Cutibacterium acnes strain improvement and establishment of skin microbiome sequencing technologies

Miquel Rozas Belmonte

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Thesis supervisor

Bernhard Paetzold,

S-Biomedic

Thesis cosupervisor:

Marc Güell Cargol,

Translational Synthetic Biology, UPF

upf. Universitat Pompeu Fabra Barcelona

S-BIOMEDIC

Abstract

Certain skin disorders are associated with skin microbiome dysbiosis, highlighting the importance of the microbiome in the maintenance of the skin's healthy state. Modulating the microbiome to reestablish its healthy state is a novel strategy to treat such skin diseases. One strategy to modulate the skin microbiome and obtain dermatological benefits is through the topical application of probiotics. Identifying and selecting the most suitable bacterial strains to treat specific skin diseases is key to success. [redacted] Furthermore, taxonomic identification of skin microbiome samples using Nanopore sequencing was benchmarked and improved, contributing to advancements in both diagnosis and novel treatments. [redacted] In summary, this research contributed to the advancement in treating skin diseases by [redacted], benchmarking and improving bacterial taxonomic identification using Nanopore sequencing and [redacted]

Ciertas enfermedades de la piel están asociadas a un desbalance en el microbioma de la piel, resaltando la importancia del microbioma en mantener la piel sana. Modular el microbioma para restablecer su estado sano es una estrategia novedosa para tratar esas enfermedades de la piel. Una estrategia para modular el microbioma de la piel y obtener beneficios dermatológicos es la aplicación tópica de probióticos. Identificar y seleccionar las bacterias que más se adecuan para tratar especificas enfermedades de la piel es clave para el éxito en su tratamiento. [redacted] Además, hemos evaluado y mejorado la clasificación taxonómica del microbioma de la piel usando Nanopore sequencing, contribuyendo así a avances en el diagnóstico y el desarrollo de nuevas terapias para tratar problemas dermatológicos. [redacted] En resumen, esta investigación ha contribuido al avance en el tratamiento de enfermedades de la piel [redacted], evaluando y mejorando la clasificación taxonómica del microbioma de la piel usando Nanopore sequencing y [redacted].

Thesis introduction

Skin microbiome

The skin is the largest ($2m^2$) and most visible organ of the human body[1]. Constituted of three immunologically active layers[2], the epidermis (75 to 150µm), the dermis (<2mm) and the hypodermis (1-2mm)[3], the skin is also the largest epithelial surface for interaction with microbes[4]. The outer part (the stratum corneum), the appendages (sweat and sebaceous glands, hair follicle)[5], and the subepidermal compartments[6] are indeed colonized by a myriad of microorganisms including, on the skin surface, eukaryotes (10%), viruses (30%), and bacteria (60%) (Figure 1A)[7]. Within the dominant bacterial kingdom, representing on the skin a total of 4.10^{10} individuals[8], four major phyla compose the microbial communities: Actinobacteria (36 – 51%), Firmicutes (24 – 34%), Proteobacteria (11 – 16%), and Bacteroidetes (6 – 9%) (Figure 1B)[9,10]. However, among the different skin sites, these proportions are strongly shaped by different skin physiological characteristics such as temperature, pH, UV light exposure, moisture/humidity and sebum content[11]. Thus, three major topographical categories provide specific environmental niches: the dry sites (e.g. volar forearm and palm) colonized by a majority of Betaproteobacteria, the moist/humid areas (e.g. bend of the elbow) predominated by the gena *Staphylococcus* and *Corynebacterium* and the oily/sebaceous sites (e.g. face and back) largely predominated by the genus *Propionibacterium* and followed by *Staphylococcus* and *Corynebacterium* (Figure 1C)[12]. In these lipid rich microenvironments, but not exclusively, relative abundance of bacterial populations is influenced by gender, age, and geographical origins. Nonetheless, *Propionibacterium* still predominates with a relative stability in sebaceous sites of healthy individuals (Figure 1D)[13–15]. This genus, recently reclassified and renamed *Cutibacterium*[16], is a sentinel of the healthy human skin microbiome[17].

The human microbiome plays a crucial role in human health[18], even though defining a healthy microbiome is not a trivial task[19]. Different gut and skin diseases have been associated with microbiome dysbiosis, ''elucidating by contraposition'' its importance in the maintenance of the healthy state[20]. The gut microbiome is the most extensively studied human microbiome[21]. It has essential functions for the healthy state of the gut, such as protection against pathogen invasion, nourishing the host cells with their metabolic products, reinforcing the intestinal barrier and, training and modulating the immune system[22].

Skin diseases and microbiome dysbiosis

Compared to the gut, lesser is known about the role of the skin microbiome for skin homeostasis, but skin microbiome alterations are associated with skin diseases such as atopic dermatitis (AD), psoriasis, rosacea and acne vulgaris[23,24].

Acne vulgaris is the most common dermatological condition worldwide[25]. Aggregating several risk factors (e.g. age, skin type) and prevalent for the sebaceous/lipid-rich skin sites (e.g. face and back)[26], acne is a chronic inflammation of the pilosebaceous unit[27] often persisting into adulthood[28]. Extensively described and studied over the past centuries[29], firmly associated with the skin microbiome and *Cutibacterium acnes* (*C. acnes)* proliferation[30,31], the paradigm of this dysbiosis is now changing. Recently, many authors observed that relative abundance and

bacterial load of *C. acnes* are not significantly different between acne and healthy patients (Figure 1E and F)[32–37]. Conjointly to these findings, the advances in sequencing technologies and research made in the past decades have revealed the diversity of *C. acnes* at subspecies (*C. acnes* subsp. *acnes* (phylotype I), *C. acnes* subsp. *defendens* (phylotype II), *C. acne* subsp. *elongatum* (phylotype III)), and subtypes levels (phylogenetic groups IA_1, IA_2, IB, IC, II, III)[38]. This is how the recent paradigm shift occurs: a decrease of the diversity between the 6 *C. acnes* phylogenetic groups, rather than *C. acnes* proliferation has been associated with acne progression (Figure 1G and H)[39,40]. *C. acnes* populations are also disturbed, among others, in AD, rosacea, psoriasis, actinic keratosis and aged skin[23,41,42].

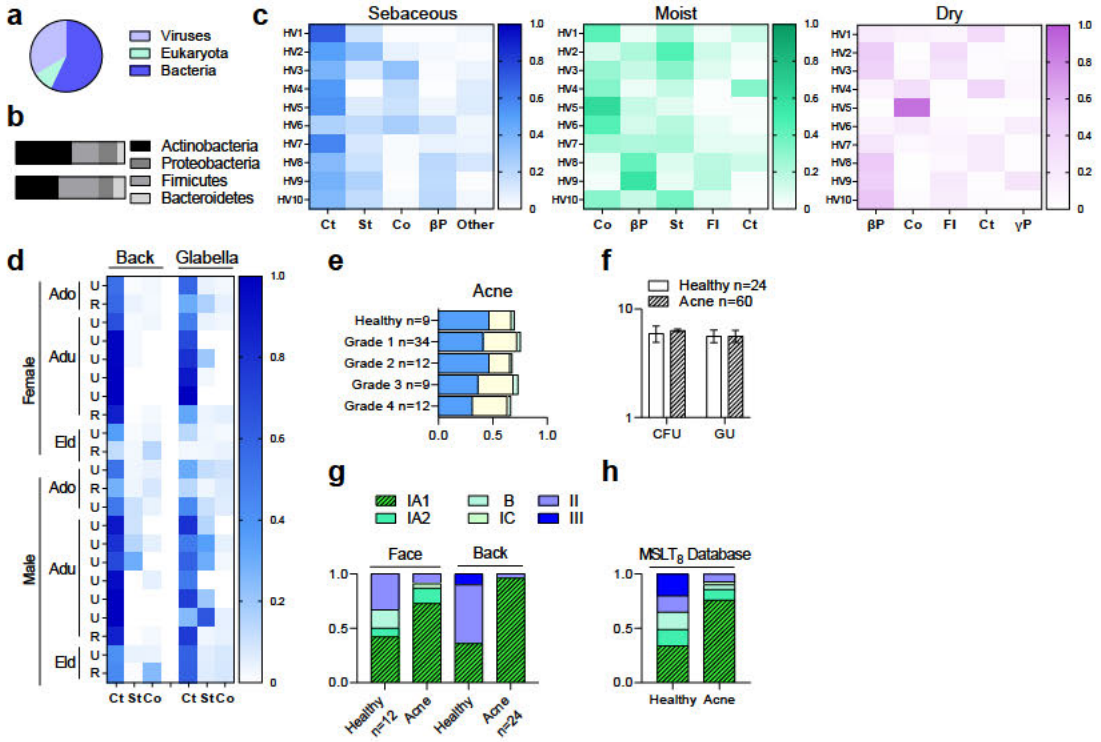

Figure 1. C. acnes in the human skin microbiome. (a) Relative abundance of skin microbiota across kingdoms in 15 healthy volunteers (HV) (9 males, 6 females) sampled in 18 different skin sites, adapted from Oh et al., 2014[7]. Bacterial genomes predominate at most sites. (b) The analysis of 16S ribosomal RNA of 10 HV (20 sites sampled, upper bar graph) and 9 HV (27 sites sampled, lower bar graph) shows that most of the sequences are attributed to four phyla, adapted from Grice et al., 2009[9] and Costello et al., 2009[10], respectively. Over this, skin microenvironments also vary drastically in their level of bacterial diversity. (c) In 10 HV sampled in sebaceous, moist, and dry sites, the relative abundance of Cutibacterium (Ct, formerly Propionibacterium), Staphylococcus (St), Corynebacterium (Co), Betaproteobacteria (βP), Flavobacteria (Fl) and Gammaproteobacteria (γP) is strongly shaped by the niche, adapted from Grice et al., 2009[9]. (d) The predominating relative abundance of Cutibacterium in sebaceous sites is relatively stable over the 22 HV from different genders, ages (Ado: teenager, Adu: adult, Eld: elderly) and locations (U: urban, R: rural), adapted from Findley et al., 2013[13] and Ying et al., 2015[14]. However, this relative abundance of Cutibacterium genus is significantly decreased (: p-value < 0.05) in (e) Interestingly, no significant alteration of the relative abundance of Cutibacterium genus was observed over 67 patients (22-23yo) with no and/or different acne severity sampled on the cheek, adapted from Li et al., 2019[51]. (f) No difference neither for the bacterial load (log Colony Forming Units (CFU) and log Genomic Units (GU) per strip) between 15-30yo males and females healthy or acne patient sampled on the face, adapted from Pecastaings et al., 2018[52]. The sharp difference between healthy and acne patient is in the diversity decrease of C. acnes phylotypes. (g) Comparison of the relative abundance of the 6 phylotypes of C. acnes between 16-35yo male and female healthy and acne patients, adapted from Dagnelie et al., 2017[55]. (h) Relative abundance and statistically significant enrichment of type IA1 with acne is confirmed by the analysis of the current $MSLT_8$ isolate database, adapted from McLaughlin et al., 2019[54].*

Cutibacterium acnes in skin homeostasis

Since some skin diseases are related to a dysbiosis of their natural microbiome, it is also expected that the skin microbiome plays a central role in skin homeostasis. One of the key players in skin homeostasis, reflected by its dysbiosis in most skin diseases, is the most abundant bacteria of the skin microbiome, *C. acnes*. It is a Gram-positive rod bacterium, aerotolerant anaerobic that does not produce spores[43]. *C. acnes* has co-evolved with the host to live in the pilosebaceous units, where oxygen and easily accessible nutrients are scarce[44]. To survive in the harsh and lipid-rich environment of the pilosebaceous units, *C. acnes* acquired genes to modulate and metabolize, *inter alia*, host skin lipids[45]. Through host lipids modulation and other mechanisms, *C. acnes* has been shown to contribute to skin homeostasis[46].

Lipid modulation

To obtain energy from the abundant triacylglycerols in the sebum, *C. acnes* secretes a triacylglycerol lipase GehA[47]. As a product of triacylglycerol fermentation, *C. acnes* secretes short-chain fatty acids (SCFAs). *C. acnes* predominantly produces the SCFA propionic acid, where its former name *Propionibacterium* comes from[44]. The role of propionic acid on the skin is yet to be uncovered, although it contributes to maintaining the acidic layer on the skin[48]. Increased pH has been reported in different skin disorders[49] and skin enzymes involved in maintaining skin homeostasis are pH dependent[49].

Follicular niche competition

C. acnes is highly adapted to live in specific skin niches, where it thrives and outcompetes pathogens for nutrient acquisition[11]. Phylotypes IB and III have been described to contain a biosynthetic gene (BSG) cluster capable of producing the antimicrobial thiopeptide cutimycin[50]. It was only expressed when *C. acnes* was co-cultured with strains from the genus *Staphylococcus* and it has shown *in-vitro* antimicrobial activity for *Staphylococcus aureus*. Furthermore, *C. acnes* produced SCFA restored *S.epidermidis* antibiotic sensitivity by reducing its capacity to form biofilms[51], usually associated with skin disorders[52].

Immune modulation

Different skin-resident immune cells contribute to tissue homeostasis[2]. The immune cells on the skin have tight interactions with the skin microbiome to keep the skin healthy[53]. *C. acnes* phylotypes associated with healthy skin or with acne showed very different immune responses[54]. Immune interactions among *C. acnes* with keratinocytes and sebocytes would not trigger immune response unless environmental changes would trigger higher production of SCFAs, highlighting the immune tolerance of the skin towards *C. acnes* on homeostasis[55,56]. Furthermore, *C. acnes* has been shown to stimulate skin defences against pathogen invasion by enhancing autophagic activity in keratinocytes[57] and promoting activation of T helper type 1 (Th1) cells *in vivo*[58].

Oxidative stress mitigation

The skin is constantly exposed to UV radiation, which triggers the formation of reactive oxygen species (ROS)[59], leading to cell damage and contributing to skin carcinogenesis[60]. In addition to the ROS mitigating strategies of epithelial cells, the most abundant secreted protein of *C. acnes*, the radical oxygenase of *Propionibacterium acnes* (RoxP*)*, has been reported with antioxidant activity[47,61]. RoxP is the first extracellular bacterial antioxidant enzyme characterized[61], which

showed to increase the viability of ROS stressed monocytes and keratinocytes *in vitro*[62]. Furthermore, in actinic keratosis (AK), an initial stage of non-melanoma skin cancer, host cells antioxidant function is shown to be deficient [63] and *C. acnes* abundance and RoxP levels were lower compared to healthy areas[42,64].

Microbiome modulation strategies

Dysbiosis of microbiome composition and function and the adapted metabolic variety of commensal microbial strains make microbiome-modulating strategies an interesting avenue for the treatment of dysbiotic conditions. Microbiome-modulating strategies have been mainly aimed at modulating the gut microbiota to redress dysbiotic patterns of the microbiome associated with disease[65]. Strategies aimed at modulating the gut microbiota involve using probiotics, prebiotics, symbiotic and faecal microbiota transplants[66]. Microbiota transplantations are based on transferring the microbiome from a healthy subject to the dysbiotic receiver. Faecal microbiota transplants (FMT) have been proven effective for restoring the phylogenetic richness of the recipient's intestinal microbiota, effectively treating gastric *Clostridium difficile* infections[67].

Microbiome modulation through the usage of probiotics already has a long history of health claims through oral usage. Probiotics are defined as "live microorganisms which when administered in adequate amounts confer a health benefit on the host"[68]. In light of the research mainly conducted in the last 10 years, health benefits of these probiotics administered through the gastrointestinal tract act through four different mechanisms of action: (i) improvement of the epithelial barrier function, (ii) interference with pathogenic bacteria, (iii) immunomodulation and (iv) influence on other organs of the body through the immune system[69]. Different strains of microbial species have specialized enzymatic activities and varied metabolic strategies, even within one species[70,71].

Skin microbiome modulation

Dysbiosis of the skin microbiome associated with skin disorders could be changed via multiple mechanisms: skin microbiome transplant, prebiotics, probiotics, synbiotics and putatively postbiotics. Whole skin microbiome transplantation would require collecting the skin microbial community, which is challenging to obtain compared to faecal microbiota samples, as a culturing step is always required. One remains challenged by the uncultivability of microorganisms in-vitro, also known as the "great plate count anomaly"[72]. Therefore, performing skin microbiome transplantation analogous to FMT is not scalable nor industry applicable.

Recent years have seen a sharp increase in clinical investigations of probiotics and postbiotics used in dermatology. Processes to produce such non-viable fermentation products (postbiotics) are industrially scalable, can easily be formulated into products and are widely used in the cosmetic industry. Probiotics, on the other hand, pose a challenge for formulation and packaging to ensure the viability of the micro-organisms[73]. As a result, most suppliers formulate with postbiotics. Nevertheless, while postbiotics of skin commensals may protect against UV-induced oxidative damage, hyperpigmentation[74] and pathogens such as *S. aureus*[75], reintroducing viable microorganisms to their adapted niche have the potential to modulate the microbiome [76]. Skin microbiome modulation could mitigate or potentially eliminate pathological skin conditions, analogous to strategies in the gut. Callewaert et al 2021 recently reviewed efforts undertaken in

skin microbiome modulation strategies[76] and reintroduced the term bacteriotherapy to describe probiotics and postbiotics.

S-Biomedic approach

S-Biomedic targets skin conditions by modulating the skin microbiome using prebiotics or bacteriotherapy, focusing on the most abundant skin commensal, *C. acnes.* As previously described, *C. acnes* contributes to skin homeostasis by modulating host lipids, competing with pathogens for specific skin niches, training and enhancing the host immune system and protecting skin cells from oxidative stress. Several skin diseases have been described to correlate with a dysbalanced *C. acnes* population at species or strain level. Acne vulgaris is the first skin disease that S-Biomedic focused its research and development program on. Acne vulgaris was historically associated with an increase of *C. acnes* relative abundance, but later advancements showed that acne vulgaris is rather associated with an imbalance at *C. acnes* sub-species level, specifically through an increase of *C. acnes* strains of phylotype IA_1[39]. The new insights on microbiome dysbiosis in acne vulgaris, allowed S-Biomedic to design a *C. acnes* strain-specific probiotic cocktail to modulate the dysbiosis associated with acne.

The following sections highlight the process that has been followed in this thesis from selecting a suitable *C. acnes* strain to target a skin disease, its production in the laboratory, testing in clinical trials and analysis of microbiome modulation. Furthermore, the relatively new Nanopore sequencing technology was benchmarked, improved and used for skin microbiome sequencing of clinical samples.

Strain selection

A key parameter to target skin diseases through microbiome modulation strategies using prebiotics or bacteriotherapy is the selection of the bacterial strain or strains to be used in the treatment. Understanding the microbiome dysbiosis in disease and the contributions of the microbiome commensals to skin homeostasis is fundamental in the design of a successful microbiome modulation strategy. For example, facial acne vulgaris has been described to be linked with a *C. acnes* strain diversity reduction, through a relative abundance increase of *C. acnes* phylotype IA_1 and relative abundance reduction of phylotypes IB and II[40]. Therefore, applying probiotics of *C. acnes* phylotypes IB and II could restore the *C. acnes* diversity on the skin of acne vulgaris patients and reduce its dermatological symptoms. These hypotheses must be tested in clinical studies to assess their safety and efficacy. To test the hypothesis, first, the laboratory growth and downstream processes of the selected strains must be mastered to obtain a successful product.

Production

To obtain a product based on bacterial strains to be tested in human clinical trials some requirements must be met. The pipeline of bacterial production has to be highly controlled and reproducible, and each produced batch is then assessed through quality control (QC). A good QC has to ensure that each produced batch complies with the determined specifications regarding the quality and safety of the product. When producing a bacterial product, the main specifications to comply with are: [redacted]

[redacted]. To obtain a successful production pipeline with robust quality control, deep knowledge of microbiology, fermentation and sequencing are essential.

Clinical trial

Once the bacterial product has passed the QC it can be tested in human clinical trials. The design of a clinical trial is at least as much important as obtaining a good product to ensure success when testing our hypothesis. Some parameters to consider when designing the clinical trial are: (i) have a large enough number of patients to test the proposed Hypothesis, (ii) have enough groups (for example placebo vs active) to test the proposed Hypothesis, (iii) carefully select the patients for the disease we want to target and their demographic data, (iv) have clear measurable outputs to validate or decline our hypothesis, (v) decide the timelines of treatment application and outputs measurements, (vi) randomize and anonymize the patients and the different groups, (vii) have a robust system of patients data protection and (viii) ensure the ethicality of the process by obtaining approval of the ethics committee for the designed clinical trial. Designing a clinical trial is a laborious and complex task, where knowledge of statistics, data management, human medicine and human ethics are required to be successful.

Microbiome modulation assessment

After the clinical trial, it can be assessed If the applied strain is significantly improving the addressed dermatological condition. To better understand the dermatological outputs of the clinical trial they are paired with additional metadata. This metadata can be profound characteristics such as age, usage of skin care products or complex parameters like skin microbiome analysis. The effect of skin microbiome modulation can be assessed by determining the microbiome populations of the patients at different timepoints of the clinical trial. To do so, molecular biology techniques and sequencing technologies are used, to obtain the evolution of microbiome profiles over different timepoints. The generated information can help to further evaluate the tested hypothesis from the perspective of microbiome modulation. The obtained knowledge can be used to formulate a new hypothesis and improve the pipeline of strain selection, production, and clinical trial design.

MinION™ Nanopore Sequencing of Skin Microbiome 16S and 16S-23S rRNA Gene Amplicons

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Introduction

Precise characterization of the different human microbiomes is a critical first step towards understanding the host-microbe interactions in human health and disease[19,46,124]. Characterization of bacterial communities was revolutionized by the development of next-generation sequencing techniques, which allowed microorganisms discrimination to deeper taxonomic levels[125].

Due to its simplicity and reliability, the most standardized sequencing strategy to identify bacteria is based on the analysis of their 16S rRNA gene[126]. The 16S rRNA gene is essential in the bacterial domain and consists of ~1500bp containing 9 hypervariable regions (V1 to V9) scattered among highly conserved sequences[127]. All or some of the 16S rRNA gene V1-V9 regions are amplified by polymerase chain reaction (PCR) using complementary primers to the conserved sequences[128]. The resulting amplicons are sequenced and assigned to a bacterial taxonomic group by nucleotide sequence comparison with a reference nucleotide database (e.g. BLASTn)[129].

The first available sequencing technique, Sanger sequencing[130], enabled 16S rRNA identification of bacterial clonal populations[131]. Technical difficulties to maintain bacterial diversity when obtaining clonal populations, effect known as the great plate count anomaly[132], limited the detectable species with Sanger sequencing[133].

Overcoming these limitations, Next generation sequencing (NGS) techniques enabled the direct analysis of complex bacterial communities by parallel high throughput generation of reads, providing faster and cheaper sequencing costs per sample[134,135]. However, the most popularized NGS technique Illumina is limited to short fragments (<600bp) and does not allow sequencing of the entire 16S rRNA gene[136]. Using NGS, taxonomic relative abundances are determined by analyzing subregions of the 16S rRNA gene, but the obtained results are biased by the selected subregion due to distinct primer binding affinities to each template[137–139]. Therefore it is not recommended to compare microbiome studies based on different 16S rRNA regions[140–143].

In 2014 Oxford Nanopore Technologies (ONT) released a single-molecule sequencing technology that allows sequencing of DNA fragments without a theoretical length limit[144]. High throughput generation of reads is achieved in a pocket-sized portable device such as MinION™[145]. MinION™ instrument made nanopore sequencing widely accessible, allowing research centers to perform real-time data analysis, drastically reducing sequencing turnaround times, and lowering the cost per sequenced base[146]. Nanopore technology allows entire 16S gene sequencing in samples with bacterial mixtures, overcoming at the same time, the main limitations of Sanger sequencing and NGS[147]. Nonetheless, Nanopore sequencing still has higher base calling error rates than established NGS technologies[148].

In this study, a defined human skin bacterial genomic mock community and a skin microbiome sample were used to analyze the performance of ONT sequencing kits on taxonomic relative abundance and species level determination. Recent studies focusing on other human

microbiomes (e.g. gut) have already described bias of ONT sequencing kits towards certain genera and species[149,150]. To the best of our knowledge, no study has focused on analyzing the performance of ONT sequencing kits on the skin microbiome. Understanding the bias and limitations of ONT kits in taxonomizing bacteria of skin microbiome samples is crucial for future experimental designs and data interpretation[143]. Obtaining insights into the skin microbiome composition to genus and species level in skin health and disease will help to develop more effective prebiotic, probiotic, or drug therapies to treat skin diseases associated with microbiome dysbiosis.

Materials & methods

Skin microbiome genomic mix

Skin genomic mock community ATCC MSA-1005 was used in this study. It consists of an even mixture of six bacterial species each representing 16.7% (*Acinetobacter johnsonii* (ATCC 17909D-5), *Corynebacterium striatum* (ATCC 6940D-5), *Micrococcus luteus* (ATCC 4698D-5), *Cutibacterium acnes* (ATCC 11828D-5), *Staphylococcus epidermidis* (ATCC 12228D-5) and *Streptococcus mitis* (ATCC 49456D-5)).

Skin microbiome standard

An artificial skin standard was created by mixing 72 extracted DNA samples of cheek skin swabs. Cheek swabs were collected, stored and transported at -20°C, using the eNAT collection and transport system (Copangroup, USA). DNA was isolated and purified using DNAeasy 96 PowerSoil Pro Kit (Qiagen, UK) following its Quick-Start Protocol. In essence, samples were disrupted by mechanical bead-beating and DNA was isolated and purified using silica membrane spin columns. A DNA skin standard was then obtained by combining 5µL of each of the 72 extracted samples.

16S V1-V9 Nanopore sequencing and reads taxonomic assignation

16S rRNA barcoded amplicons were produced in a single four-primer PCR reaction following Matsuo protocol[150]. The following inner primers for amplification of V1-V9 of the 16S rRNA gene, with complementary region underlined and anchor region were used: forward primer (27F) 5'-TTTCTGTTGGTGCTGATATTGCAGAGTTTGATCMTGGCTCAG-3' and reverse primer (1492R) 5'-ACTTGCCTGTCGCTCTATCTTCCGGTTACCTTGTTACGACTT-3'. Barcoded outer primers containing the complementary anchor sequence to inner primers from PCR Barcoding Expansion Pack 1-96 EXP-PBC096 (Oxford Nanopore Technologies, UK) were used. DNA amplification was performed using Veriti 96 Well Fast Thermo Cycler in a reaction mix containing 200nM of inner primers, 200nM of outer primers 12.5µL of LongAmp polymerase mix and 5uL of template in a total volume of 25µL. The cycling program used from the Matsuo protocol was adapted to LongAmp polymerase. It consisted of 3 min denaturation at 95°C, 5 cycles (95°C – 15s, 55°C – 15s, 65°C – 90s), 30 cycles (95°C – 15s, 62°C – 15s, 65°C – 90s) and a final extension step of 65°C for 2min. Samples were also amplified using a KAPA HiFi HotStart PCR Kit KK2502 (Roche, Switzerland), using the same primer concentrations, mastermix prepared following manufacturer recommendations and following Matsuo PCR conditions. PCR amplicons were run in 1% agarose gel in an electrophoresis chamber, pooled together and purified using DNA clean & concentrator kit (Zymoresearch, USA). Purified samples were then quantified with Accublue Broad Range dsDNA quantification kit (Biotium, USA) and further processed using SQK-LSK110 kit (Oxford

Nanopore Technologies, UK). The library was sequenced using flow cell R9.4.1 (FLO-MIN106D) until the sample was exhausted or the desired number of reads was achieved. Basecalling was performed on MinION Mk1C using Guppy (version 5.0.13) with fast basecalling model and read filtering of min_score=8. Epi2me (version v2021.09.09) was used to demultiplex the samples, filter reads retaining size range of 1.2-1.8kb and assign the reads to its taxonomic group with default parameters using NCBI 16S database[151].

16S-23S Nanopore sequencing and reads taxonomic assignation

NanoID kit is designed to produce 16S-23S amplicons of 2.5kb. It contains a forward primer complementary to the 16S gene (27F): 5'-AGRRTTYGATYHTDGYTYAG-3', and a reverse primer complementary to the 23S gene (23SR): 5'-AGTACYRHRARGGAANGR-3'. 16S-23S amplicons were produced using NanoID kit (Shoreline Biome, USA) and following the manufacturer's instructions except for using DNA clean & concentrator (Zymoresearch, USA) instead of magnetic beads for the clean-up step. The library was prepared for sequencing using LSK-110 (Oxford Nanopore Technologies, UK) and sequenced using flow cell R9.4.1 (FLO-MIN106D) until the sample was exhausted or the desired number of reads was achieved. Basecalling was performed on MinION Mk1C using Guppy (version 5.0.13) with fast basecalling model and read filtering of min_score=8. SBanalyser was used to demultiplex the samples, discard reads <200bp and assign the reads to its taxonomic group using Athena 16S-23S database.

Illumina sequencing and OTU classification

16S rRNA hypervariable regions V1 and V3 were amplified and sequenced using Illumina MiSeq system by BaseClear B.V. (The Netherlands). Initial quality assessment was based on data passing the Illumina Chastity filtering and reads containing PhiX control signal were removed using a self-developed filtering protocol. Afterwards, reads containing (partial) adapters were clipped (up to a minimum read length of 50 bp). A second quality assessment was performed based on the remaining reads using the FASTQC quality control tool (version 0.11.8). Paired-end sequence reads were collapsed into so-called pseudo reads using sequence overlap with USEARCH (version 9.2)[152]. Classification of these pseudo reads was performed based on the results of alignment with SNAP (version 1.0.23)[153] against the RDP database (version 11.5)[154] for bacterial organisms.

Whole genome shotgun sequencing

Whole genome shotgun sequencing was performed using Illumina HiSeq system by BaseClear B.V., The Netherlands. Initial quality assessment was based on data passing the Illumina Chastity filtering and reads containing PhiX control signal were removed using a self-developed filtering protocol. Afterwards, reads containing (partial) adapters were clipped (up to a minimum read length of 50 bp). A second quality assessment was performed based on the remaining reads using the FASTQC quality control tool (version 0.11.8). Alignment-based filtering was performed by aligning the Illumina reads against the reference sequence using BBmap (version 38.79). Kraken2[155] (version 2.0.8) was used to taxonomically classify the metagenomic reads based on a reference database enriched with skin-specific genomes. Species and genus-level relative abundance profiles were obtained using Bracken (version 2.6.0)[156].

Statistical analysis

Statistical analysis on bacterial compositions was performed with Prism9 (GraphPad Software Inc, USA) for the Pearson correlation coefficient.

Results

Genomic skin mock community taxonomic classification

Our study aimed to verify whether nanopore sequencing is an accurate technique to investigate the skin microbiome. In order to do so, we aimed to generate and sequence a library of V1-V9 16S rRNA gene amplicons using ONT library prep kits and the defined genomic skin mock community. Duplicates of V1-V9 amplicons were successfully generated for the mock community using a four-primer PCR. The library was sequenced and basecalled with MinION Mk1C, generating more than 25,000 reads in the length range of 1.2-1.8kb with a quality score ≥ 8 that afterwards were classified to its taxonomic group with Epi2me (Table 20 in Appendix). Duplicates statistical comparison and taxonomic relative abundances obtained using LongAmp polymerase are shown in Figure 12A. Statistically significant similarities (Pearson correlation) were found in the genus relative abundances across the duplicates. Each genus in the mock community was expected to be 16.7%. We found in our analysis *Staphylococcus* (~55.2%) and *Streptococcus* (~23.9%) were respectively highly and mildly overrepresented. *Acinetobacter* (~13.3%) was slightly underrepresented and *Cutibacterium* (~0.7%), *Corynebacterium* (~0.5%) and *Micrococcus* (~0.1%) were highly underrepresented. ~6,5% of the classified reads were not assigned to any of these six genera.

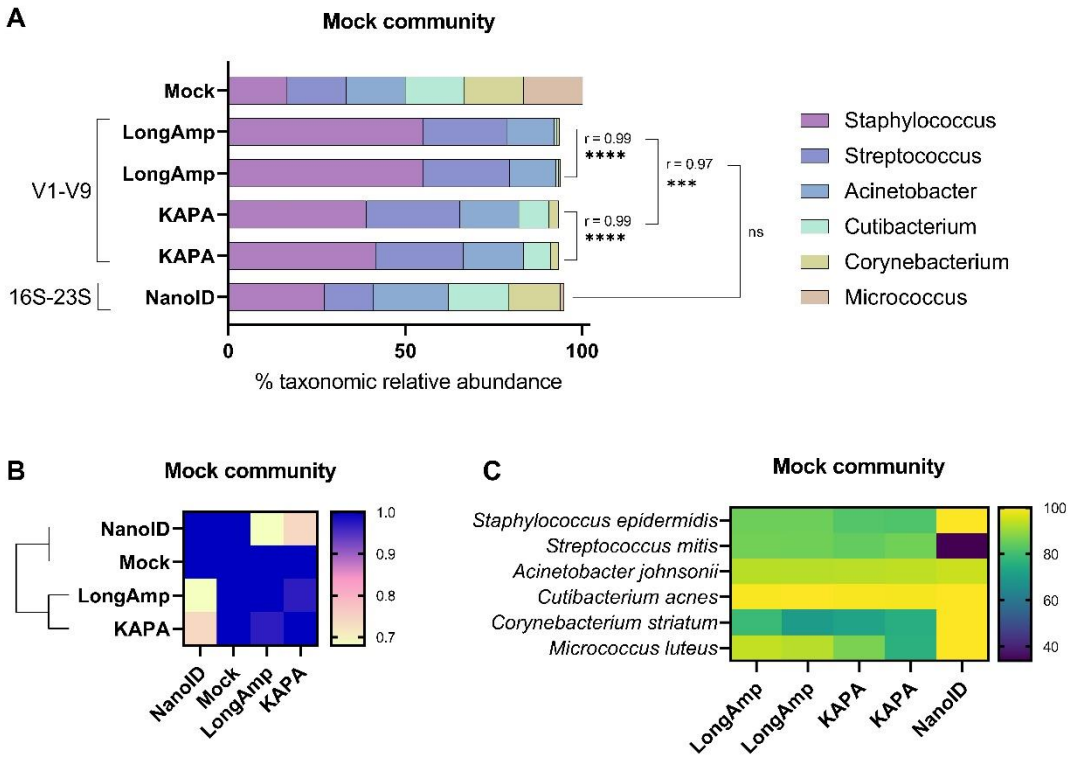


Figure 12. Testing of different amplification methodologies for MinION™ sequencing of human mock skin microbial communities. ***(A)****, Comparison of taxonomic profiles of classified reads of the mock community. The Pearson coefficient (r) between sequencing methods was computed to highlight significant correlation between samples and/or methodologies, ns, not significant; *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001; ****P ≤ 0.0001.* ***(B)****, Similarity matrix and hierarchical clustering of the methodologies based on their relative abundance profiles.* ***(C)****, Heat map showing percentage of classified reads to the correct species between the sequencing methods in the mock community.*

After assessing the possibilities causing this large bias in some genera, we found that the three underrepresented genera have higher GC content (*Cutibacterium* 60.1%, *Corynebacterium* 59.3% and *Micrococcus* 73.1%) compared to the overrepresented genera (*Staphylococcus* 32.2% and *Streptococcus* 40.1%). We hypothesized that the polymerase recommended in the ONT protocol LongAmp could be one of the reasons for the underrepresentation of genera with high GC content. Therefore, we assessed the performance of KAPA, a polymerase widely used for NGS applications, to estimate bacterial relative abundance for the mock community. Applying the same pipeline but using KAPA instead of LongAmp polymerase, more than 25,000 reads in the length range of 1.2-1.8kb with a quality score ≥ 8 were obtained and Epi2me assigned them to taxonomic groups (Table 20 in Appendix). Duplicates statistical comparison and taxonomic relative abundances obtained using KAPA are shown in Figure 12A. Statistically significant similarities (Pearson correlation) have been found in the genus relative abundances across the duplicates. Comparing to the previous results obtained using LongAmp, relative abundances obtained for *Staphylococcus (*~40.4%), *Cutibacterium* (~8.2%), *Corynebacterium (*~2.3%) and *Acinetobacter* (~16.7%) were significantly correlated but closer to the expected in the mock community (16.7%). *Streptococcus* (~25.7%) and *Micrococcus (*~0.2%) relative abundances were not affected by the change of polymerase neither the percentage of unclassified reads to any of these genera (~6.5%). Overall, closer relative abundances to the mock community were obtained using KAPA, but the obtained relative abundances were still poorly representing the mock community.

Differential primer affinities to 16S rRNA genes have been described to produce bias when determining relative abundances in mixed bacterial samples[157]. When comparing the previously used 1492R primer to the 16S gene sequences present in the mock community using the NCBI database, we observed that 1492R does not completely bind any of the genera. To see if primers with affinity to a broader range of bacteria would improve the relative taxonomic abundances obtained, we used the NanoID kit from Shoreline Biome. NanoID uses a degenerated version of 27F primer and a reverse degenerated primer complementary to the 23S gene, which is ~1kb downstream of the binding site of 1492R. 16S-23S amplicons were successfully generated following NanoID guidelines and sequenced and basecalled with MinION Mk1C. Sbanalyzer filtered reads below 200bp and successfully assigned more than 95.000 reads to a bacterial taxonomic level (Table 20 in Appendix). The obtained taxonomic relative abundances and statistical comparisons are shown in Figure 12A. Comparing to V1-V9 results obtained using KAPA and LongAmp polymerase, NanoID shows non-significant similarities. Relative abundances obtained with NanoID for *Staphylococcus* (~27.3%), *Cutibacterium* (~17.2%), *Corynebacterium* (~14.5%) and *Streptococcus* (~13.9%) were considerably closer to the mock community. *Acinetobacter* (~21.1%) estimation was less accurate and *Micrococcus* (~1.1%) was improved but still largely underrepresented. A slightly lower percentage of reads (~5%) were not classified to any of the genera from the mock community. Even though *Micrococcus* was largely underrepresented, NanoID showed the better overall performance to determine bacterial relative abundance in the mock community than the previously tested protocols (see similarity matrix Figure 12B).

Another relevant parameter to analyze is the percentage of reads in each genus that were classified to the proper specie. All V1-V9 reads were classified to a species while a small fraction of 16S-23S reads were classified to a species level. In the mock community, each genus is exclusively composed of a single bacterial species and the number of reads assigned to the

correct species was analyzed to calculate the percentage of correctly identified species in each genus (Figure 12C). No differences were observed in V1-V9 sequencing runs between LongAmp and KAPA and are shown together as V1-V9. The obtained percentages of correctly identified species for V1-V9 and 16S-23S were respectively the following: *Staphylococcus epidermidis* (~83.6%, ~99.9%), *C. acnes* (~99.5%, ~100%), *Corynebacterium striatum (*~74%, ~99.9%), *Streptococcus mitis* (~85.4%, 33.8%), *Acinetobacter johnsonii* (~93.3%, ~94.6%). This value was not determined for *Micrococcus luteus* due to the low number of reads obtained. Overall 16S-23S amplicons resulted in a more accurate species determination with the sole exception of *Streptococcus mitis*, for which ~66.1% of the reads were classified as *Streptococcus pneumoniae*.

Skin standard taxonomic classification

We tested if the described observations in the skin mock community would also apply to a real skin microbiome sample. First, since our skin microbiome standard had an unknown composition, we analyzed the bacterial relative abundance by sequencing its V1-V3 16S rRNA region with Illumina MiSeq and by whole genome shotgun (WGS) sequencing (Figure 13A). The relative abundances obtained with MiSeq and WGS were, respectively, the following: *Staphylococcus* (~14.6%, ~3.4%), *Cutibacterium* (~63.3%, ~80.1%), *Corynebacterium* (~2%, ~2%), *Streptococcus* (~1.3%, 0.4%), *Acinetobacter* (~0.8%, 0.1%) and *Micrococcus* (0.1%, 0.1%). Then we processed the skin microbiome standard with the three conditions previously tested (V1-V9 with LongAmp, V1-V9 with KAPA and 16S-23S with NanoID). We generated, 16,617 and 2,610 reads for LongAmp duplicates, more than 15,000 for KAPA duplicates and more than 78,000 reads for NanoID (Table 20 in Appendix). Afterwards, with its corresponding software and database, reads were assigned to a taxonomic group. The obtained taxonomic relative abundances and statistical comparisons between duplicates and different methods are shown in Figure 13A. Statistically significant similarities (Pearson correlation) have been found in the genus relative abundances across the duplicates. Relative abundances obtained using LongAmp, KAPA and NanoID were respectively the following: *Staphylococcus* (~44,9%, ~18,4%, ~21,2%) , *Cutibacterium* (~14,8%, ~58,2%, ~66,5%), *Corynebacterium (*~2,1%, ~0,4% , ~2,7%), *Streptococcus* (~3,9%, ~1,1%, ~1,3%), *Acinetobacter* (~1,8%%, ~0,16%, ~0,15%) and *Micrococcus (<0,1%, <0,1%, <0,1%)*. As can be seen in Figure 13A and taking WGS as a reference, *Staphylococcus* was largely overrepresented when using the recommended polymerase by ONT kits LongAmp and to a lesser extent, still overrepresent for all other techniques. Except for LongAmp polymerase, the other techniques have a significant Pearson correlation compared to WGS. The similarity matrix (Figure 13B) shows similarities for all the techniques except for LongAmp. Altogether, these results suggest that the biases observed in the mock community also apply to real skin microbiome samples and this bias can be reduced by changing the polymerase or primers used.

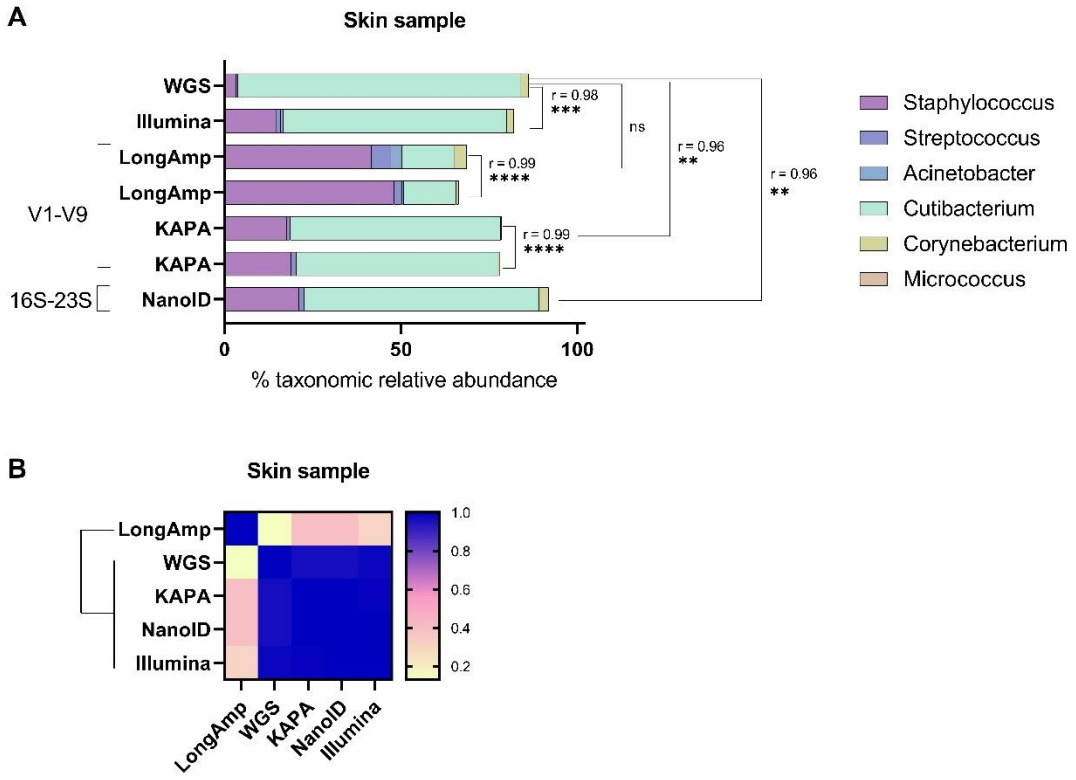


Figure 13. Testing of different amplification methodologies for MinION™ sequencing of human skin samples microbial communities. ***(A)****, Comparison of taxonomic profiles of classified reads of the skin samples communities. The Pearson coefficient (r) between sequencing methods was computed to highlight significant correlation between samples and/or methodologies, ns, not significant; *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001; ****P ≤ 0.0001.* ***(B)****, Similarity matrix and hierarchical clustering of the methodologies based on their relative abundance profiles.*

Discussion

Nanopore is revolutionizing sequencing in laboratories by generating high throughput reads that can be analyzed in real-time, reducing total processing time and sequencing costs per sample. Nevertheless, its lower basecall accuracy (85-93%) and described biases towards certain genera and species in complex bacterial samples[149,150] urged us to investigate if ONT is ready to be used in skin microbiome analysis. Using a defined genomic skin mock community, we show that recommended polymerase (LongAmp) and 16S primer sequences in ONT kits have a strong bias toward the most prevalent skin bacterial genera and towards low GC content bacteria (Figure 14). Furthermore, we show that using a different polymerase (KAPA) and primer selection (NanoID) can reduce this bias and improve the overall results. These improvements were demonstrated on a bacterial skin mock community and confirmed in a real skin microbiome sample.

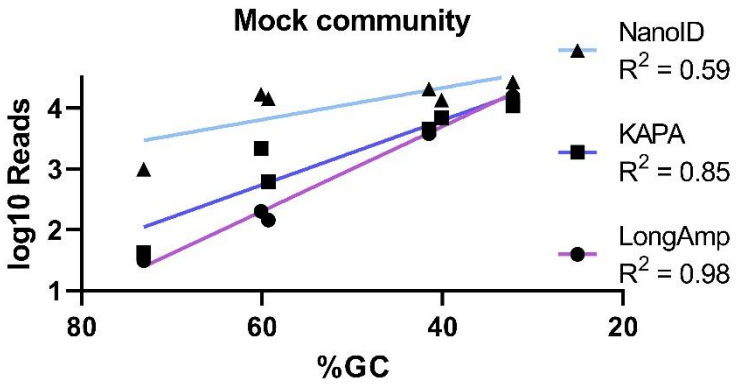


Figure 14. Basic linear regression analysis used to correlate the GC content (%) of mock community skin genera in sequenced samples (x-axis) compared to the number of reads in the MinION™ sequenced samples (y-axis).

Upon studying the performance of the recommended polymerase by Nanopore, LongAmp, on the skin mock community, we observed a strong bias towards certain genera (Figure 12A). *Staphylococcus* was highly and *Streptococcus* mildly overrepresented while *Cutibacterium*, *Corynebacterium* and *Micrococcus* were strongly underrepresented. Overrepresented and underrepresented genera had respectively low and high genomic GC contents. It has been described that polymerase performance can be negatively influenced by high GC content[158]. To assess this problem, we tested KAPA, a widely used polymerase for NGS studies which has improved performance on GC-rich templates[159]. Using KAPA, *Staphylococcus* abundance overrepresentation decreased and *Cutibacterium*, *Corynebacterium* and *Acinetobacter* abundances increased, obtaining for the four genera, closer bacterial relative abundances to the defined mock community. *Streptococcus* overrepresentation and *Micrococcus* vast underrepresentation did not improve with KAPA polymerase (Figure 12A). *Micrococcus* is the genus with the highest GC content (73.1%) in the mixture which makes it a complicated target. It is known that GC-rich DNA double strands require higher energy for strand dissociation, reducing their availability for primer binding and resulting in lower PCR amplification[160]. Other factors such as the differences in 16S gene copy number mean in the genomes of *Staphylococcus epidermidis* (5.9), *Streptococcus mitis* (3.9), *Acinetobacter johnsonii* (7) *Cutibacterium* acnes (3.1), *Corynebacterium striatum* (4) and *Micrococcus luteus* (2.1) can influence the amplicon amounts produced on PCR[161,162].

Another important variable described to cause bias in amplification of mixed genomic samples is the primer binding affinity to each target, which decreases with lower sequence similarity[157]. The NCBI database show differences in sequence similarity of primer 1492R among the genera in the used mock community. A different primer pair was used in an attempt to improve the obtained relative abundance. These primers, included in the NanoID kit, were designed by Shoreline biome to have a higher affinity to a broader variety of bacterial species. It is important to notice that the polymerase used by NanoID kit is not disclosed by the provider. When using NanoID kit to amplify the 16S-23S region, *Staphylococcus*, *Cutibacterium*, *Corynebacterium* and

Streptococcus relative abundances were closer to the mock community than the ones obtained with V1-V9 amplifications. NanoID have slightly poorer performance in determining *Acinetobacter* relative abundance than the V1-V9 region, and even if NanoID performed better for *Micrococcus*, this genus was still vastly underrepresented (Figure 12A). Primers used in NanoID are a degenerated version of 27F primer and instead of the 1492R primer, it contains a degenerated primer complementary to the downstream 23S rRNA gene. Degenerated primers have already been shown to be a good alternative when targeting a broad taxonomic range of bacteria[128]. Overall NanoID obtained better relative abundances than any of the tested polymerases amplifying the region V1-V9. Even though, it is not clear if the improvement in relative abundances using NanoID is caused by their degenerated primers, their undisclosed polymerase, or a combined effect of both.

V1-V9 sequencing data was analyzed with Epi2me assigning all reads to species level, a much larger percentage compared to NanoID kit data analyzed with Sbanalyzer. This large difference in assigned reads percentages is due to software restrictions for species level read classification. Default criteria for reads assignation to species level are less restrictive on Epi2me than on Sbanalyzer, resulting in a higher percentage of false positive results for species identification and making Sbanalyzer species results more reliable. This observation is consistent for all the genera except for *Streptococcus* where species *S. pneumoniae,* a well-recognized human pathogen, accounted for more than half of the genus assigned reads. Besides, it is important to keep in mind that NanoID generates 16S-23S amplicons, which are longer than the V1-V9 amplicons, allowing a more precise reads discrimination to species level[163]. Differentiation at the species level of a skin commensal such as *Staphylococcus epidermidis* from the skin pathogen *Staphylococcus aureus* can be crucial in diagnostic procedures[164]. Therefore, depending on the aim of the study and the impact of false positives, more permissive or restrictive analysis criteria should be chosen accordingly.

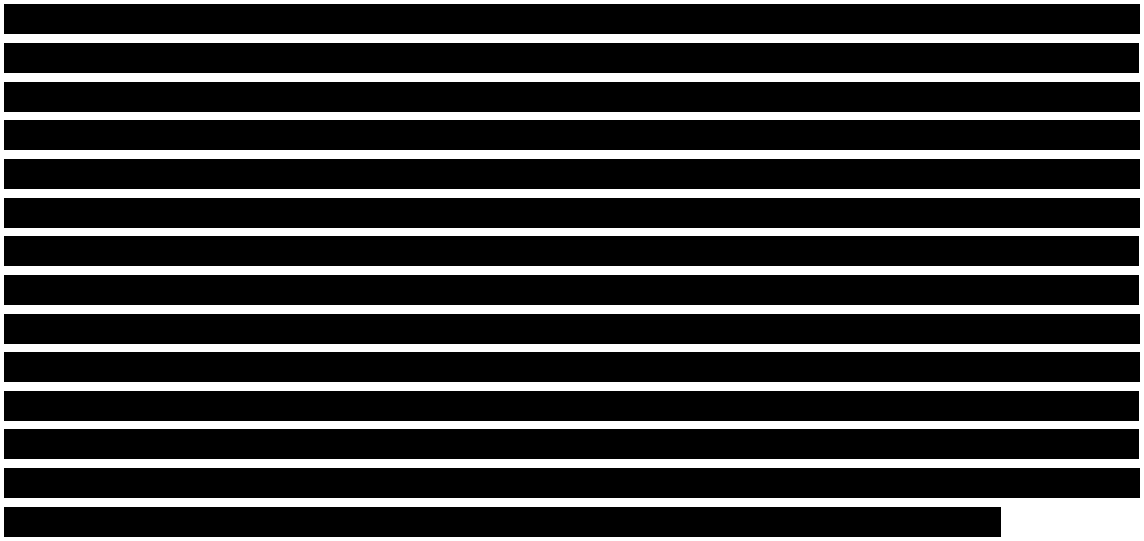
In order to assess if the described relative abundance biases for the mock community using ONT kits also apply to real skin samples, a real skin microbiome sample was analyzed. Since the actual taxonomic composition of the skin sample was unknown, shotgun whole genome sequencing (WGS) and V1-V3 MiSeq were performed as a means of comparison. WGS has been described as the most accurate technique for determining bacterial taxonomic relative abundances on skin samples, while V1-V3 MiSeq gives a close estimation[143]. Taking WGS as the more realistic estimation, LongAmp performed very poorly on determining *Cutibacterium* and *Staphylococcus* abundances compared to all other methods. V1-V9 amplified with KAPA, NanoID and V1-V3 MiSeq, show similar levels of *Cutibacterium* underrepresentation and *Staphylococcus* overrepresentation. V1-V3 MiSeq and NanoID gave a better approximation of *Corynebacterium* than V1-V9 methods. For the rest of the genera in the mock community, it is difficult to assess the performance of the different conditions since each of these genera represent less than 0.5% in the WGS data. Overall, we showed that the observed bias in a defined skin mock community also applies to a real skin microbiome sample and can also be reduced by using alternative polymerases and primers.

Conclusions

Human skin microbiome dysbiosis can have clinical consequences. Characterizing taxonomic composition of bacterial communities associated with skin disorders is important for dermatological advancement in both diagnosis and novel treatments. This study aimed to analyze and improve the accuracy of taxonomic classification of skin bacteria with MinION™ nanopore sequencing using a defined skin mock community and a skin microbiome sample. Strong bias was observed on the main skin genera abundances when recommended polymerase (LongAmp) and 16S primers by Oxford Nanopore Technologies kits were used. We suggested an alternative polymerase (KAPA) and primers (NanoID) that generated better results in a defined skin mock community and a skin microbiome sample. Nonetheless, variables such as polymerase and primers selection, PCR conditions and bioinformatic analysis should be further improved to obtain more reliable data with this technology. Once these issues are addressed, nanopore sequencing will allow precise, faster, and cheaper generation of data in skin microbiome studies.

Thesis conclusions

The human microbiome plays a crucial role in human health, even though defining a healthy microbiome is not a trivial task. New developments in sequencing technologies allowed more precise characterization of the skin microbiome, which alterations have been associated with common skin diseases such as acne vulgaris, psoriasis, rosacea, and atopic dermatitis. S-Biomedic targets skin diseases associated with skin microbiome alterations by modulating the skin microbiome, focusing on the most abundant skin commensal *Cutibacterium acnes. C. acnes* contributes to skin homeostasis by modulating host lipids, impeding skin pathogens colonization, training and enhancing the host immune system, and protecting skin cells from oxidative stress. The benefits that *C. acnes* bring to skin homeostasis are strain dependent, and dysbiosis at this taxonomic level has been described for some skin diseases. Therefore, selecting or generating *C. acnes* strains with the desired phenotype is a key step toward successfully treating each different skin disease. To test the hypothesis of whether the chosen strain can improve the dermatological condition it is tested in a clinical trial. Afterwards, the patients skin microbiome can be assessed to understand If the dermatological improvement is linked to a microbiome modulation towards a healthy state.



The second project of this research consisted in benchmarking and improving the accuracy of skin microbiome samples taxonomic classification using Nanopore sequencing. Identifying bacterial communities in skin health and disease is important for dermatological advancement in both diagnosis and novel treatments. A strong bias in determining the relative abundance on a defined genomic skin mock community and a skin microbiome sample was observed when using the recommended reagents by Oxford Nanopore Technologies. Alternative reagents were suggested and tested, resulting in an improvement in taxonomic classification for the defined mock and microbiome samples. Even though improvements were established, there is still a bias to be addressed. This bias ranges from the scope of sample processing, bioinformatics analysis and Nanopore sequencing technology. However, Nanopore sequencing is a fast-evolving technique, and it can be expected that these biases can be addressed soon. Once these biases are addressed, Nanopore sequencing will allow a precise, faster, and cheaper generation of data in skin microbiome studies.

[redacted]

Summarizing, in the performed research [redacted] Nanopore sequencing was benchmarked and improved for skin microbiome samples sequencing, [redacted]. All these advancements will contribute to improving current diagnoses and novel treatments in the dermatology field.

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Appendix

Basecalling using Google Colab

Using Google Colab, the following scripts will do a high accuracy basecalling of the obtained fast5 files from Nanopore sequencing. In red are displayed the scripts to be executed.

Download Guppy

Once you have access and can navigate to the 'Software Downloads' section of the ONT community forum you will see a listing for Guppy. I recommend grabbing the pre-compiled binaries, i.e. the version listed as Linux x64-bit GPU, it should have a file name similar to ont-guppy_X.X.X_linux64.tar.gz - where the X's denote the version number. You can copy the link to this download and paste it into the code block below, i.e. replace the section [paste_guppy_link_here]

```
%%shell

GuppyBinary=[paste_guppy_link_here]

wget $GuppyBinary
```

Extract the compressed Guppy binaries

Before we can use the Guppy binaries we need to extract the file we downloaded. Replace the X's in the below code block with the version you downloaded and then run the code block. If we use version 4.5.3 as an example:

```
%%shell

tar -xzvf ont-guppy_4.5.3_linux64.tar.gz
```

Check Guppy version

We should now be able to run the Guppy binaries we downloaded. They are located in ./ont-guppy/bin. The below code block should run guppy_basecaller and report the version of the software.

```
%%shell

./ont-guppy/bin/guppy_basecaller --version
```

Mount your Google Drive

By mounting your Google Drive you will be able to upload fast5 files which can be processed and the output can be written back to the same location within Drive.

The below chunk performs the mounting. You will be asked to authenticate, just follow the instructions and things should go smoothly.

```
from google.colab import drive

drive.mount('/content/gdrive', force_remount=True)
```

For this example, I created a directory within My Drive called ONT and then within this folder another directory called example_data. I then uploaded a few fast5 files to this location.

We can check that the mounted drive and files are identified in the notebook environment below.

```
%%shell

ls gdrive/MyDrive/ONT/example_data
```

Basecalling with Guppy

With all the above working then we can now basecall our data. First we will set a few variables. The below code block creates shell variables for input and output locations, the guppy binary (basecaller) and several model files for basecalling (i.e. fast, hac and modified bases).

HAC model run

This basecalling run performs high accuracy calling. I was actually very surprised with the speed of the GPU that generated this output (Nvida T4). I feel it would be a decent option if you wanted to turn around a small amount of data using the hac model.

The below code block will perform hac:

```
%%shell
inputPath="gdrive/MyDrive/ONT/example_data"
outputPath="gdrive/MyDrive/ONT/example_data"
guppy_bc=./ont-guppy/bin/guppy_basecaller                                  # set
guppy_basecaller binary location
guppy_cfg_fast=./ont-guppy/data/dna_r9.4.1_450bps_fast.cfg                 #
fast model calling
guppy_cfg_hac=./ont-guppy/data/dna_r9.4.1_450bps_hac.cfg                   #
high accuracy calling
guppy_cfg_mod=./ont-guppy/data/dna_r9.4.1_450bps_modbases_5mc_hac.cfg      #
base modification calling

$guppy_bc -i $inputPath -s $outputPath  \
--recursive \
--config $guppy_cfg_hac \
--gpu_runners_per_device 16 \
--cpu_threads_per_caller 2 \
--device cuda:0
```

Table 20. Reads assigned to each genus for the different methods tested in the mock community and skin standard

Mock community							
Method	**No. Classified reads**	***Staphylococcus***	***Cutibacterium***	***Corynebacterium***	***Acinetobacter***	***Streptococcus***	***Micrococcus***
V1-V9 LongAmp.1	29,623	16,347	202	140	3,897	7,204	34
V1-V9 LongAmp.2	26,946	14,865	200	147	3,640	6,340	28
V1-V9 KAPA.1	27,698	11,553	2,164	589	4,681	6,879	38
V1-V9 KAPA.2	25,456	9,948	2,186	650	4,218	6,745	46
NanoID	97,950	26,713	16,810	14,226	20,566	13,584	986
Skin Standard							
Method	**No. Classified reads**	***Staphylococcus***	***Cutibacterium***	***Corynebacterium***	***Acinetobacter***	***Streptococcus***	***Micrococcus***
WGS	17,078,491	579,005	13,686,529	346,759	20,150	73,456	21,091
V1-V3 Illumina	14,145	2,153	8,957	1,230	11	183	8
V1-V9 LongAmp.1	16,617	7,992	2,452	127	90	370	0
V1-V9 LongAmp.2	2,610	1,087	388	91	78	146	1
V1-V9 KAPA.1	15,986	3,036	9,099	94	32	205	1
V1-V9 KAPA.2	18,290	3,248	10,886	53	21	154	2
NanoID	78,378	16,607	52,114	2,122	120	1,058	15