50 years old, while the spring samples belonged to women over 30 years old: we should therefore keep an eye out for fluctuations involving Kocuria (*cf.* § II.2.1.2) and Acetobacter and Streptococcus (*cf.* § II.2.1.1).



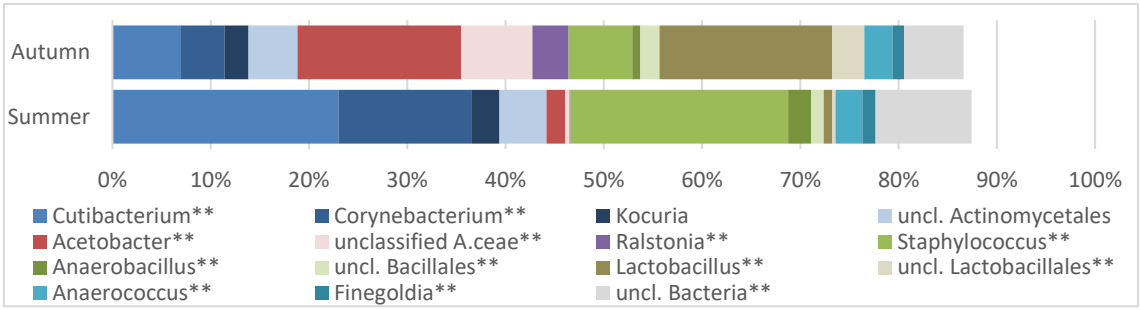
Graph 48. *Bar graph of the 10 most abundant genera genera and the 5 most abundant parent taxa, on the cheek and based on the season (women, Biella POC). ** both original p and corrected value <0.05; * only original p <0.05.*

Similar to the results from the Pavia POC, the autumn group was the more different while the two others were more alike, and seemed in both cases to be characterised by lower percentages of *Cutibacteria* spp. Because *Acetobacter* is virtually absent from the winter and spring groups, we do not believe its variation to be related to the age factor. In the case of *Streptococcus* however, we clearly see that it is depleted in the spring samples (collected only in women > 30 y.o.), suggesting this behaviour is more likely due to skin ageing than to seasonality.

Overall, each genus (except *Staphylococcus*) displayed statistically different populations and all biodiversity calculations came out relevant (LEfSe analysis, Annex 9), with the graphical representations suggesting that the winter samples had the lower taxa richness and evenness, followed by the spring group, and with the autumn specimens showing the higher biodiversity (data not shown). This would go with our above hypothesis that weather-induced microbiome variations are triggered by the summer conditions and effective in autumn, and that microbiota composition returns to its baseline during the winter and remains overall stable across the spring and summer.

III.1.1.2. Other districts

Since some cutaneous areas are more exposed than others, we were curious to see if the seasonality similarly affected more sheltered niches. We focused on leg samples that were collected on the upper thigh (therefore rarely exposed to the environment) and could arrange together 70 samples collected in Autumn and summer in our Pavia POC. Taxa richness and evenness showed $p < 0.05$ only for lower taxonomic levels but the two groups showed differences in community structure at all taxonomic levels (data not shown). Again, autumnal samples showed an overall higher diversity, in line with the results obtained both in the same POC but on the face (§ II.2.1.1) and on the face in a different POC (§ II.2.1.2). This higher biodiversity and different microbiota composition is evidenced in Graph 49 and supported by LEfSe analysis (Annex 10), which picked up almost all genera (except Kocuria).



Graph 49. *Bar graph of the 10 most abundant genera genera and the 5 most abundant parent taxa, on the legs and based on the season (women, Pavia POC). ** both original p and corrected value <0.05; * only original p <0.05.*

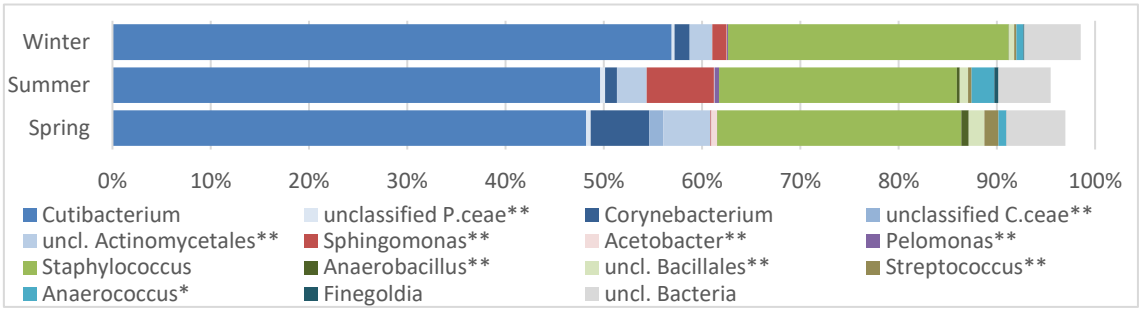
Initially, we also postulated that warmer weather might lead to higher proportions of hydro- and halophilic bacteria through increased sweat production. The leg microbiome composition strongly supports this assumption with much higher levels or *Corynebacterium* and *Staphylococcus* in summer. Similarly, we can see higher percentages for both these taxa in summer (Graph 47) and spring (Graph 48), suggesting that the seasonality variations might be due more to a physiological response to the temperatures rather than to an increased/decreased exposure to the environment.

III.1.2. Men

Logically, the next step was to compare the effect of seasonality on women and on men. Due to our much scantier male samples' library, in this case we could assemble only one subset, and 30 cheek samples collected in our Pavia POC in spring, summer, and winter were subjected to statistical analysis.

This time, all *p* values calculated for the Shannon index were lower than 0.05 but the Simpson and PERMANOVA calculations revealed relevant differences only at the family and genus levels. In line with our previous observations, the winter samples have lower overall biodiversity and similar values were obtained for the spring and summer groups (data not shown). Curiously, the diversity of the spring specimens closely resembles that of the winter specimens for higher taxonomic levels but is alike the summer samples at the lower levels.

If we can observe higher levels of *Corynebacterium* in spring, the summer and winter samples have the same proportion and *Staphylococcus* holds the same importance across all three seasons. The lack of correspondence with the season influence on the women cheek microbiome might be due to the overall less diverse – and thus more stable – male facial microbiome. Nevertheless, most of the genera showed statistically different populations depending on the season (LEfSe analysis, Annex 11) except the three major ones: *Cutibacterium*, *Corynebacterium*, and *Staphylococcus*, further highlighting the male facial microbiome's consistency and robustness towards stress factors.



Graph 50. *Bar graph of the 10 most abundant genera genera and the 5 most abundant parent taxa, on the cheek and based on the season (men, Pavia POC). ** both original p and corrected value <0.05; * only original p <0.05.*

III.2. Living area

By studying the effect of seasonality on the cutaneous microbiota, we also noticed some discrepancies between the bacterial distribution of groups sampled in the same season but in different POCs (Graph 47 vs. Graph 48, winter and autumn groups). This time, our work was limited to the cheek area for either sex as not enough samples from other body districts could be assembled into a rational cohort. In the same manner as the previous approach, we grouped the samples by season before analysing the eventual differences between our various collection points. Again, the results presented below should be understood as trends rather than

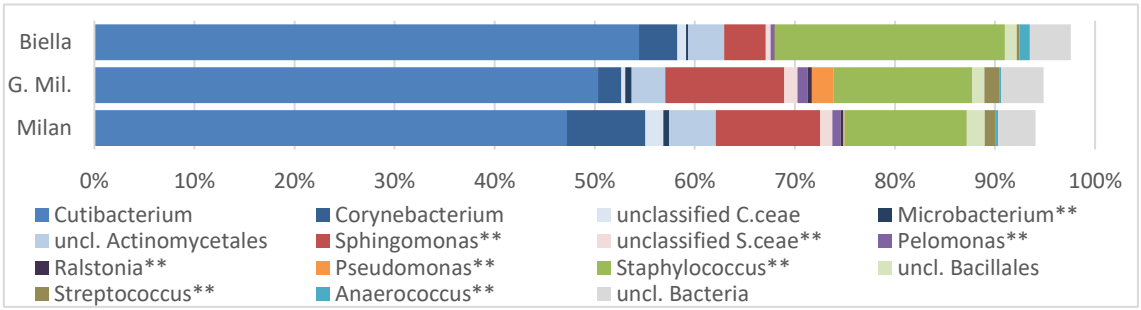
definitive conclusions, since no comparison including all 4 locations could be carried out and the total number of samples remained a bit low to yield statistically reliable results.

Our client studies were so far conducted in four locations with different environments. First, our laboratories in Pavia (40 km south of Milan) and Garbagnate Milanese (15 km north of Milan) can be considered both rural (agricultural area) and polluted (high aerial microparticles concentrations) environments. Then, our laboratory in Biella was deemed 100% rural (agricultural, mountainous area) and the one in Milan 100% urban, as it is situated in the city centre, far from industrial complexes.

III.2.1. Women

Of all the samples collected in the spring and on the cheek, we could match 63 specimens, 21 from each of our Biella, Garbagnate Milanese, and Milan laboratories. Despite not showing alphadiversity contrasts, the Biella samples displayed an overall lower taxa richness and dispersion, while all betadiversity calculations suggest community differences between the three groups (data not shown). These structural variations seem to regard all genera except Cutibacterium and Corynebacterium (Annex 12), highlighting the stability of the major members compared to the less preponderant taxa.

The general lower biodiversity of the Biella samples is visible on Graph 50, with high percentages of *Cutibacterium* and *Staphylococcus* while the other all have low representation, and is in line with the expected robustness of microbiota exposes to less pollution (L. Wang et al. 2021). The smaller percentage of *Sphingomonas* (compared to the G. Mil. and Milan samples) is an indication of limited atmospheric pollution as well, since members of this genus are suspected to play a role in the microbiome's balance and the fight against pollution (Cosseau et al. 2016).



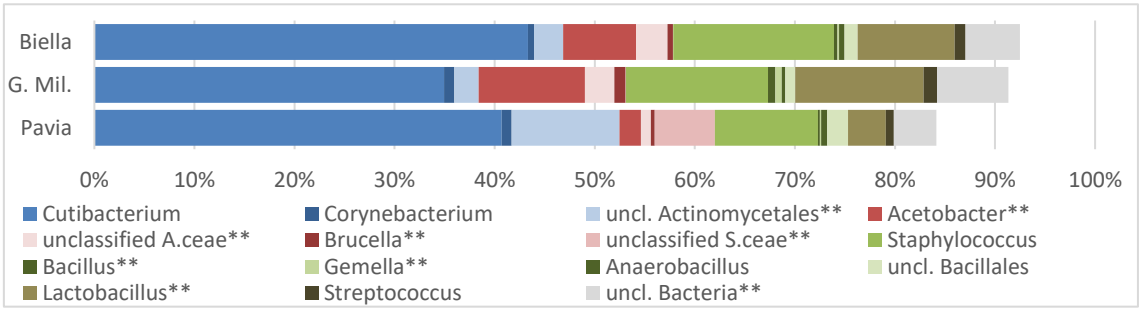
Graph 51. *Bar graph of the 10 most abundant genera genera and the 5 most abundant parent taxa, on the cheek and based on the living area (women, spring). ** both original p and corrected value <0.05; * only original p <0.05.*

In this graph, we can also note that the microbiotas of the G. Mil. and Milan volunteers are quite similar, except maybe for *Corynebacterium* and *Pseudomonas*. On one hand, urban pollution (and consequent oxidative stress) has been shown to alter barrier integrity and to oilier skin, supporting the increased percentage of Corynebacterium in the Milan group (Passeron et al. 2021). On the other hand, many *Pseudomonades* spp. are

associated to plant pathogens, soil bacteria, and plant-growth promoters (Padda, Puri, and Chanway 2018) further justifying our previous choice of area categorisation (rural + urban, 100% rural, 100% urban). In fact, our Pavia and G. Milanese POCs are situated in largely residential areas in close proximity with industrial areas and within the same, highly atmospherically polluted Po basin.

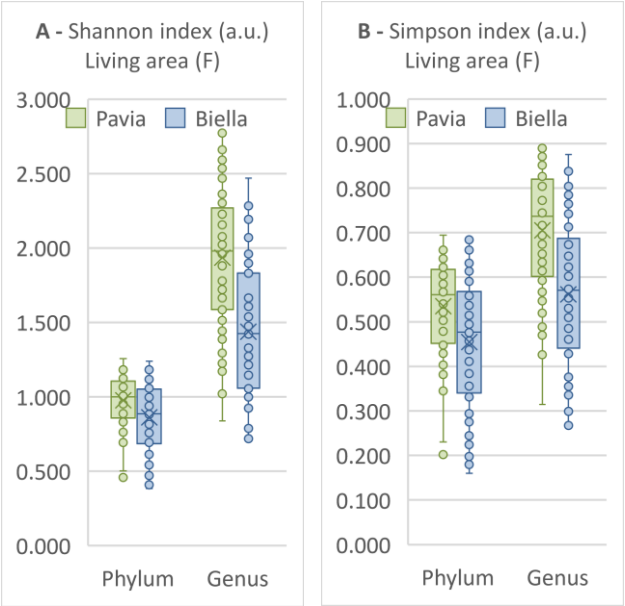
We then pursued our investigations by combining 54 female cheek samples from our three rurality-associated POCs and gathered in Autumn. Coincidentally, all the specimens were collected in subjects under 50 years of age, eliminating the risk of menopausal states to affect our results. Some differences in both taxa richness and evenness could be observed at the phylum and family levels – with the Biella samples always displaying lower absolute values – and the PERMANOVA analysis determined that the communities were different to one another at every taxonomic level (data not shown). The LEfSe analysis of the signature taxa, however, highlighted much less genera than previously (Annex 13) and suggests that the Pavia, G. Mil. and Biella are closer in composition and structure than any with Milan.

If this assumption should be true given the proximity to agricultural and natural resources of all three, we previously observed that the G. Mil. microbiota might resemble more the Milan area than the Biella area, but we also see now that the Pavia and G. Mil. structures seem quite different (Graph 52). On one hand these discrepancies could be related to the natural hypervariability of the facial microbiome – especially because urban environments tend to induce more dissimilarities (Lehtimäki et al. 2017) – but the seasonality might also be the culprit. In fact, these samples were collected in Autumn, and we already saw earlier that it was the season with the less structural stability (III.1.1).



Graph 52. *Bar graph of the 10 most abundant genera genera and the 5 most abundant parent taxa, on the cheek and based on the living area (women, autumn). ** both original p and corrected value <0.05; * only original p <0.05.*

Finally, we conducted an analysis on the two locations where we collected the most samples (Biella and Pavia) and could assemble 140 samples, all collected in winter and on women younger than 50. Both POCs can be considered rural as their surroundings are mainly agriculture, but the Pavia area is more affected by the notorious northern Italy pollution blanket than Biella – which lies at the foot of the mountains and > 100km from dense urban centres. All the biodiversities showed significant differences between the two POCs (Table 31) and we can, again, clearly see that the rural area has overall lower taxa richness and evenness (Graph 53).

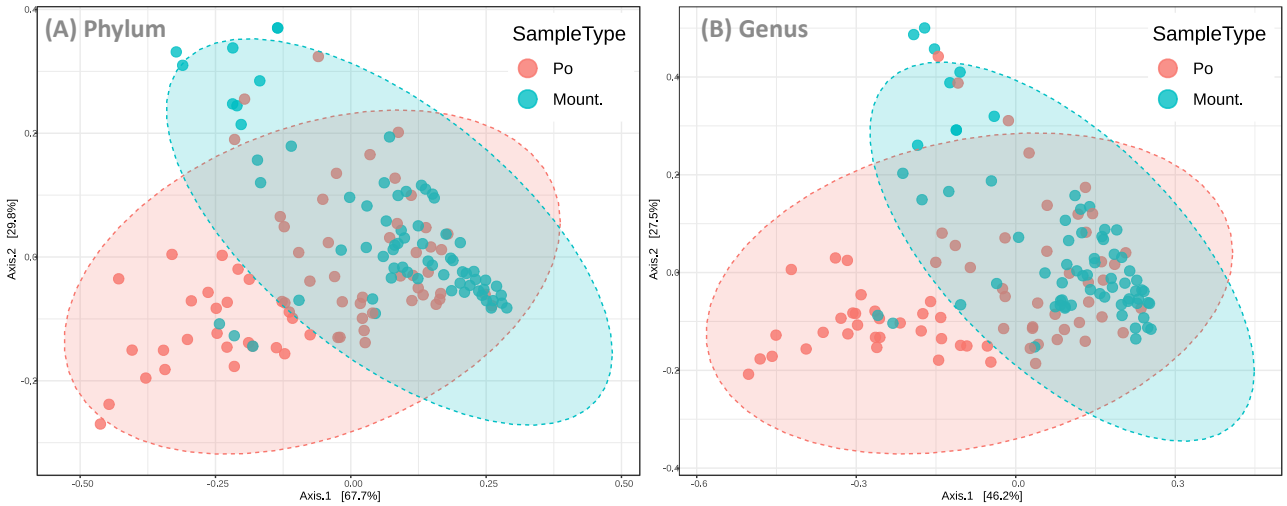


	alpha		beta
	Shannon	Simpson	
PHYLUM	1.56E-04	2.01E-03	0.008
CLASS	1.18E-03	5.01E-03	0.005
ORDER	5.48E-04	4.62E-03	0.008
FAMILY	2.83E-03	1.22E-02	0.009
GENUS	5.41E-03	1.61E-02	0.009

Table 31. *Statistical diversity values, at the sample level* (alphadiversity) and the group level (betadiversity).

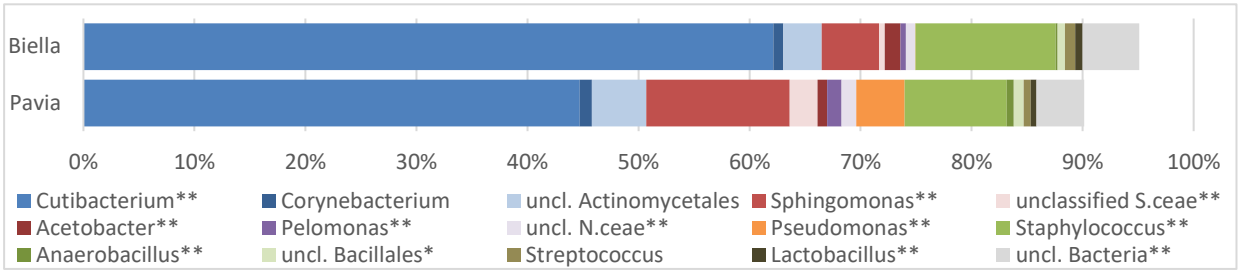
Graph 53. *Graphic representation of the alphadiversity at the phylum and genus taxonomic levels, calculated using the Shannon (A) and Simpson (B) indices and depending on the sex. a.u.=arbitrary unit.*

The lower alphadiversities and the significant differences in community structure (betadiversity, Table 31 and Graph 54) are in line with both the literature and our previous observations and assumptions about 100% rural *vs.* hybrid rural + urban/industrial environments. In fact, Graph 55 shows higher abundances of *Sphingomonas* (protection against pollutants) and *Pseudomonas* (linked to soil bacteria and plant-growth promoting factors) for the Pavia group, while the taxa distribution of the Biella group strongly resembles the one in Graph 51 (spring samples) and thus supporting the rural microbiota stability hypothesised in paragraph III.1.1. The LEfSe analysis (Annex 14) confirmed that the structural differences concerned mainly the *Proteobacteria* and *Firmicutes* phyla.



Graph 54. *Graphic representation of β-diversity at the phylum and genus taxonomic levels, on the cheek, in winter and depending on the living location.*

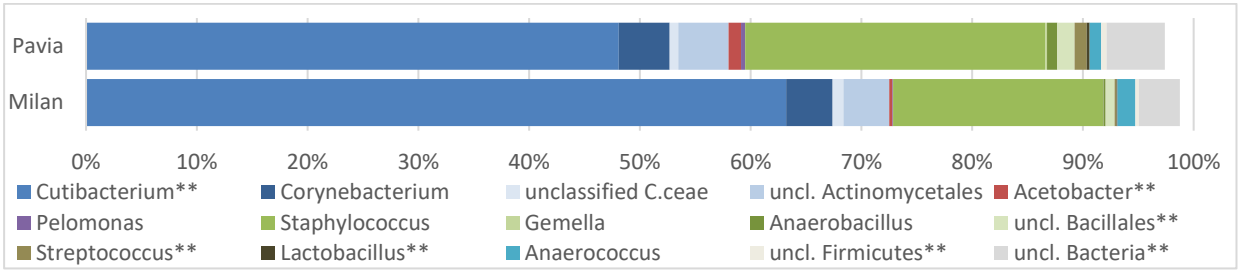
Interestingly, *Corynebacterium* was never found to be affected by the living area, suggesting that its variations are not triggered by the sole decrease in *Cutibacterium* preponderance, but rather by changes in humidity levels in the sampled area – as demonstrated by our results based on the age and on the seasonality.



Graph 55. *Bar graph of the 10 most abundant genera genera and the 5 most abundant parent taxa, on the cheeks and based on the POC (women, winter). ** both original p and corrected value <0.05; * only original p <0.05.*

III.2.2. Men

Although our male samples' pool is reduced compared to the women's, we still could assemble 44 samples collected in the same season (spring) and body niche (cheek) but in different POCs. In this case, the specimens were either from Milan and Pavia, which should allow us to better define the differences previously observed for women between 100% urban and urban + rural environments (Graph 56 and Annex 15).



Graph 56. *Bar graph of the 10 most abundant genera genera and the 5 most abundant parent taxa, on the cheeks and based on the POC (women, winter). ** both original p and corrected value <0.05; * only original p <0.05.*

First, we should note that the male facial microbiota composition and structure are fundamentally different between an urban and urban-associated surrounding, contrarily to what suggested by Graphs 51, 52, and Annexes 12, 13. In this case, all alpha- and betadiversity *p*-values were below the 0.05 threshold thus proving the strong dissonances in taxa richness, evenness, and overall distribution (data not shown). The general biodiversity trend however remains the same as in women (urban + rural > 100% urban > 100% rural) and suggesting i) that atmospheric pollution exposure might not be as harmful (towards the microbiota) as usually suspected, and ii) the cutaneous microbiome of men and women are similarly impacted by the same atmospheric conditions.

The LEfSe analysis (Annex 15), however, revealed that much less members were affected in men than in women, with only 4 genera affected: this might be explained by the lower microbiota variability in men, already established in paragraph II.1. Again, *Corynebacterium* was not impacted by the difference in living location, further confirming the absence of pollution impact of its relative abundance. Interestingly, the *Firmicutes* variations concerned exclusively *Lactobacillales* while the previous comparison in women (Graph 55) showed changes mainly in the *Bacillales* order. Because *Lactobacillales* are more characteristic of the female microbiota (and *Bacillales* for men), we believe this difference to be linked to the POC's environment rather than to physiological differences.

IV. Conclusions and Outlooks

The access to a large number of samples collected in different time points and locations, from volunteers of both sexes and various ages, allowed us to try and pinpoint how different factors influence the microbiota composition of a given body site. In fact, we could observe that the physiological differences between men and women lead to specific variations of the commonly described *Cutibacterium*, *Staphylococcus*, *Corynebacterium*, *Microbacterium*, and *Streptococcus* and thus to different microbial signatures genera (Byrd, Belkaid, and Segre 2018; Grice et al. 2009; Baldwin et al. 2017)(Skowron et al. 2021). Women generally demonstrated higher biodiversity, which is believed to stem from the more regular use of cosmetics and the consequent increased moisturisation, overall improving the barrier function but also acting as fertiliser to the "microbial garden" (Y. Belkaid and Segre 2014).

In this first approach, we successfully demonstrated that sex is a crucial factor in shaping the microbial community of the human face, and the identification of low-abundance, high-relevance entities suggests that factor-driven microbiome variations do not necessarily depend on the most abundant members of the microbial flora. The expansion of the analysis to other body sites determined that, while niche-specific physiologic differences induces different microbial assemblies (Grice et al. 2009; Costello et al. 2009) (Findley et al. 2013) (Grice and Segre 2011), not all areas are similarly impacted and the local differences in skin structure are more fundamental than the overall physiological factor.

Because the cutaneous microbiota is notoriously sensitive to many intrinsic and extrinsic factors (Y. Belkaid and Segre 2014; Chen, Knight, and Gallo 2023; Townsend and Kalan 2023), we then focused on describing the microbiome's variations depending on the subject's age. We already obtained preliminary results with the multivariate analysis (§ II.1.1.), but our results did not align with the literature, and we prepared different cohorts to try and estimate age-dependent shifts by sex, in different cohorts, and from several body sites. The obtained results were (again) mostly inconclusive, and we suspect the reason to be linked to the various time points and/or POCs in which the samples were collected – as suggested by the work of Lehtimäki *et al.* (2017) and Ying *et al.* (2015).

Since the climatic conditions supposedly have a larger impact on the composition of the human skin microbiota than its subject's living habits (Harel et al. 2022), we focused first on the season in which the specimens were recovered. While some trends could be discerned (higher biodiversity and variability in Autumn, *Corynebacterium* more expressed in Spring and summer), we were unable to pinpoint specific correlations as each comparison was somewhat contradicting the previous one. On one hand, the bacteria adaptation to their niche may explain why the majority of the human skin microbiome remained stable despite the meteorologic variations (Harel et al. 2022).

On the other hand, each comparison was performed on samples from a given POC, and microbiome differences linked to the geographical origins are already widely reported: *Dermacoccus* is described as major genera in the Singapore microbiota (Chng et al. 2016) but we detected it only in very low proportions in our Italian cohort; Egyptians often demonstrate high abundances of *Proteobacteria* (Ramadan et al. 2016) but Cameroonians preferably host *Staphylococcus* and *Micrococcus* (Ogai et al. 2022); individuals in south-east Asia tend to express high levels of *Corynebacterium* and *Streptococcus* (Chaudhari et al. 2020), but Japanese show more *Cutibacterium* (Ogai et al. 2022); Americans and Europeans are expected to host large proportions of *Corynebacteria* (Cho and Eom 2021), but our results indicate only very moderate amounts despite always being one of the major members (1^{st}-4^{th} place depending on the approach).

The geographical variations might indeed explain the difficulties we met in characterising the cutaneous microbiota depending on the season and the age, since the living area is believed to have a major impact compared to the age or even the sex of the subjects (Ying et al. 2015; Lehtimäki et al. 2017; Harel et al. 2022). Despite the small size of our cohorts, we were in fact able to confirm the better connectivity or microbial networks in rural communities along with the increased proportions of soil and agriculture associated microbes in populations living in mixed environments (L. Wang et al. 2021; Peng and Biswas 2020; Ying et al. 2015; Hospodsky et al. 2014). Thanks to our continuous sample collection in the frame of client-ordered studies, we should soon be able to compose more robust cohorts and thus obtain more accurate results on the weight of each factor on the microbiota of different body sites, and pinpoint how they affect the taxon types, distribution, and the community structure.

GENERAL CONCLUSIONS AND OUTLOOKS

Since the advent of NGS and the possibilities their offer to accurately study the human microbiota, the focus was put mainly on characterising the host-microbes relationships and their implications in diseased states. Today, many cosmetics manufacturers are also interested in understanding how beauty products may influence the cutaneous microbiota to benefit the skin's quality. To this end, Complife (Garbagnate Milanese, Italy) opened a laboratory dedicated to microbiome study in the beginning of 2020, in collaboration with the Centre for Allergic and Autoimmune Diseases of the University of Eastern Piedmont (Novara, Italy).

The first objective of this PhD thesis was therefore to establish a full pipeline allowing to study the skin microbiome upon cosmetic products application, in relatively short times and with moderate costs. The swabbing method was chosen for sample collection because it is non-invasive, quick, economic, and the samples are easily conserved over long periods of time. Microbiome analysis was performed by sequencing the V1-V3 hypervariable regions of the 16S rRNA bacterial gene, and the resulting data was treated with the MicrobAT (SmartSeq, Novara, Italy) and MicrobiomeAnalyst (McGill University, Canada) informatic tools. First, a pilot study conducted on 18 samples demonstrated the reliability, reproducibility, and repeatability (both inter- and intra-run) of our approach. Then, a preliminary study performed on 60 samples confirmed the robustness of our workflow also at bigger scale.

Nevertheless, the primary goal of the laboratory remained to offer efficient, state of the art analyses to the Complife's clients. To this end, we first collaborated with Copan for testing and validating innovative sampling devices and demonstrated that, for skin microbiome studies, their new Smart eNAt® and ADS® products performed equally well as their classic FLOQswab® + eNAt® kit. Furthermore, they allow for self-collection by the study subjects, which may relieve some pressure from the points of collection and lower the overall study costs. Through this collaboration, we also validated an area-defining template allowing for consistent material collection, a particularly important aspect when dealing with low-biomass specimens such as skin microbiome samples. Finally, the joined work with Copan consented to optimise the sample preparation protocol prior to DNA handling: the use of their novel NAO® Basket device showed to yield better quality results by recovering more microbiota extract than more classical methods.

Parallel to the work on raw sample quality, we looked to optimise the DNA extraction step. To this end, we compared the results obtained with the QIAamp DNA Microbiome kit from (Qiagen, Hilden, Germany) against results obtained with the PureLink™ Genomic DNA (Invitrogen, Altham, MA, USA) and the ZymoBIOMICS™ DNA Miniprep Kit (ZymoResearch, Irvine, CA, USA). We demonstrated that the results obtained with the Invitrogen and ZymoResearch products were suitable for analysis, although of slightly lower quality than the Qiagen's ones. Because of their lower costs and shorter protocols, they still should be considered for cutaneous microbiome analysis – particularly when looking to adjust the studies' timeline and budget.

Thanks to the many clients interested in this technology, we were able to collect over 3000 samples in about three years, and around 1000 of them came from untreated areas of the body. As such, they could be used for fundamental research purposes and the second focus of this PhD project was put on the factors that might influence the composition on the cutaneous microbiota. The preliminary study was conducted on the face of both men and women of various ages, and since the results already showed differences linked to the subjects' physiology, we chose to investigate the microbiome variations first based on the sex.

We could observe that the variations of the commonly described *Cutibacterium*, *Staphylococcus*, *Corynebacterium*, *Microbacterium*, and *Streptococcus* genera reflected the physiological and anatomical distinctions between the two sexes, hereby demonstrating that sex is a crucial factor in shaping the microbial community of the human face. We were also able to confirm the presence as resident taxa of rarely described members belonging to the phyla *Proteobacteria* (*Sphingomonas* and *Acetobacter*, *Pelomonas*, *Pseudomonas* genera) and *Firmicutes* (genus *Anaerococcus* from the *Clostridia* class), that presented statistically relevant population differences as well. In fact, the latter suggests that factor-driven microbiome variation might depend on both the most prevalent and the lower abundance members. The impact of the sex on the cutaneous microbiota on other body areas was investigated as well, and we could note that the chest – another sebaceous area – overall showed similar variations on the same members, while variations in the armpit were less marked (although consistently and primarily regarding *Staphylococcus* and *Sphingomonas*).

We then looked to apply the same approach to other microbiome-influencing factors, and re-organised the samples based on either the volunteers' age, their living area, and the season in which the samples were collected. Such sub-classifications however lead to much smaller study groups, and while some trends could be observed, the overall inconsistency of our results underlines how microbiome shifts with environment changes can be person- and situation-specific.

In an effort to further expand Complife's commercial offer, we are now looking at additional analytical approaches. First, we applied our actual pipeline to the fungal component of the microbiota by targeting the ITS-1 gene instead of the bacterial 16s rRNA gene, starting with evaluating the efficacy of different kits from Qiagen in extracting fungal genetic material. Preliminary results indicate good correlation of our results with the literature (independently of the DNA extraction kit), and we believe the workflow to be operational.

Since our clients are equally interested in monitoring particular species as they are in the overall microbiota composition, we also started to implement a Digital Droplet PCR protocol (Bio-Rad, Hercules, CA, USA) which will complement our current offer. At the moment, feasibility studies are conducted on 15 typical skin commensals: 10 bacteria (*C. acnes, C. granulosum, S. epidermidis, S. hominis, S. caprae, S. aureus, C. tuberculostearicum, A. nagyae, S. pyogenes*) and 5 fungi (*M. furfur, M. globosa, M. restricta, C albicans, C. herbarum*).

Finally, continuous sample collection in our centres will allow us to consolidate our samples' pool and continue our work on characterising the influence of endo- and exogenous factors on the microbial populations, therefore refining our understanding of the normal, healthy human cutaneous microbiota.

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ANNEXES

PHYLUM	CLASS	ORDER	FAMILY	GENUS	MEDIAN	V1				V2				V3			
						Copan			Norgen	Copan			Norgen	Copan			Norgen
						1R	2R	1L	2L	1R	2R	1L	2L	1R	2R	1L	2L
Actinobacteria	Actinobacteria	Actinomycetales	Propionibacteriaceae	Propionibacterium	35.11%	24.00%	17.29%	44.04%	18.40%	26.18%	25.20%	72.49%	23.00%	76.71%	88.26%	85.65%	87.00%
			Corynebacteriaceae	Corynebacterium	0.63%	0.48%	0.52%	0.63%	0.34%	0.85%	0.82%	0.63%	0.44%	1.28%	0.46%	1.08%	2.03%
				Other Actinobacteria	*4.29%*	*4.40%*	*5.21%*	*4.17%*	*4.44%*	*4.58%*	*4.51%*	*3.97%*	*4.11%*	*4.05%*	*3.77%*	*3.85%*	*4.43%*
Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Sphingomonas	5.61%	6.65%	9.27%	3.58%	9.14%	12.64%	13.70%	4.56%	7.62%	3.69%	1.27%	3.02%	0.97%
		Rhodobacterales	Rhodobacteraceae	Paracoccus	0.25%	0.30%	0.63%	0.73%	0.07%	0.20%	0.47%	0.20%	0.58%	0.59%	0.05%	0.04%	0.00%
				Other Alphaproteobacteria	*3.93%*	*9.80%*	*11.19%*	*1.40%*	*9.59%*	*6.45%*	*7.06%*	*1.23%*	*11.78%*	*0.74%*	*0.29%*	*0.61%*	*0.13%*
	Betaproteobacteria	Burkholderiales	Comamonadaceae	Pelomonas	0.42%	0.51%	0.60%	0.23%	0.74%	1.67%	1.11%	0.26%	0.92%	0.32%	0.04%	0.18%	0.06%
				Other Betaproteobacteria	*2.93%*	*11.63%*	*4.17%*	*23.38%*	*4.25%*	*2.10%*	*2.70%*	*3.17%*	*3.76%*	*0.71%*	*0.12%*	*0.17%*	*0.11%*
	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Enhydrobacter	1.68%	1.75%	2.77%	4.56%	2.12%	1.60%	1.97%	0.83%	3.76%	0.17%	0.11%	0.11%	0.35%
			Pseudomonadaceae	Pseudomonas	1.03%	1.22%	1.65%	0.65%	1.28%	2.90%	2.47%	0.84%	1.59%	0.52%	0.28%	0.44%	0.16%
		Vibrionales	Vibrionaceae	Photobacterium	0.26%	0.76%	0.41%	0.08%	0.08%	0.40%	0.67%	0.25%	0.27%	0.39%	0.09%	0.02%	0.00%
				Other Gammaproteobacteria	*1.85%*	*2.63%*	*5.30%*	*0.71%*	*3.71%*	*3.84%*	*4.03%*	*0.72%*	*3.47%*	*1.08%*	*0.12%*	*0.15%*	*0.06%*
Firmicutes	Bacilli	Bacillales	Staphylococcaceae	Staphylococcus	7.96%	6.47%	12.59%	9.44%	17.79%	14.54%	13.43%	4.61%	11.10%	2.16%	1.64%	0.67%	1.29%
		Lactobacillales	Streptococcaceae	Streptococcus	0.89%	0.84%	0.93%	0.51%	1.23%	1.90%	2.47%	1.69%	1.08%	0.82%	0.23%	0.42%	0.24%
				Other Bacilli	*1.69%*	*2.79%*	*2.57%*	*0.89%*	*3.87%*	*3.11%*	*2.49%*	*0.80%*	*2.75%*	*0.47%*	*0.13%*	*0.24%*	*0.10%*
	Clostridia				0.33%	0.83%	1.55%	0.40%	0.98%	0.25%	0.89%	0.10%	1.00%	0.02%	0.07%	0.07%	0.07%
unclassified Bacteria					*7.73%*	*18.94%*	*16.61%*	*3.68%*	*15.96%*	*11.73%*	*11.56%*	*3.18%*	*15.53%*	*3.91%*	*2.94%*	*3.14%*	*2.88%*
Bacteroidetes					0.46%	1.44%	0.82%	0.26%	0.67%	2.19%	0.83%	0.24%	2.46%	0.04%	0.01%	0.10%	0.04%
Cyanobacteria/Chloroplasts					0.19%	0.22%	0.48%	0.17%	0.34%	0.78%	0.96%	0.04%	0.42%	0.03%	0.06%	0.00%	0.03%
TOTAL					**97.81%**	**95.67%**	**94.55%**	**99.51%**	**95.00%**	**97.92%**	**97.34%**	**99.81%**	**95.66%**	**97.70%**	**99.95%**	**99.96%**	**99.96%**

Annex 1. *10 most abundant genera found on the face and their parent taxa, as determined by the pilot study.*

PHYLUM	CLASS	ORDER	FAMILY	GENUS	MEDIAN	V1		V2		V3	
						run 1	run 2	run 1	run 2	run 1	run 2
Actinobacteria	Actinobacteria	Actinomycetales	Propionibacteriaceae	Propionibacterium	24.54%	17.16%	17.07%	24.85%	24.24%	88.30%	87.98%
			Corynebacteriaceae	Corynebacterium	0.52%	0.53%	0.47%	0.84%	0.76%	0.45%	0.51%
				Other Actinobacteria	*4.73%*	*5.11%*	*5.10%*	*4.45%*	*5.01%*	*3.75%*	*3.56%*
Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Sphingomonas	9.52%	9.16%	9.89%	13.47%	13.35%	1.27%	1.37%
				Other Alphaproteobacteria	*7.47%*	*11.58%*	*11.40%*	*7.46%*	*7.48%*	*0.33%*	*0.37%*
	Betaproteobacteria	Burkholderiales	Comamonadaceae	Pelomonas	0.64%	0.65%	0.62%	1.18%	1.20%	0.05%	0.06%
			Burkholderiaceae	Ralstonia	0.61%	0.62%	0.72%	0.72%	0.61%	0.03%	0.02%
				Other Betaproteobacteria	*1.99%*	*3.52%*	*3.38%*	*1.94%*	*2.04%*	*0.09%*	*0.05%*
	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Enhydrobacter	1.97%	2.73%	2.54%	1.93%	2.01%	0.11%	0.16%
			Pseudomonadaceae	Pseudomonas	1.94%	2.01%	1.86%	2.99%	2.72%	0.29%	0.28%
				Other Gammaproteobacteria	*5.03%*	*5.89%*	*5.62%*	*4.99%*	*5.07%*	*0.21%*	*0.21%*
Firmicutes	Bacilli	Bacillales	Staphylococcaceae	Staphylococcus	12.35%	12.43%	12.27%	13.20%	13.81%	1.64%	1.67%
		Lactobacillales	Streptococcaceae	Streptococcus	0.88%	0.92%	0.84%	2.42%	2.21%	0.23%	0.23%
				Other Bacilli	*2.46%*	*2.50%*	*2.42%*	*2.53%*	*2.56%*	*0.11%*	*0.09%*
	Clostridia				0.89%	1.69%	1.64%	0.95%	0.83%	0.07%	0.05%
unclassified Bacteria					*11.37%*	*16.41%*	*17.12%*	*11.36%*	*11.39%*	*2.93%*	*3.27%*
Bacteroidetes					1.07%	1.14%	1.10%	1.09%	1.06%	0.04%	0.05%
Cyanobacteria/Chl	Chloroplast	Chloroplast	Chloroplast	Streptophyta	0.50%	0.47%	0.53%	0.94%	0.93%	0.06%	0.04%
	Other Cyanobacteria/Chloroplasts				-	-	-	-	-	-	-
TOTAL					**97.29%**	**94.52%**	**94.60%**	**97.31%**	**97.27%**	**99.95%**	**99.96%**

Annex 2. *10 most abundant genera found on the face and their parent taxa, interpreted for inter-run reproducibility.*

PHYLUM	CLASS	ORDER	FAMILY	GENUS	MEDIAN	V1		V2		V3	
						Og	Dp	Og	Dp	Og	Dp
Actinobacteria	Actinobacteria	Actinomycetales	Propionibacteriaceae	Propionibacterium	48.68%	22.24%	20.17%	52.46%	44.90%	63.20%	53.41%
			Corynebacteriaceae	Corynebacterium	0.55%	0.30%	0.56%	0.38%	0.63%	0.63%	0.54%
				Other Actinobacteria	*3.98%*	*4.41%*	*2.33%*	*4.28%*	*4.93%*	*3.68%*	*3.24%*
Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Sphingomonas	19.40%	32.66%	37.34%	19.28%	19.52%	15.06%	18.07%
		Rhodobacterales	Rhodobacteraceae	Paracoccus	0.32%	0.36%	0.22%	0.07%	0.28%	2.06%	1.93%
				Other Alphaproteobacteria	*4.29%*	*4.89%*	*5.55%*	*2.88%*	*5.57%*	*2.62%*	*3.69%*
	Betaproteobacteria	Burkholderiales	Comamonadaceae	Pelomonas	1.28%	1.94%	1.64%	0.92%	1.41%	1.16%	0.88%
				Curvibacter	0.85%	1.06%	0.96%	0.74%	0.04%	0.31%	1.19%
			Burkholderiaceae	Ralstonia	0.70%	1.61%	0.95%	0.53%	0.37%	0.64%	0.76%
				Other Betaproteobacteria	*1.77%*	*2.35%*	*2.21%*	*0.37%*	*1.83%*	*0.63%*	*1.71%*
	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Enhydrobacter	1.10%	1.04%	1.16%	1.17%	0.38%	0.43%	1.96%
			Pseudomonadaceae	Pseudomonas	4.04%	7.25%	6.92%	4.55%	3.54%	3.10%	3.41%
				Other Gammaproteobacteria	*1.45%*	*2.38%*	*1.60%*	*1.63%*	*1.30%*	*0.79%*	*0.68%*
Firmicutes	Bacilli	Bacillales	Staphylococcaceae	Staphylococcus	4.73%	7.77%	8.73%	3.92%	5.53%	0.54%	1.07%
		Lactobacillales	Streptococcaceae	Streptococcus	1.23%	1.11%	2.11%	1.68%	1.35%	0.37%	0.42%
				Other Bacilli	*1.25%*	*1.46%*	*1.86%*	*0.78%*	*1.24%*	*1.21%*	*1.26%*
	Clostridia				0.23%	0.89%	0.25%	0.32%	0.20%	0.21%	0.01%
				unclassified Bacteria	*3.67%*	*4.47%*	*4.13%*	*3.66%*	*3.67%*	*2.99%*	*3.54%*
				Bacteroidetes	0.53%	0.40%	0.66%	0.10%	2.44%	0.09%	1.54%
				Cyanobacteria/Chloroplasts	0.05%	0.01%	0.04%	0.00%	0.11%	0.05%	0.09%
				TOTAL	**99.41%**	**98.59%**	**99.40%**	**99.74%**	**99.25%**	**99.78%**	**99.41%**

Annex 3. *10 most abundant genera found on the face and their parent taxa, interpreted for intra-run reproducibility.*

PHYLUM	CLASS	ORDER	FAMILY	GENUS	<30	30-50	LEfSe *p*-value	FDR-corrected	LDA score
Actinobacteria	Actinobacteria	Actinomycetales	Propionibacteriaceae	Cutibacterium	52.04%	56.41%	1.68E-01	3.24E-01	5.39
			Corynebacteriaceae	Corynebacterium	0.85%	1.23%	9.58E-01	9.81E-01	4.36
			Microbacteriaceae	Microbacterium	0.47%	0.25%	1.25E-01	2.95E-01	-4.03
				uncl. Actinomycetales	*3.90%*	*4.97%*	*2.96E-01*	*4.45E-01*	*4.71*
Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Sphingomonas	9.75%	6.10%	1.20E-01	2.95E-01	-5.21
				uncl. S.ceae	*1.51%*	*1.25%*	*4.34E-01*	*5.81E-01*	*-4.00*
		Rhodospirillales	Acetobacteraceae	Acetobacter*	0.82%	0.58%	5.35E-02	2.50E-01	-4.26
	Betaproteobacteria	Burkholderiales	Comamonadaceae	Pelomonas	0.95%	0.53%	8.91E-02	2.83E-01	-4.29
	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	1.68%	0.86%	8.36E-01	9.03E-01	-4.55
Firmicutes	Bacilli	Bacillales	Staphylococcaceae	Staphylococcus	11.95%	13.72%	2.43E-01	4.21E-01	4.99
			Bacillaceae 1	Anaerobacillus	0.44%	0.22%	7.31E-02	2.63E-01	-3.99
		Lactobacillales	Streptococcaceae	Streptococcus**	1.36%	0.86%	**3.81E-05**	**2.05E-03**	-4.58
				*unclassified Bacteria**	*5.06%*	*4.49%*	***6.36E-03***	*5.72E-02*	*-4.62*
				TOTAL	**90.79%**	**91.46%**			

Annex 4. *Relative abundance results of the 10 most abundant genera and of their parent taxa of interest (Σ>2%). ** both original* p *and corrected value <0.05; * only original* p *<0.05.*

PHYLUM	CLASS	ORDER	FAMILY	GENUS	30-50	>50	LEfSe *p*-value	FDR-corrected	LDA score
Actinobacteria	Actinobacteria	Actinomycetales	Propionibacteriaceae	Cutibacterium	52.00%	44.94%	1.57E-01	6.14E-01	5.56
			Corynebacteriaceae	Corynebacterium	5.24%	3.46%	3.76E-01	7.32E-01	4.75
			Micrococcaceae	Kocuria*	0.16%	1.05%	**1.49E-02**	6.14E-01	-4.64
			Microbacteriaceae	Microbacterium	0.39%	0.71%	2.10E-01	6.14E-01	-4.19
				uncl. Actinomycetales	*3.79%*	*3.48%*	*4.69E-01*	*7.32E-01*	*3.97*
Proteobacteria	Alphaproteobacteria	Rhodospirillales	Sphingomonadaceae	Sphingomonas	8.61%	11.95%	5.06E-01	7.32E-01	-5.19
				unclassified S.ceae	*1.02%*	*1.25%*	*6.93E-01*	*8.21E-01*	*-4.00*
	Betaproteobacteria	Burkholderiales	Comamonadaceae	Pelomonas	0.63%	0.99%	3.45E-01	7.32E-01	-4.25
		Neisseriales	Neisseriaceae	*unclassified N.ceae*	*0.99%*	*0.68%*	*3.20E-01*	*7.32E-01*	*4.09*
	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	1.06%	0.58%	1.27E-01	6.14E-01	4.17
Firmicutes	Bacilli	Bacillales	Staphylococcaceae	Staphylococcus	13.73%	18.43%	1.60E-01	6.14E-01	-5.25
				uncl. Bacillales	*1.14%*	*1.46%*	*3.98E-01*	*7.32E-01*	*-4.18*
		Lactobacillales	Streptococcaceae	Streptococcus	1.51%	1.26%	2.53E-01	6.65E-01	-4.19
	Clostridia	Clostridiales	C. Incertae Sedis XI	Anaerococcus	0.31%	0.85%	8.47E-02	6.14E-01	-4.41
				unclassified Bacteria	*4.45%*	*4.06%*	*4.63E-01*	*7.32E-01*	*4.29*
				TOTAL	**95.00%**	**95.15%**			

Annex 5. *Relative abundance results of the 10 most abundant genera and of their parent taxa of interest (Σ>2%). ** both original* p *and corrected value <0.05; * only original* p *<0.05.*

PHYLUM	CLASS	ORDER	FAMILY	GENUS	< 30	30-50	> 50	LEfSe *p*-value	FDR-corrected	LDA score
Actinobacteria	Actinobacteria	Actinomycetales	Propionibacteriaceae	Cutibacterium*	81.58%	42.44%	40.96%	**4.63E-03**	5.31E-02	6.28
			Corynebacteriaceae	Corynebacterium*	0.73%	14.20%	22.87%	**1.76E-02**	7.36E-02	5.95
			Micrococcaceae	Micrococcus*	0.05%	0.92%	0.85%	**3.38E-03**	5.31E-02	4.64
				Kocuria	0.14%	0.37%	0.34%	6.53E-01	7.54E-01	3.98
				uncl. Actinomycetales	*5.55%*	*6.20%*	*7.86%*	*8.05E-01*	*8.05E-01*	*4.95*
Proteobacteria	Alphaproteobacteria	Rhodospirillales	Acetobacteraceae	Acetobacter*	0.43%	1.69%	1.94%	**1.48E-02**	6.59E-02	4.84
				*unclassified A.ceae**	*0.19%*	*0.60%*	*0.66%*	***2.68E-02***	*9.97E-02*	*4.33*
Firmicutes	Bacilli	Bacillales	Staphylococcaceae	Staphylococcus*	2.79%	19.86%	7.51%	**1.20E-02**	5.72E-02	5.93
				*uncl. Bacillales**	*0.27%*	*1.29%*	*0.79%*	***7.19E-03***	*5.31E-02*	*4.72*
		Lactobacillales	Lactobacillaceae	Lactobacillus	0.58%	1.94%	1.74%	6.00E-02	1.55E-01	4.81
			Streptococcaceae	Streptococcus*	0.14%	0.67%	0.23%	**3.89E-03**	5.31E-02	4.46
	Clostridia	Clostridiales	C. Incertae Sedis XI	Anaerococcus*	0.16%	1.10%	1.78%	**4.46E-02**	1.30E-01	4.95
				Finegoldia*	0.04%	0.74%	0.35%	**2.87E-02**	1.01E-01	4.62
				uncl. Clostridiales	*0.02%*	*0.18%*	*1.26%*	*1.64E-01*	*3.17E-01*	*4.84*
				unclassified Bacteria	*5.45%*	*4.39%*	*5.87%*	*3.09E-01*	*4.45E-01*	*4.99*

				TOTAL	**98.12%**	**96.57%**	**95.01%**			

Annex 6. *Relative abundance results of the 10 most abundant genera and of the 5 most abundant parent taxa of interest, on the chest area. ** both original* p *and corrected value <0.05; * only original* p *<0.05.*

PHYLUM	CLASS	ORDER	FAMILY	GENUS	< 30	30-50	> 50	LEfSe *p*-value	FDR-corrected	LDA score
Actinobacteria	Actinobacteria	Actinomycetales	Propionibacteriaceae	Cutibacterium	5.49%	7.01%	3.70%	2.00E-01	3.85E-01	5.34
			Corynebacteriaceae	Corynebacterium	15.00%	1.78%	19.85%	3.43E-01	5.78E-01	5.84
			Micrococcaceae	Kocuria	0.05%	0.21%	0.04%	8.59E-01	8.92E-01	2.78
				uncl. Actinomycetales	*5.43%*	*5.82%*	*9.52%*	*6.07E-01*	*7.44E-01*	*5.37*
Proteobacteria	Alphaproteobacteria	Rhodospirillales	Acetobacteraceae	Acetobacter	0.18%	0.02%	0.01%	3.97E-01	5.78E-01	4.34
	Betaproteobacteria	Burkholderiales	Comamonadaceae	Pelomonas*	0.05%	0.30%	0.13%	**1.42E-02**	5.48E-02	3.98
Firmicutes	Bacilli	Bacillales	Staphylococcaceae	Staphylococcus	53.98%	39.29%	44.52%	6.34E-01	7.44E-01	5.71
			Bacillaceae 1	Anaerobacillus	0.22%	0.76%	0.28%	1.56E-01	3.24E-01	4.32
				*uncl. Bacillales**	*3.00%*	*2.17%*	*1.72%*	***2.65E-02***	*8.95E-02*	*5.06*
		Lactobacillales		*uncl. Lactobacillales*	*2.02%*	*0.17%*	*0.10%*	*7.11E-01*	*7.68E-01*	*5.27*
	Clostridia	Clostridiales	C. Incertae Sedis XI	Anaerococcus*	0.74%	21.28%	9.47%	**1.28E-04**	**8.66E-04**	5.96
				Finegoldia*	0.21%	4.02%	0.07%	**7.18E-03**	**3.23E-02**	5.45
			Peptoniphilaceae	Peptoniphilus*	0.01%	5.34%	0.50%	**1.95E-05**	**3.31E-04**	5.43
				*uncl. Firmicutes**	*1.30%*	*0.97%*	*0.74%*	***4.20E-02***	*1.13E-01*	*4.70*
				unclassified Bacteria	*7.92%*	*7.67%*	*6.99%*	*3.98E-01*	*5.78E-01*	*4.94*
				TOTAL	**95.59%**	**96.80%**	**97.63%**			

Annex 7. *Relative abundance results of the 10 most abundant genera and of the 5 most abundant parent taxa of interest, on the armpit area. ** both original* p *and corrected value <0.05; * only original* p *<0.05.*

PHYLUM	CLASS	ORDER	FAMILY	GENUS	Summer	Autumn	Winter	LEfSe *p*-value	FDR-corrected	LDA score
Actinobacteria	Actinobacteria	Actinomycetales	Propionibacteriaceae	Cutibacterium	40.82%	39.18%	43.13%	5.50E-01	6.30E-01	5.62
				*uncl. P.ceae**	*0.43%*	*3.10%*	*0.40%*	***4.22E-07***	***9.51E-06***	*5.12*
			Corynebacteriaceae	Corynebacterium	1.81%	0.39%	0.45%	1.54E-01	2.43E-01	4.74
			Microbacteriaceae	Microbacterium	0.64%	0.42%	0.69%	6.43E-01	7.08E-01	3.89
				*uncl. Actinomycetales**	*2.87%*	*11.99%*	*3.16%*	***5.36E-07***	***9.51E-06***	*5.66*
Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Sphingomonas*	15.57%	8.78%	17.43%	**3.85E-02**	9.42E-02	5.61
				*uncl. S.ceae**	*1.72%*	*5.96%*	*1.66%*	***3.75E-04***	***2.05E-03***	*5.37*
	Betaproteobacteria	Burkholderiales	Comamonadaceae	Pelomonas	0.82%	1.20%	1.84%	2.82E-01	4.09E-01	4.62
			Burkholderiaceae	Ralstonia	0.43%	0.35%	0.50%	5.00E-01	5.92E-01	3.65
	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	6.96%	3.69%	6.71%	5.15E-02	1.13E-01	5.32
				*uncl. P.ceae**	*0.72%*	*3.27%*	*0.68%*	***2.72E-04***	***1.61E-03***	*5.13*
Firmicutes	Bacilli	Bacillales	Staphylococcaceae	Staphylococcus*	11.19%	4.54%	8.11%	**4.79E-02**	1.10E-01	5.53
			Bacillaceae 1	Anaerobacillus	0.71%	0.52%	0.93%	6.55E-01	7.08E-01	4.16
		Lactobacillales	Streptococcaceae	Streptococcus	1.17%	0.53%	0.65%	6.58E-01	7.08E-01	4.95

				*uncl. Bacteria***	*5.43%*	*2.94%*	*4.58%*	***1.03E-05***	***8.39E-05***	*5.11*
				TOTAL	**91.30%**	**86.85%**	**90.93%**			

Annex 8. *Relative abundances and LefSe analysis results of the 10 most abundant genera and of the 5 most abundant parent taxa of interest, on the cheek area. ** both original* p *and corrected <0.05; * only original* p *<0.05.*

PHYLUM	CLASS	ORDER	FAMILY	GENUS	Spring	Autumn	Winter	LEfSe *p*-value	FDR-corrected	LDA score
Actinobacteria	Actinobacteria	Actinomycetales	Propionibacteriaceae	Cutibacterium**	54.61%	46.65%	64.14%	**1.71E-02**	**2.34E-02**	5.94
				*unclassified P.ceae**	*0.44%*	*0.64%*	*0.36%*	***7.84E-05***	***1.88E-04***	*4.14*
			Corynebacteriaceae	Corynebacterium**	3.84%	0.76%	0.99%	**3.07E-02**	**3.88E-02**	5.18
				uncl. Actinomycetales	*3.63%*	*3.13%*	*3.47%*	*4.15E-01*	*4.33E-01*	*4.37*
Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Sphingomonas**	4.16%	0.01%	5.50%	**5.59E-10**	**8.90E-09**	5.43
		Rhodospirillales	Acetobacteraceae	Acetobacter**	0.12%	7.46%	0.01%	**3.19E-12**	**1.53E-10**	5.52
				*unclassified A.ceae**	*0.05%*	*3.74%*	*0.01%*	***3.39E-11***	***8.15E-10***	*5.20*
	Betaproteobacteria	Burkholderiales	Comamonadaceae	Pelomonas**	0.27%	0.14%	0.47%	**2.70E-03**	**4.81E-03**	4.13
Firmicutes	Bacilli	Bacillales	Staphylococcaceae	Staphylococcus	22.82%	19.83%	14.48%	6.45E-02	7.37E-02	5.66
			Bacillaceae 1	Anaerobacillus**	0.16%	0.60%	0.05%	**7.63E-06**	**2.44E-05**	4.59
				*uncl. Bacillales**	*1.17%*	*1.49%*	*0.54%*	***1.07E-04***	***2.41E-04***	*4.79*
		Lactobacillales	Streptococcaceae	Streptococcus**	0.31%	1.48%	1.25%	**9.99E-03**	**1.45E-02**	4.82
			Lactobacillaceae	Lactobacillus**	0.01%	1.44%	0.53%	**1.49E-08**	**7.14E-08**	4.91
	Clostridia	Clostridiales	C. Incertae Sedis XI	Anaerococcus**	1.01%	0.29%	0.50%	**2.84E-02**	**3.68E-02**	4.52
				*uncl. Bacteria**	*4.14%*	*6.32%*	*4.08%*	***7.44E-07***	***2.56E-06***	*5.11*
				TOTAL	**96.73%**	**94.00%**	**96.37%**			

Annex 9. *Relative abundances and LefSe analysis results of the 10 most abundant genera and of the 5 most abundant parent taxa of interest, on the cheek area. ** both original* p *and corrected <0.05; * only original* p *<0.05.*

PHYLUM	CLASS	ORDER	FAMILY	GENUS	Spring	Autumn	LEfSe *p*-value	FDR-corrected	LDA score
Actinobacteria	Actinobacteria	Actinomycetales	Propionibacteriaceae	Cutibacterium**	23.00%	6.97%	**3.30E-06**	**8.51E-06**	5.86
			Corynebacteriaceae	Corynebacterium**	13.59%	4.45%	**5.79E-09**	**2.84E-08**	5.68
			Micrococcaceae	Kocuria	2.80%	2.42%	3.19E-01	3.40E-01	4.65
				uncl. Actinomycetales	*4.77%*	*5.01%*	*1.49E-01*	*1.66E-01*	*-4.96*
Proteobacteria	Alphaproteobacteria	Rhodospirillales	Acetobacteraceae	Acetobacter**	1.90%	16.65%	**1.57E-08**	**5.92E-08**	-5.75
				*unclassified A.ceae**	*0.47%*	*7.24%*	***9.31E-13***	***1.14E-11***	*-5.59*
	Betaproteobacteria	Burkholderiales	Burkholderiaceae	Ralstonia**	0.08%	3.67%	**8.19E-09**	**3.65E-08**	-5.23
Firmicutes	Bacilli	Bacillales	Staphylococcaceae	Staphylococcus**	22.16%	6.51%	**1.47E-10**	**1.03E-09**	5.90
			Bacillaceae 1	Anaerobacillus**	2.35%	0.81%	**3.20E-04**	**5.41E-04**	4.87
				*uncl. Bacillales**	*1.26%*	*1.94%*	***5.68E-04***	***8.98E-04***	*-4.83*

		Lactobacillales	Lactobacillaceae	Lactobacillus**	0.89%	17.55%	4.42E-13	1.08E-11	-5.92
				*uncl. Lactobacillales***	*0.31%*	*3.28%*	***2.07E-11***	***2.03E-10***	*-5.34*
	Clostridia	Clostridiales	C. Incertae Sedis XI	Anaerococcus**	2.73%	2.88%	2.88E-06	7.83E-06	4.98
				Finegoldia**	1.34%	1.18%	2.71E-05	5.78E-05	4.72
				*uncl. Bacteria***	*9.79%*	*6.07%*	***2.21E-04***	***3.86E-04***	*5.15*
				TOTAL	**87.44%**	**86.63%**			

Annex 10. *Relative abundances and LefSe analysis results of the 10 most abundant genera and of the 5 most abundant parent taxa of interest, on the upper thigh. ** both original* p *and corrected <0.05; * only original* p *<0.05.*

PHYLUM	CLASS	ORDER	FAMILY	GENUS	Spring	Summer	Winter	LEfSe *p*-value	FDR-corrected	LDA score
Actinobacteria	Actinobacteria	Actinomycetales	Propionibacteriaceae	Cutibacterium	48.20%	49.65%	56.90%	8.05E-02	9.79E-02	5.70
				*uncl. P.ceae***	*0.46%*	*0.50%*	*0.30%*	***4.26E-03***	***7.66E-03***	*3.97*
			Corynebacteriaceae	Corynebacterium	5.97%	1.19%	1.52%	9.65E-02	1.11E-01	5.39
				*uncl. C.ceae***	*1.43%*	*0.06%*	*0.04%*	***8.33E-03***	***1.39E-02***	*4.84*
			*uncl. Actinomycetales***		*4.79%*	*2.98%*	*2.29%*	***1.33E-03***	***3.33E-03***	*5.08*
Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Sphingomonas**	0.08%	6.82%	1.47%	1.25E-05	1.87E-04	5.53
		Rhodospirillales	Acetobacteraceae	Acetobacter**	0.61%	0.08%	0.02%	4.52E-04	1.57E-03	4.53
	Betaproteobacteria	Burkholderiales	Comamonadaceae	Pelomonas**	0.00%	0.44%	0.11%	6.73E-06	1.52E-04	4.37
Firmicutes	Bacilli	Bacillales	Staphylococcaceae	Staphylococcus	24.86%	24.23%	28.55%	4.86E-01	4.97E-01	5.26
			Bacillaceae 1	Anaerobacillus**	0.74%	0.29%	0.01%	1.38E-04	6.89E-04	4.63
			*uncl. Bacillales***		*1.60%*	*0.83%*	*0.57%*	***1.13E-03***	***3.00E-03***	*4.71*
		Lactobacillales	Streptococcaceae	Streptococcus**	1.43%	0.37%	0.22%	3.03E-02	4.27E-02	4.80
	Clostridia	Clostridiales	C. Incertae Sedis XI	Anaerococcus*	0.77%	2.30%	0.72%	3.73E-02	5.09E-02	4.94
				Finegoldia	*0.05%*	*0.45%*	*0.10%*	*7.72E-02*	*9.65E-02*	*4.14*
				uncl. Bacteria	*5.97%*	*5.29%*	*5.71%*	*9.01E-01*	*9.01E-01*	*4.46*
				TOTAL	**96.96%**	**95.47%**	**98.53%**			

Annex 11. *Relative abundances and LefSe analysis results of the 10 most abundant genera and of the 5 most abundant parent taxa of interest, on the cheek area. ** both original* p *and corrected <0.05; * only original* p *<0.05.*

PHYLUM	CLASS	ORDER	FAMILY	GENUS	Milan	G. Mil.	Biella	LEfSe *p*-value	FDR-corrected	LDA score
Actinobacteria	Actinobacteria	Actinomycetales	Propionibacteriaceae	Cutibacterium	47.24%	50.34%	54.44%	6.10E-01	6.61E-01	5.52
			Corynebacteriaceae	Corynebacterium	7.84%	2.34%	3.83%	2.97E-01	3.37E-01	5.33
				unclassified C.ceae	*1.78%*	*0.37%*	*0.84%*	*1.60E-01*	*1.99E-01*	*4.77*
			Microbacteriaceae	Microbacterium**	0.58%	0.65%	0.20%	2.69E-04	1.44E-03	4.37
			uncl. Actinomycetales		*4.65%*	*3.34%*	*3.61%*	*3.25E-01*	*3.60E-01*	*4.80*
Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Sphingomonas**	10.43%	11.93%	4.15%	1.08E-03	3.53E-03	5.60
				*unclassified S.ceae***	*1.24%*	*1.31%*	*0.49%*	***1.11E-03***	***3.53E-03***	*4.63*
	Betaproteobacteria	Burkholderiales	Comamonadaceae	Pelomonas**	0.82%	1.01%	0.29%	4.45E-04	1.89E-03	4.57
			Burkholderiaceae	Ralstonia**	0.26%	0.41%	0.12%	1.21E-03	3.64E-03	4.16

	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas**	0.13%	2.14%	0.01%	7.33E-08	3.74E-06	5.13
Firmicutes	Bacilli	Bacillales	Staphylococcaceae	Staphylococcus**	12.21%	13.90%	23.00%	2.80E-03	5.50E-03	5.78
				uncl. Bacillales	*1.80%*	*1.21%*	*1.17%*	*8.97E-02*	*1.17E-01*	*4.61*
		Lactobacillales	Streptococcaceae	Streptococcus**	1.04%	1.47%	0.30%	3.13E-04	1.45E-03	4.89
	Clostridia	Clostridiales	C. Incertae Sedis XI	Anaerococcus**	0.29%	0.22%	1.01%	7.21E-03	1.31E-02	4.56
				uncl. Bacteria	*3.76%*	*4.21%*	*4.12%*	*7.69E-01*	*7.69E-01*	*4.40*
				TOTAL	**94.07%**	**94.85%**	**97.58%**			

Annex 12. *Relative abundances and LefSe analysis results of the 10 most abundant genera and of the 5 most abundant parent taxa of interest, on the cheek area. ** both original* p *and corrected <0.05; * only original* p *<0.05.*

PHYLUM	CLASS	ORDER	FAMILY	GENUS	Pavia	G. Mil.	Biella	LEfSe *p*-value	FDR-corrected	LDA score
Actinobacteria	Actinobacteria	Actinomycetales	Propionibacteriaceae	Cutibacterium	40.72%	34.95%	43.33%	3.34E-01	3.77E-01	5.70
			Corynebacteriaceae	Corynebacterium	0.96%	1.02%	0.59%	2.44E-01	2.80E-01	4.44
				*uncl. Actinomycetales**	*10.78%*	*2.41%*	*2.92%*	***6.47E-05***	***8.83E-04***	*5.62*
Proteobacteria	Alphaproteobacteria	Rhodospirillales	Acetobacteraceae	Acetobacter**	2.16%	10.63%	7.30%	7.34E-05	8.83E-04	5.65
				*uncl. A.ceae**	*0.99%*	*2.94%*	*3.11%*	***2.23E-02***	***3.32E-02***	*5.00*
		Rhizobiales	Brucellaceae	Brucella**	0.37%	1.13%	0.57%	1.39E-03	4.47E-03	4.56
		Sphingomonadales	Sphingomonadaceae	*uncl. S.ceae**	*6.04%*	*0.02%*	*0.02%*	***4.41E-04***	***1.92E-03***	*5.53*
Firmicutes	Bacilli	Bacillales	Staphylococcaceae	Staphylococcus	10.28%	14.21%	16.07%	7.06E-02	8.97E-02	5.58
				Bacillus**	0.21%	0.72%	0.34%	1.59E-03	4.63E-03	4.40
				Gemella**	0.12%	0.66%	0.16%	1.31E-04	8.83E-04	4.52
			Bacillaceae 1	Anaerobacillus	0.61%	0.35%	0.55%	6.07E-02	8.61E-02	4.22
				uncl. Bacillales	*2.07%*	*1.00%*	*1.30%*	*6.51E-02*	*8.63E-02*	*4.77*
		Lactobacillales	Lactobacillaceae	Lactobacillus**	3.77%	12.80%	9.69%	3.29E-04	1.55E-03	5.64
			Streptococcaceae	Streptococcus	0.82%	1.36%	1.09%	4.33E-01	4.81E-01	4.46
				*uncl. Bacteria**	*4.25%*	*7.11%*	*5.44%*	***1.59E-03***	***4.63E-03***	*4.40*
				TOTAL	**84.14%**	**91.32%**	**92.48%**			

Annex 13. *Relative abundances and LefSe analysis results of the 10 most abundant genera and of the 5 most abundant parent taxa of interest, on the cheek area. ** both original* p *and corrected <0.05; * only original* p *<0.05.*

PHYLUM	CLASS	ORDER	FAMILY	GENUS	Pavia	Biella	LEfSe *p*-value	FDR-corrected	LDA score
Actinobacteria	Actinobacteria	Actinomycetales	Propionibacteriaceae	Cutibacterium**	44.72%	62.14%	5.56E-06	1.56E-05	5.86
			Corynebacteriaceae	Corynebacterium	1.09%	0.89%	3.86E-01	4.41E-01	-4.34
				uncl. Actinomycetales	*4.88%*	*3.47%*	*5.00E-01*	*5.49E-01*	*-4.81*
Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Sphingomonas**	12.93%	5.16%	1.95E-06	6.08E-06	-5.56
				*unclassified S.ceae**	*2.51%*	*0.54%*	***3.61E-09***	***3.37E-08***	*-4.95*
		Rhodospirillales	Acetobacteraceae	Acetobacter**	0.86%	1.41%	2.67E-02	3.69E-02	4.59

	Betaproteobacteria	Burkholderiales	Burkholderiaceae	Pelomonas**	1.29%	0.47%	**2.87E-06**	**8.45E-06**	-4.55
		Neisseriales	Neisseriaceae	*uncl. N.ceae***	*1.33%*	*0.84%*	***6.13E-04***	***1.14E-03***	*-4.33*
	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas**	4.36%	0.03%	**9.35E-18**	**2.62E-16**	-5.34
Firmicutes	Bacilli	Bacillales	Staphylococcaceae	Staphylococcus**	9.20%	12.63%	**5.17E-03**	**7.82E-03**	5.29
			Bacillaceae 1	Anaerobacillus**	0.62%	0.17%	**4.52E-11**	**6.33E-10**	-4.21
				*uncl. Bacillales**	*0.90%*	*0.66%*	***4.46E-02***	*5.94E-02*	*-3.72*
		Lactobacillales	Streptococcaceae	Streptococcus	0.63%	0.92%	7.77E-01	8.06E-01	4.27
			Lactobacillaceae	Lactobacillus**	0.53%	0.68%	**1.29E-05**	**3.14E-05**	4.28
				*uncl. Bacteria***	*4.30%*	*5.10%*	***3.28E-04***	***6.55E-04***	*4.76*
				TOTAL	**90.15%**	**95.11%**			

Annex 14. *Relative abundances and LefSe analysis results of the 10 most abundant genera and of the 5 most abundant parent taxa of interest, on the upper thigh. ** both original* p *and corrected <0.05; * only original* p *<0.05.*

PHYLUM	**CLASS**	**ORDER**	**FAMILY**	**GENUS**	**Milan**	**Pavia**	**LEfSe *p*-value**	**FDR-corrected**	**LDA score**
Actinobacteria	Actinobacteria	Actinomycetales	Propionibacteriaceae	Cutibacterium**	63.22%	48.10%	**6.95E-03**	**1.52E-02**	5.77
			Corynebacteriaceae	Corynebacterium	4.17%	4.58%	8.69E-01	8.69E-01	4.33
				unclassified C.ceae	*0.99%*	*0.79%*	*4.53E-01*	*5.66E-01*	*4.32*
				uncl. Actinomycetales	*4.13%*	*4.54%*	*5.89E-01*	*6.88E-01*	*-3.67*
Proteobacteria	Alphaproteobacteria	Rhodospirillales	Acetobacteraceae	Acetobacter**	0.29%	1.11%	**4.85E-03**	**1.13E-02**	-4.61
	Betaproteobacteria	Burkholderiales	Comamonadaceae	Pelomonas	0.07%	0.40%	3.85E-01	4.99E-01	-3.88
Firmicutes	Bacilli	Bacillales	Staphylococcaceae	Staphylococcus	18.95%	27.09%	3.13E-01	4.21E-01	-5.36
				Gemella	0.03%	0.17%	1.59E-01	2.42E-01	-4.06
			Bacillaceae 1	Anaerobacillus	0.20%	0.91%	5.14E-02	9.47E-02	-4.51
				*uncl. Bacillales***	*0.80%*	*1.57%*	***7.25E-04***	***5.07E-03***	*-4.56*
		Lactobacillales	Streptococcaceae	Streptococcus**	0.19%	1.09%	**1.30E-03**	**5.69E-03**	-4.92
			Lactobacillaceae	Lactobacillus**	0.07%	0.28%	**2.46E-03**	**7.83E-03**	-4.11
	Clostridia	Clostridiales	C. Incertae Sedis XI	Anaerococcus	1.62%	1.01%	7.96E-01	8.45E-01	4.51
				*uncl. Firmicutes***	*0.33%*	*0.50%*	***3.89E-03***	***1.05E-02***	*-3.98*
				*uncl. Bacteria***	*3.69%*	*5.25%*	***1.02E-03***	***5.33E-03***	*-5.04*
				TOTAL	**98.75%**	**97.39%**			

Annex 15. *Relative abundances and LefSe analysis results of the 10 most abundant genera and of the 5 most abundant parent taxa of interest, on the cheek area. ** both original* p *and corrected <0.05; * only original* p *<0.05.*