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Characterisation of the influence of Nitrosomonas eutropha on the skin regulation of NO metabolism and the microbiome

by

Rfeef Alyami

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ABSTRACT

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The skin is the largest organ and demonstrates a wide spectrum of physiological functions which facilitate its various biological characteristics including host defence, water regulation, thermoregulation and micronutrient synthesis. However, in settings of cutaneous inflammation, these processes are significantly disrupted and can result in systemic inflammation and altered biochemistry. Previous studies using microbiological methodology based on plate culture identification, were unable to fully characterise the skin microbiome. However, with the advent of culture independent techniques such as ribosomal RNA 16s analysis, the true diverse structure of human microbiome systems on epithelial surfaces has been uncovered. In this thesis I describe the optimal methodology for 16s rRNA skin microbiome sampling which has not been investigated comprehensively.

Nitric oxide (NO) has recently been shown to be a key regulator of biofilm function in respiratory mucosa, and has been shown to regulate the microbiome at epithelial surfaces. In this study of healthy human volunteers (n=23), I set out to characterise the skin NO physiology in different body sites and after exercise. I show that NO emanation from the skin is enhanced by sweating and exercise. In contrast to previous work, I found no evidence that skin pH provided significant regulation of NO emanation.

The common soil bacteria, Nitrosomonas eutropha, which is not found on human skin, facilitates the oxidation of ammonia to form nitrite (hence ammonia oxidising bacteria, AOB). Nitrite is a key component of the pathway to NO formation by further reduction in the epithelial surface. Recently Nitrosomonas eutropha has become commercially available as a spray for skin application. However, the question of whether the microbe can survive on human skin, or whether it has any physiological effect has not been addressed.

I show for the first time that Nitrosomonas can survive on the skin surface and become part of the skin microbiome. In addition, enhanced skin NO emanation and increased skin pH ($p=0.011$) were noted following Nitrosomonas application.

Furthermore, the impact of perturbation of the skin microbiome with Nitrosomonas was examined using 16s rRNA analysis. I show that the presence of Nitrosomonas in the skin microbiome did not lead to a significantly altered diversity or microbiome structure at a genus or phylum level. Despite recent work suggesting loss of skin microbiome diversity following S. aureus infection in atopic dermatitis, these findings point to a robustness of the skin microbiome. Whilst no significant differences pre and post AOB application were seen in an extensive panel of markers of systemic NO physiology, a significant elevation of total free thiols (TFTs) was detected in plasma ($p=0.006$). This is of note, because TFT elevation has been associated with reduced cardiovascular morbidity and mortality which may suggest that investigation of AOB application to the skin should be further explored.

The findings presented here demonstrate that sweating, pH, and microbiome are likely to be important in regulating skin NO emanation and potentially systemic inflammation. The delivery of Nitrosomonas eutropha to the skin in a human clinical study as reported here, offers a unique opportunity to study the skin microbiome and demonstrates a resilience in the microbiome community to new pathogens. Overall this study suggests that application of AOBs as a cutaneous 'probiotic' is safe and offers significant clinical potential.

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Academic Thesis: Declaration Of Authorship

I, Rfeef Alyami .....

declare that this thesis and the work presented in it are my own and has been generated by me as the result of my own original research.

Characterisation of the influence of Nitrosomonas eutropha on the skin regulation of NO metabolism and the microbiome.

I confirm that:

1. This work was done wholly or mainly while in candidature for a research degree at this University;
2. Where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;
3. Where I have consulted the published work of others, this is always clearly attributed;
4. Where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;
5. I have acknowledged all main sources of help;
6. Where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
7. None of this work has been published before submission

Signed: .....

Date: .....

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Definitions and Abbreviations

16S rRNA	16S ribosomal RNA
AMP	Antimicrobial peptides
AOB	Ammonia-oxidising bacteria
cGMP	Cyclic guanosine monophosphate
cNOS	Constitutive nitric oxide synthase
E. coli	*Escherichia coli*
eNOS	Endothelial nitric oxide synthase
GCP	Good clinical practice
GSNO	S-nitrosoglutathione
HMP	Human microbiome project
iNOS	inducible nitric oxide synthase
IFN-γ	Interferon gamma
IL-1	Interleukin-1 family
IRAS	Integrated research application system
ITS	Internal transcribed spacer
LPS	Bacterial lipopolysaccharide
mRNA	Messenger RNA
N_2O	Nitrous oxide
NEM	N-ethylmaleimide
NIH	National institute of health
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NOSs	Nitric oxide synthases

O_2	Oxygen
O_2^-	Superoxide
O	Ozone
$OONO^-$	Peroxynitrite anion
OUT	Operational taxonomic units
P. acnes	*Propionibacterium acnes*
PCA	Principal components analysis
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PSM	Pheno-soluble modulins
PPRs	Pattern recognition receptors
PSM	Pheno-soluble modulins
RNA	Ribonucleic acid
RNOS	Reactive nitrogen oxide species
S. aureus	*Staphylococcus aureus*
S. epidermidis	Staphylococcus epidermidis
sGC	Soluble guanylyl cyclase
SNOs	S-nitrosated proteins
SSTI	Skin and soft tissue infections
TNF-α	Tumour necrosis factor alpha
VMM	Mean maximum voltage
VRE	Vancomycin-resistant enterococci

Chapter 1 Introduction

1.1 Human skin

The skin is the largest organ of the body (Kanitakis, 2002) and is composed of three main layers: the epidermis, the dermis and subcutaneous tissue (Figure 1.1). The epidermis is the outermost layer of the skin that lies in direct contact with the environment and is constantly being renewed; it comprises a specific constellation of cells including keratinocytes (Xu et al., 2013). The keratinocytes are the key cells of the epidermis that manufacture and store keratin, an intracellular fibrous protein that provides structural integrity to the cells (Figure 1.1) (Albanna and Holmes, 2016). The stratum corneum layer (Figure 1.2) is composed of corneocytes, which are keratinocytes that passed through full differentiation to become non-viable cells and undergo autophagy to become completely devoid of nuclei and organelles (Fitzpatrick and Morelli, 2010). Corneocytes become increasingly flattened as they move towards the surface to form a lamellar structure (James et al., 2016). These cells retain their internal keratin proteins and become enveloped in a layer of lipid to form the stratum corneum, which is an important regenerative and durable keratinized protective barrier against the external environment (Albanna and Holmes, 2016).

The dermis lies below the epidermis and is adherent to it at 'the basement membrane zone' (BMZ). The dermis is formed of connective tissue that is vascularised and contains various appendages including; sweat glands (important for temperature regulation), hair follicles and sebaceous glands (that produce sebum, and oil) (Marks and Miller, 2017). The connective tissue element of the dermis is made up of collagen (70%), elastin and extra-fibrillarly matrix and provides structural support for the epidermis. Blood, nerves and lymphatic channels pass through the dermis to supply the epidermis.

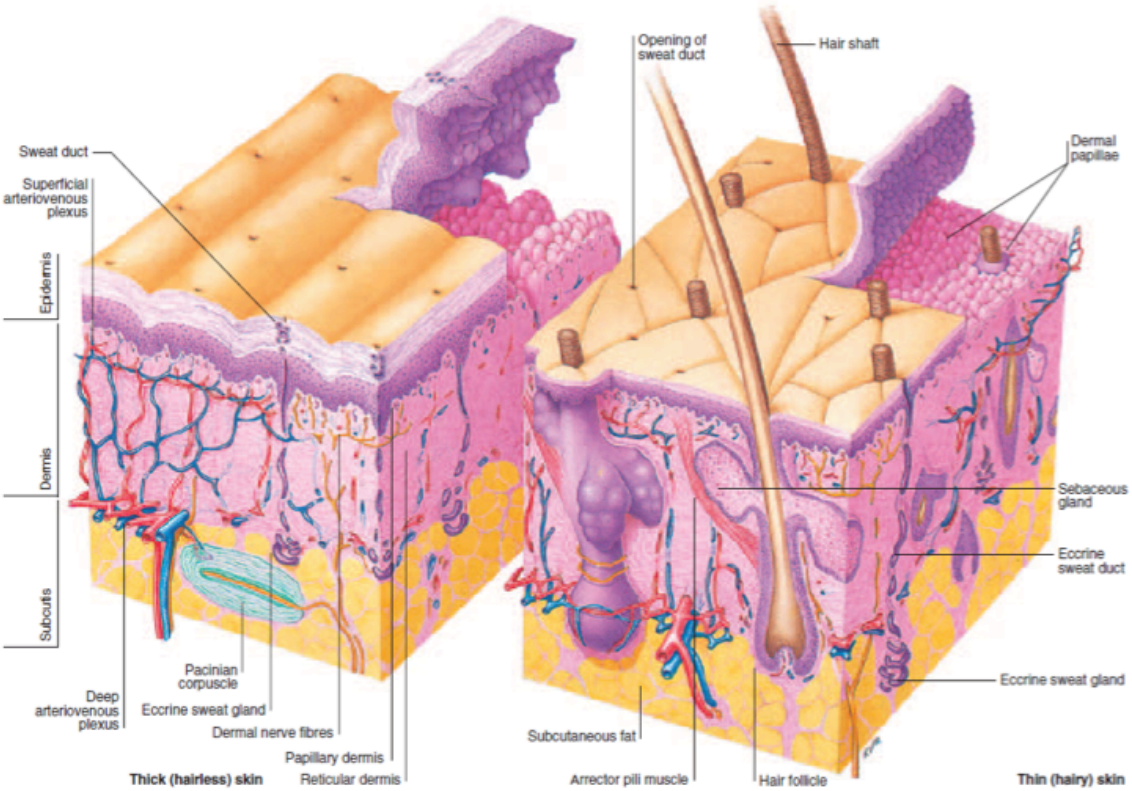


Figure 1.1 Structure of the skin. Schematic diagram showing thick (hairless) and thin (hairy) skin. Taken from (Gawkrodger and Ardern-Jones, 2012).

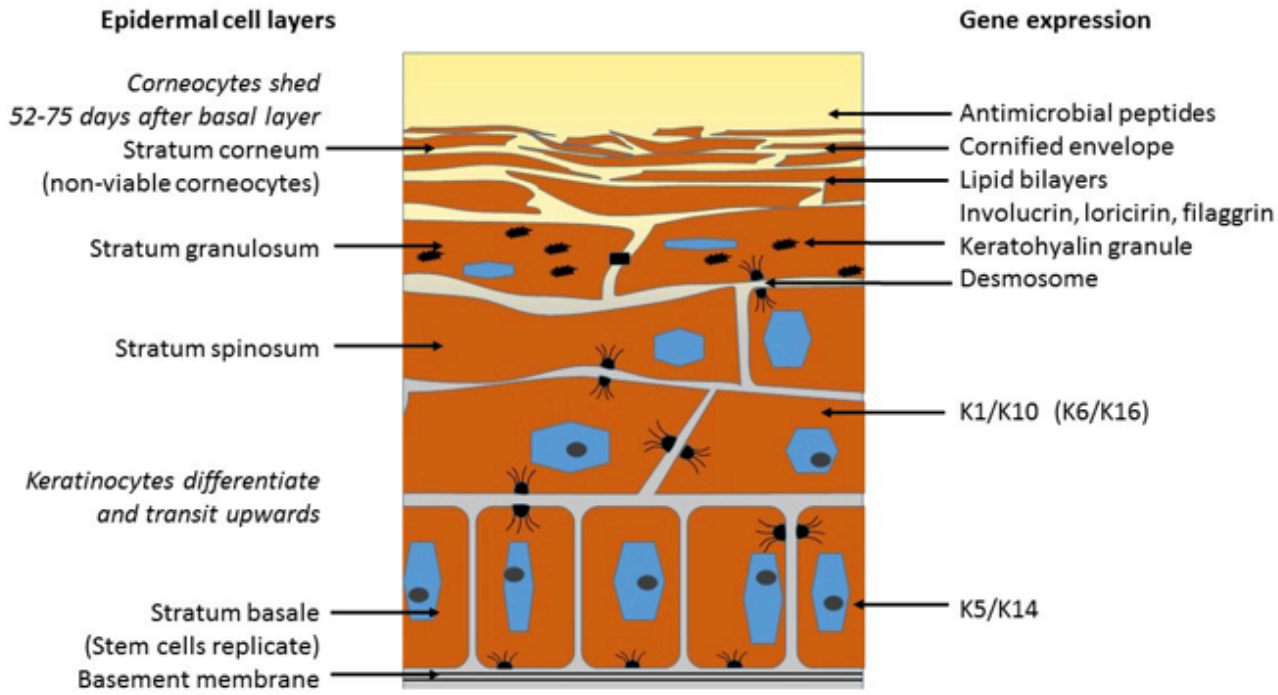


Figure 1.2 Epidermal cell layers and gene expression. Reproduced from (Arden-Jones, 2019)

The epidermal and dermal layers harbour a complex immunological barrier comprised of cells including macrophages, lymphocytes and dendritic cells (Figure 1.3) that respond in different inflammatory skin conditions (Gawkrodger and Ardern-Jones, 2012). Resident immune cells in the skin provide long-lived memory and rapid immunity against pathogens. Dendritic cells efficiently capture and present antigens to T cells, driving different biological responses. Innate lymphoid cells release type-2 cytokines on activation and can help regulate immune responses (Gawkrodger and Ardern-Jones, 2012).

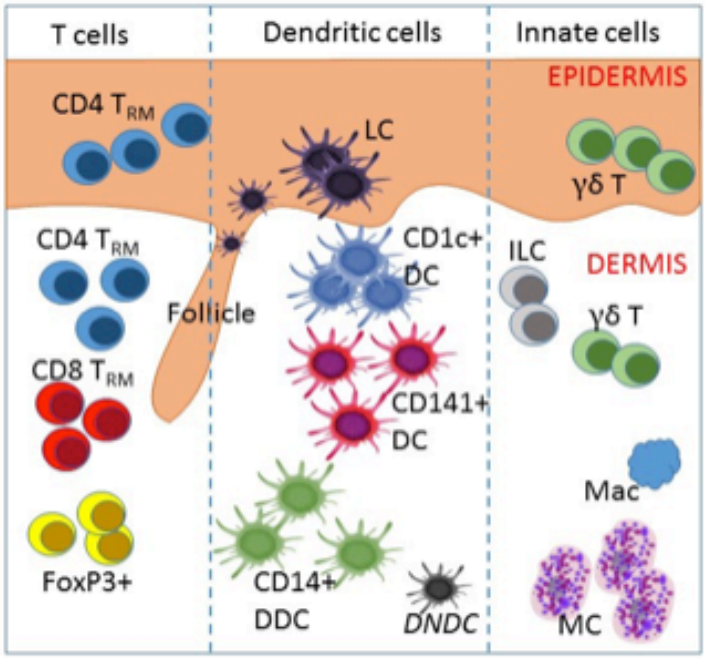


Figure 1.3 Immune cells in the skin. CD4 TRM - CD4 Resident Memory T cells; CD8 TRM - CD8 Resident Memory T cells; FoxP3+, FoxP3+ CD4+ T regulatory cells (Treg); LC -Langerhans cells. Drive Th17 responses and regulate tolerance; DC - Dendritic cells (CD1c+ / CD141+ / CD14+) drive Th1/Th2 responses; DNDC- Double negative DC (as yet only characterised in mice); ILC - Innate lymphoid cells, release type 2 cytokines on activation; γδ T, γδ T Cells (syn. dendritic epidermal T cells, DETCs); Mac -macrophages; MC - Mast cells. Reproduced from (Arden-Jones, 2019).

In addition to the well-conserved layers of the skin, body sites contain unique microenvironments that vary in temperature, moisture, pH, topography, sebum content and ultraviolet (UV) light exposure (Grice and Segre, 2011). These characteristics have been used to categorise different body sites as shown in Table 1.1. These environments are heavily influenced by sweat glands, hair follicles and sebaceous glands. In the moist regions of the body, sweat glands are most abundant and critical for thermoregulation. This is maintained through evaporation of water, which contributes to the acidification of skin. These acidic conditions serve as unfavourable conditions for the growth and colonisation of particular microorganism, thus influencing the environment. This is also achieved through antimicrobial molecules within sweat (e.g. fatty acids and antimicrobial peptides) that can inhibit microbial colonization (Grice and Segre, 2011, Gallo and Hooper, 2012). In the oily regions of the body, sebaceous glands are most dense and are connected to the hair follicle. These glands secrete lipid-rich sebum that serves as an antibacterial shield to the hair and skin through its hydrophobic coating.

Table 1.1 Microenvironments in different body sites.

Microenvironment		Body site	Appendages influencing environment
1	**Sebaceous/oily**	Face, chest and back	Sweat glands, hair follicles and sebaceous glands
2	**Moist**	Bend of elbow, back of knee and groin	
3	**Dry**	Volar forearm and palm	

Studying the composition of the microbiota in different body sites is therefore critical for elucidating the aetiology of common skin disorders that often have a preference for specific skin sites. For example, atopic eczema has a predilection for the inner elbow (flexor surface) (Kong et al., 2012) whereas psoriasis mainly affects the outer elbow (extensor surface) (Byrd et al., 2018).

1.2 Human microbiome

A healthy human body is comprised not only of human cells but also an extensive range of microbes. The collection of microbes in their entirety (archaea, bacteria, fungi, mites and viruses) are known as the microbiome. These microbes are resident in and on different niches including the oral cavity, throat, oesophagus, lower respiratory tract, stomach, colon, urogenital tract and the skin (Cenit et al., 2014). It has been estimated that the ratio of bacteria to nucleated human cells in the body is 10:1 (Costello et al., 2012, Huttenhower et al., 2012, Ley et al., 2006, Savage, 1977).

1.2.1 Importance of human microbiome research

The human microbiota play a critical role in human health and contribute to nutrition, resistance against pathogens, immune homeostasis and metabolic activity (Dethlefsen et al., 2007). Increasing studies revealed that host-indigenous microbiota importance, have led to an increased focus on the role of complex microbial communities in health and disease (Backhed et al., 2004, Cash et al., 2006, Guarner et al., 2006, Kelly et al., 2004, Martin et al., 2007, Mazmanian et al., 2005, Rakoff-Nahoum et al., 2004, Dethlefsen et al., 2007).

The study of the human microbiome has expanded considerably in the past fifteen years and this is largely due to the developments in ‘omics technologies’, the growing availability of data and the computational approaches to analysis (Cleary and Clarke, 2017). The first human microbiome niche to be explored was the alimentary canal, which is composed of almost 10 trillion bacterial cells and more than 2000 different species (Gerritsen et al., 2011). Nutritional intake, environmental and genetics are factors that radically affect the composition of these species and regulate the human microbiome (Wang et al., 2017). Analysis of the skin

microbiome has also shown similar dependency on host environment interactions. The Human Microbiome Project (HMP) revealed extensive inter- and intra-individual diversity (variation among, and within individuals) linked to body site. The majority of this diversity is yet to be accounted for, but factors including: diet, environment, host genetics and early microbial exposure appear to have an apparent role (HMP, 2012b). Numerous studies have followed on from the HMP to examine the role of specific microbiomes in health and disease. Studies revealed links between gut microbiomes and inflammatory bowel disease, obesity and diabetes (Halfvarson et al., 2017, Yassour et al., 2016). Links were also made between gut dysbiosis and cognitive decline, Alzheimer's disease, multiple sclerosis and depression (Cattaneo et al., 2017, Jangi et al., 2016, Kelly et al., 2015). Disturbances in microbial populations are also thought to contribute to a number of other diseases including: colorectal cancer, Clostridium difficile infections and psychological conditions including anxiety (Kinross et al., 2011, Cho and Blaser, 2012, Hsiao et al., 2013). While the gut microbiome is undoubtedly a widely researched area (Stecher et al., 2013, Kamada et al., 2013), the role of the microbiome in mediating health or disease in the skin (acne and atopic dermatitis) were investigated as well. As a result of the relationships between gut microbiome and other organs, terms such as "gut-brain axis", "gut-lung axis" and "gut-skin axis" were invented. (Watanabe et al., 2003, Mah et al., 2006, Zhang et al., 2008, Bowe and Logan, 2011, Nylund et al., 2015, Song et al., 2016, Lee et al., 2018a, Lee et al., 2018b) The composition and bacterial niches within the skin are significantly different from those found in the gut and their proportions differ vastly. Whilst Actinobacteria members are more abundant on the skin, Firmicutes and Bacteroidetes members are more abundant in the gastrointestinal tract. A common feature of gut and skin microbial communities seems to be low diversity at the phylum level, but high diversity at the species level (Grice and Segre, 2011).

High-throughput next-generation sequencing (NGS) has only recently gained traction as a popular method for the analysis of skin microbiota. Until relatively recently, it was widely considered that the use of culture-based methods, through swabbing the surface of the skin and short incubation periods, were the most reliable for skin microbiota analysis (Grice et al., 2008, Gao et al., 2007). However, culture-based methods have proven limited in the extent to which they can capture the full diversity of skin microbiota. It is, nonetheless, considered that nearly ≈75% species of skin microbiota can be obtained from culture methods, which is significantly greater than other parts of the body such as the gut (Gao et al., 2007).

1.2.2 High-throughput 16S rRNA sequencing

The majority of microbes thrive in large communities and only a minority of bacterial species flourish in isolation. Culture-based laboratory techniques select specific microbes using different nutritional and physiological conditions for diagnostic purposes. However, those selected using these conditions may not be the most abundant or representative microbes within the community. The anoxic environments in hair follicles and sebaceous glands for example, harbour anaerobic microbes that grow very slowly and require

special conditions to grow. It is therefore no surprise that Next Generation (NGS) techniques demonstrated greater diversity of skin bacteria compared to culture-based methods (Grice and Segre, 2011).

In the case of skin, the four most prominent phyla included: Actinobacteria, Firmicutes, Bacteroidetes and Proteobacteria. The proportions of these bacterial phyla were largely dependent on the physiology of the skin site with specific bacteria being associated mostly with moist, dry and sebaceous environments (Table 1.2) (Gao et al., 2007, Grice et al., 2009, Costello et al., 2009). In the dry sites of skin that are most diverse and contain a mixture of microorganisms from all four phyla, the most abundant organisms were the gram-negative organisms despite them rarely being thought to colonize the skin and as contaminants from the gastrointestinal tract. Interestingly, the microbiome of the nares, plantar heel, antecubital fossa and back were more similar between individuals for the same skin site compared to different skin sites on the same individual. This indicates that skin site is a more important factor for determining the composition of the microorganism environment compared to individual genetic variation among healthy volunteers. The site of skin sampled also determines the temporal variability of the skin microbiome, as revealed by molecular analysis. In healthy adults, the composition of the microenvironment in regions like the glabella, nares and external auditory canal are more stable than regions like heel, which are dry. Additionally, contralateral sites on the same individual are more similar to each other than the corresponding site on another individual (Kong and Segre, 2012).

Table 1.2 Dominant microorganisms in sebaceous, moist and dry areas of the skin.

	Type of environment	Skin site	Dominant organisms
1	**Sebaceous**	Face, chest and back	*Propionibacterium spp.*
2	**Moist**	Inner forearm	*Staphylococcus and Corynebacterium spp.*
3	**Dry**	Heel	Mixed from all four phyla (Actinobacteria, Firmicutes, Bacteroidetes and Proteobacteria)

The HMP project was conducted by the US National Institute of Health (NIH) in 2007 using pyrosequencing-based profiling of the 16S ribosomal RNA (rRNA) gene to investigate the microbial populations within healthy individuals. A total of 242 healthy volunteers were involved in the study and body sites like the distal gut, mouth, skin and vagina were examined (HMP, 2012a, Hannigan and Grice, 2013). This uncovered both the multiplicity and variations in human microbiota depending on body site and skin depth. The bacterial species in the human intestine are predominantly genera *Bacteroides*, *Prevotella* and *Lactobacillus.* There are approximately 800 bacterial species present in the oral cavity and they are mainly *Streptococcus*. Approximately 300 bacterial species exist in the vagina and they are composed predominantly of

Lactobacillus (Grice et al., 2008). On the skin, 1,200 bacterial species were identified and these were prominently *Corynebacterium*, *Propionibacterium* and *Staphylococcus*.

In order to study these microbiotas, there must be a system that unveils the diversity between different species. To overcome the limitations of standard culture-based techniques, technologies that are more representative are required to study human microbiota (Riesenfeld et al., 2004).

DNA sequencing is a useful and unbiased tool for determining the microorganism populations within a sample. It is not possible to discriminate between dead and live colonies using this technique, in contrast to culture-based techniques where only viable microbes can be detected. However, culture-based techniques are limited by culture conditions, which significantly affect microbe growth in a selective manner, and therefore modify microbial detection. These issues can, to some extent, be addressed by RNA sequencing, which can be amplified selectively to only show data from skin microorganisms. Although the approach has technical challenges including difficulty in obtaining adequate biomass from organisms sampled from the skin, it may be a more representative method for the study of microbiota (Korem et al., 2015).

Numerous DNA sequencing techniques can be used to study microbiota ranging from basic to advanced sequencing methods. The basic sequencing methods include: Maxam-Gilbert sequencing and Sanger sequencing. Advanced methods of sequencing such as shotgun sequencing. The next level of revolution in sequencing technology is the next-generation sequencing including SOLiD, Pyrosequencing and Illumina. The Illumina platform uses bridge amplification PCR. The 16S rRNA gene sequencing technique was recently used to describe different bacteria in the environment. This method utilises the PCR to amplify the prokaryotic small sub-unit 16S rRNA gene directly from bacteria (Dethlefsen et al., 2007, Turnbaugh et al., 2007). The 16S rRNA gene is composed of preserved and variable regions. The invariable regions allow binding of PCR primers, which do not need to be species specific, for sequencing of the entire gene. However, the variable regions are unique to each species of bacteria and therefore mediate taxonomic categorisation subsequent to the PCR products being subjected to high-throughput sequencing (Hugenholtz and Pace, 1996). Because the 16S rRNA gene is present in all bacteria and archaea but not in eukaryotes, this process can build a picture of the entire microbial composition of the sampled area in a semi-quantitative manner.

Sequence alignment determines that sequences with a similarity greater than 97% are categorised within one species. Strains are identified by variation in sequences within one species. In addition to taxonomic categorisation, the number of sequences found from one species provides an indication of level of abundance of the species in a given skin sample. Thus, metagenomics offers a comprehensive overview of microbiota by establishing semi-quantitative abundance of the existing species identified.

1.2.3 Human microbiome function

The microbiota in different body regions have different functions and some of these have been reported in the literature. Gut microbes can ferment carbohydrates and enable their absorption; they also enable the host to use some normally indigestible carbohydrates. Additionally, they can synthesise and excrete vitamins, which can be absorbed as nutrients by the host. An example includes the human gut bacteria that secret Vitamin K and Vitamin B12 (Lactic acid bacteria can also produce certain B-vitamins) (Conrad and Vlassov, 2015). Whilst precise functions of skin microbiota are yet to be established, it has been shown that skin species can antagonise competitors and foreign bacteria by the release of substances, such as non-specific fatty acids and peroxides or highly-specific bacteriocins.

1.2.4 The skin microbiome

Human skin microbiota displays a wider variety compared to oral microbiota or the gut due to the overall diversity from all skin locations (Hannigan and Grice, 2013). Similar to their function in the gut, skin microbes play an important role in protecting against pathogens by serving as a protective barrier and the breakdown of natural products (Scharschmidt and Fischbach, 2013, Belkaid and Segre, 2014, Grice, 2015). Approximately 90% or more of the 1200 bacterial species associated with human skin originate from only four of a total of 20 phyla. These phlya are: *Actinobacteria,* constituting 52% of bacteria; *Firmicutes*, 24%; *Proteobacteria,* 16% and *Bacteriodetes,* 5%. These four phyla contain the majority of skin bacteria, which includes thousands of species (Grice et al., 2009). For example, a 16s sequencing based study identified 4,792 species in total on the palms (hands) from 51 healthy subjects, with each palm comprising an average of 158 species (Fierer et al., 2008).

To characterise the normal skin microbiome, the HMP examined diversity on the skin by sampling 11 different body locations including the forehead, left and right axillae, left and right inner elbows, left and right forearms, left and right forelegs, and behind the left and right ears on healthy volunteers (Gao et al., 2010). The total numbers of bacteria were calculated with semi-quantitative PCR with 16S rRNA gene region primers and probes. Additional research has been conducted on the most frequently occurring genera *Propionibacterium, Staphylococcus, Corynebacterium,* and *Streptococcus.*

Fierer et al., (2008) showed that there appears to be a core set of phylotypes present on the skin of the adult. This group also suggested that the genomes of the representative organisms should be prioritized for sequencing to make sense of deeper metagenomic studies. However, the non-core phylotypes appear to exhibit a ‘‘long tail’’ effect (most phylotypes are rare) suggesting that exhaustive sampling is not a reasonable goal. Furthermore, the significant heterogeneity in community composition between for example the left and right hands from the same individual, suggests that careful sampling strategies will be required to obtain usable data for the International HMP.

1.2.4.1 Regulation of the skin microbiome

The heterogeneity of the resident microbial populations on different body sites and between individuals are thought to arise due to many host and environmental factors. An exhaustive list shows some of these factors in Table 1.3.

Table 1.3 Intra and inter- individual factors affecting skin microbiota.

Intra-individual factors	Inter-individual factors
Adaptive immunity (antibodies)	Environmental reservoirs
Ecological zone	Food resources
Host behaviour	Geographical location, climate and UV exposure
Host development	Health condition, injuries and antibiotics
Host genetic variation and host origin	Lifestyle e.g. hygiene, deodorant usage, occupation, pet ownership, anxiety and drugs
Humidity	Pathogen (Bd)
Innate immunity (AMPs, alkaloids, lysozymes)	Physiology e.g. gender, age and ethnicity
Intrinsic factors such as a sebum overproduction, e.g. during puberty	Season, time of the day and mode of delivery
Microbial presence within the epidermis	Skin microbial interaction Physical activity
pH	
Salinity	
Skin shedding, acne, psoriasis and atopic dermatitis	
Skin site	
Temperature and chemical attribution of the skin	

1.2.4.1.1 Host factors

There are significant differences in bacterial taxa found on sebaceous, moist and dry areas of major skin regions as shown in Figure 1.4 (Grice et al., 2009). Interestingly, sebaceous areas show the least variability, whereas dry areas, like the forearm and palm, vary more significantly over a given period, suggesting that such differences modify long-term stability of diversity. Similar species of bacteria reside in environments, which share similarities such as the popliteal fossae and the axillae (Costello et al., 2009, Grice et al., 2009). Sites that produce sebum (for example the back, forehead and retroauricular crease), have an abundance of *Propionibacterium,* whereas moist areas such as the axillae predominantly show *Corynebacterium sp.* and *Staphylococcus sp.* (Grice and Segre, 2011).

The temperature and pH of different human body sites can contribute to the expansion or reduction in the numbers of microorganisms. pH in the human body spans between 4.2 to 7.9 and the temperature ranges between 29.5 to 36.6°C (see Figure 1.4) (Dreno et al., 2016, Wilson, 2005). Skin microbiome composition is known to be affected by the temperature, pH, level of sweat. According to culture-based studies, these factors support distinct sets of microorganisms. Some regions of the skin are partially occluded like the groin, axillary vault and toe web. Temperature and humidity in these regions are higher and are therefore optimal conditions for the growth of microorganisms (e.g. Gram-negative *bacilli*, *corneforms* and *S. aureus* (Roth and James, 1988). Areas of skin on the arm and leg experience large fluctuations in surface temperature compared to other skin sites. Culture-based methods revealed that these areas harbour quantitatively fewer organisms compared to moist areas of the skin surface (Grice and Segre, 2011).

Another factor that determines the composition of skin bacteria is the number of sebaceous glands in a given area. Regions like the face, chest and back have a higher density of sebaceous glands, which promote the growth of lipophilic organisms like *Malassezia spp*. and *Propionibacterium spp* (Costello et al., 2009) (Grice and Segre, 2011)

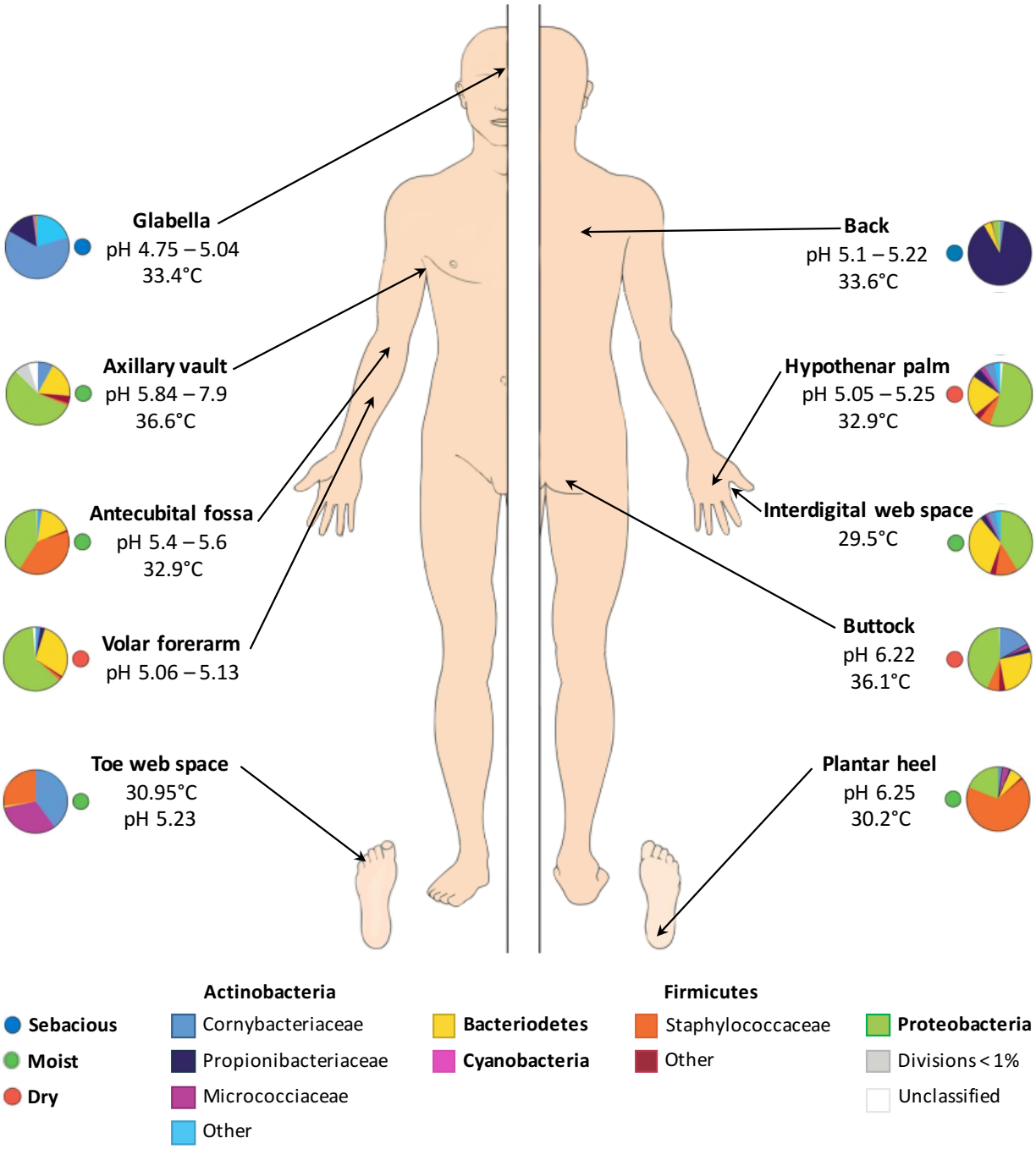


Figure 1.4 Major bacterial taxa, pH and temperature in various skin regions. Schematic re-created based on (Dreno et al., 2016) (Grice and Segre, 2011).

Several different detection methods have shown that bacteria are not only found on the surface of the skin, but are also present in the epidermis, dermal adipose tissue and dermis (Nakatsuji et al., 2013). In these layers, specific microbiome profiles can be identified as well as a number of specialised cell types including: dendritic cells, Langerhans cells and melanocytes, which express different pattern recognition receptors (PRRs), able to recognise specific microbial structures on pathogens, named pathogen-associated molecular patterns (PAMPs). The most prominent examples of PAMPs are lipopolysaccharide (LPS) of gram-negative bacteria, lipoteichoic acids (LTA) of gram-positive bacteria , peptidoglycan and lipoproteins generated by

palmitylation of the N-terminal cysteines of many bacterial cell wall proteins (Kumar and Sharma, 2008) (Miller and Modlin, 2007, Yu et al., 2009, Zouboulis, 2009, Nakatsuji et al., 2013). It has been suggested that phagocytic cells carry out translocation of the microbiota found in the appendage structures or outer layers into the sub-epidermal regions. However, this mechanism is yet to be elucidated and the means of translocation are to be proven (Nakatsuji et al., 2013).

Evidence indicates that the deeper you probe into the epidermis layers, the higher the number of bacterial cells. Grice et al. (2008) estimated bacterial populations in the epidermis using a quantitative 16S rRNA PCR method. They found that the populations (as measured by genome copies per cm^2) varied for punch biopsies from the lower epidermal sections, scrapes from the middle layers and swabs taken from the superficial layer as follows: 1,000,000; 50,000; and 10,000, respectively. Such results were not anticipated, and the authors suggested that the bacterial presence could be linked to the higher levels of moisture and nutrients in the deeper skin levels (Grice et al., 2008).

Larger numbers of bacterial species in the skin tend to reside on adult male skin compared to females. It was suggested that this is due to greater micro-colony sizes found in males as opposed to females. Different sexes show distinct bacterial populations as demonstrated by a study on hand microbiome, differences were found in *Propionibacterium* (37% greater abundance in males), *Corynebacterium* (80% greater abundance in males), *Enterobacteriales* (400% greater abundance in females), *Moraxellaceae* (180% greater abundance in females), *Lactobacillaceae* (340% greater abundance in females), and *Pseudomonadaceae* (180% greater abundance in females). Although it should be noted that the exact cause of these differences is yet to be established, it has been proposed that the lower pH in men's skin and higher sweat rate in males are possibly contributory factors (Fierer et al., 2008).

Age has great impact on the microenvironment of the skin and in turn the colonizing microbiota. Bacterial colonies found in newborn babies depend on the method by which they have been delivered (Sarkany and Gaylarde, 1968, Dominguez-Bello et al., 2010). The skin of vaginally delivered babies was shown to harbour bacteria associated with maternal vaginal and faecal microbiota and they include: vaginal *Lactobacillus, Prevotella,* and *Sneathia* species. Newborns delivered via a caesarean section were shown to have classical skin bacteria *Corynebacterium*, *Propionibacterium* and skin *Staphylococcus* species (Dominguez-Bello et al., 2010). The precise effect of such variations is still unclear. Nevertheless, after several months, the skin microbiota of infants born by vaginal deliveries and Caesarean section become increasingly alike (Blaser, 2014). It is expected that the microbial communities of the skin and other sites are established and stabilised during the first years of life as the newborn explores its environment and its immune system matures (Sarkany and Gaylarde, 1968). However, this area requires further research. During puberty, culture-based approaches showed that the changes in sebum production are parallel to the levels of lipophilic bacteria on the skin (Somerville, 1969).

In another study on the bacterial species colonizing the human intestinal tract, it was shown that the microbiome diversity is significantly affected by aging. The progression of bacterial diversity in different stages of life was observed and quantified using quantitative or real-time PCR (qPCR). After evaluating the major dominant and subdominant groups of the human faecal microbiota, it was shown that the ratio of *Firmicutes/Bacteroidetes* increases from birth to adulthood and alters again with advanced age (Mariat et al., 2009).

In a study involving twenty-eight healthy individuals aged from 2 to 40 years, the analysis of bacterial populations in Body sites sampled included skin sites (antecubital/popliteal fossae), (volar forearm) and nares demonstrated clear differences between children and adults.

In the skin, the microbiomes of young children and adolescent/post-adolescent individuals were compared. the microbiomes community in children were dominated by Gram-negatives *Moraxella, Haemophilus*, and *Neisseria* and a greater diversity of bacteria were observed including *Streptococcus spp*. In contrast, adolescent/post-adolescent individuals had few to none of these taxa found in the children skin; their microbiomes were dominated instead by lipophilic bacteria that are associated with sebaceous skin regions including *Propionibacterium, Corynebacterium*, and *Turicella*. The results comparing young children versus adolescent/post-adolescent individuals were highly concordant.

In nares, the bacterial communities that were overrepresented in children included *Proteobacteria, Bacteroidetes* and *Firmicutes* (*Streptococcaceae*) were strikingly differed from adults, the predominant populations were comprised of *Propionibacteriaceae* and *Corynebacteriaceae* (Oh et al., 2012)

1.2.4.1.2 Environmental factors

Studies on ethnicity and population-specific variation in human microbiome composition provide us with important information. However, controversies exist whether these differences are the result of race or the environment. It has been suggested that ethnicity is secondary in determining cutaneous microbiota composition and that the ecological zone of the human body is the key determining factor. This is based on a study of three skin sites (forearm, axilla and scalp) in men from six ethnic groups living in New York City. Skin samples were collected at baseline and four days after daily use of neutral soap and the discontinued use of any other hygiene products including shampoos and deodorants. 16S rRNA gene sequencing showed significant differences at phylum and genus levels between subjects of different ethnic origins at all skin sites and confirmed that the location of the skin was the main factor in determining the composition of microorganism populations. The alpha diversity (number of species observed) was greater in the arm compared to the scalp or axilla in all the groups studied (Perez Perez et al., 2016).

Studies on hand microbiota in Tanzanian and US women demonstrated more abundant populations of *Propionibacteriaceae, Staphylococcaceae* and *Streptococceacae* in US women, while bacterial populations

that are soil-related including *Rhodobacteraceae* and *Nocardioidaceae* were found in women from Tanzania (Hospodsky et al., 2014). Such a geographical difference in skin microbiome may be attributed to different lifestyles and environment in each of the countries. The participants from the Tanzanian population had a tendency to carry out their daily activities outside and made extensive contact with soil and water, whereas the US population on the whole spent most of their time indoors and made contact mostly with dry surfaces.

These findings were further supported by research in China, which also showed significant differences in skin microbiota between countryside and city populations. *Trabulsiella sp* which is a genus of the family Enterobacteriaceae that resembles Salmonella. was more prevalent in the city compared to the countryside, particularly in areas such as the back of hands and forearm (Ying et al., 2015). Most urban inhabitants spent large amounts of time inside buildings, thereby limiting skin microbiome to largely human-derived origin with little input from the natural environment.

In a study that compared forearm skin specimens from healthy Amerindians in the Venezuelan Amazon and healthy individuals in New York and Colarado, showed significant differences in the microbial communities between the two populations with different lifestyles (Blaser et al., 2013). Skin samples from the US participants were dominated by *Propionibacterium*, whilst the Amerindians were divided into two, one was dominated by *Staphylococcus* (but similar to the US community) and another contained a wide range of *Proteobacteria*. The Armerindians in the study represented a population in transition that shifted approximately two to three generations ago from a nomadic hunter-gather lifestyle to a more modern lifestyle and this has been suggested as a possible explanation for the similarity between one group of Amerindians and the US population. However, no definite association (age, gender, body mass index, bathing etc.) was found for the differences between the Amerindian groups (Gupta et al., 2017, Blaser et al., 2013).

There is also evidence for seasonality in microbial populations. For example, studies of the gut in the Hazda hunter-gatherers of Tanzania, whose microbiome profile was examined following the collection of 350 stool samples in a longitudinal study, revealed an annual cyclic configuration of the microbiome. Some taxa only appeared in certain seasons while others became undetectable. The Hadza data set was then compared with data collected from 18 populations in 16 countries with different lifestyles. It was shown that the gut microbial community corresponded with exposure to western lifestyles. For instance, the taxa that were undetectable in the Hadza microbiota in certain seasons were always absent in the industrialized populations (Smits et al., 2017). These data indicate that some dynamic lineages of microbes have reduced in prevalence and abundance in modernized populations and that the gut microbiota of urbanized people shows characteristic patterns that may be relevant for other resident bacterial populations in other regions like the skin (Sonnenburg et al., 2016).

Unsurprisingly, skin treatments, such as antibiotics, may significantly modify the skin microbiome (SanMiguel et al., 2017). SanMiguel and colleagues investigated the impact of antimicrobials on skin microbiome by

applying an array of antibiotics to the skin of hairless mice. These treatments included a broadly applicable triple-antibiotic ointment and a narrowly targeted mupirocin ointment. It was revealed that the antibiotics significantly impacted the composition of microbial populations on the skin of mice. Additionally, these alterations lasted several days after treatment (SanMiguel and Grice, 2015).

Systemic antibiotics are widely used to manage moderate to severe acne. In a longitudinal study involving 20 participants with moderate to severe facial acne, patients were prescribed oral doxycycline twice daily for six weeks and skin areas on the cheek were collected and analysed using 16S rRNA gene sequencing. To determine the effects of oral antibiotics on skin microbiota, the microbial populations were compared at baseline (pre-treatment) and after six weeks of doxycycline treatment. The two most dominant bacterial species at baseline were *Cutibacterium acnes* and *Staphylococcus epidermidis*. The severity of acne was also positively correlated with the abundance of *Cutibacterium acnes*. In all participants, the six-week treatment with doxycycline decreased the clinical acne grade and was linked to an almost 2-fold decrease in the relative abundance of *Cutibacterium acnes.* The antibiotic treatment was also linked to an increase in bacterial alpha diversity and resulted in significant changes in the levels of other bacterial species like *Cutibacterium* granulosum (~4.5 fold increase). These data provided strong evidence that systemic antibiotics is associated with changes in the composition and diversity of skin microbiota.(Park et al., 2020)

1.2.4.2 Skin diseases and associations with microbiome constituents

According the Global Burden of Disease (GBD) project, skin diseases are the 4th leading cause of non-fatal disease burden worldwide and affect millions of individuals (Seth et al., 2017, Hay et al., 2014). Aging, environmental and genetic factors, and trauma are associated with the development of different types of skin diseases and more than 3000 entities are recognized in the literature (Lim et al., 2017).

S. aureus is a gram-positive bacteria and a facultative anaerobe and is a skin commensal in approximately 30% of the population (Otto, 2014). However, it is also a major cause of skin and soft tissue infections (SSTI) (Cogen et al., 2008). *S. aureus* is also strongly implicated as pathogenic in atopic dermatitis where loss of microbial diversity is associated with an increase in the relative abundance of *S. aureus* (Kong et al., 2012). This species is considered to be overrepresented owing to low amounts of antimicrobial peptides on the skin, i.e. those produced by stimulation from other neighbouring microbes like *S. epidermidis* (Brestoff and Artis, 2013, Scharschmidt and Fischbach, 2013).

P. acnes is a gram-positive, oxygen tolerant anaerobe and one of the most common skin commensal bacteria. This bacterium resides in hair follicles and sebaceous glands where sebum triglycerides are metabolized into free fatty acids. *P. acnes* is associated with acne vulgaris, the most common adolescent skin condition.

However, it is important to note that acne vulgaris is likely to be multifactorial and caused by combination of genetic and environmental factors (Cogen et al., 2008).

Healthy skin keratinocytes synthesise antimicrobial peptides (AMP), which are key factors driving the inherent immunity of skin by inhibiting the survival of microbes. Commensal microbes stimulate AMP expression in the skin by the presence of *Propionibacterium* species and other Gram-positive bacteria and thus, demonstrate the direct contribution of skin microbiota to innate immunity (Nagy et al., 2006, Lee et al., 2008). In addition to AMPs, antimicrobial free fatty acids can be produced by sebocytes. For example, *P. acnes* and *S. epidermidis* hydrolyze sebum triacylglycerides generating peptides categorised as pheno-soluble modulins (PSM) which show selective activity against *S. aureus, Escherichia coli* and group A *Streptococcus*. Indeed, some strains of *S. epidermidis* also directly produce PSM and inhibit *S. aureus* directly (Otto, 2009, Iwase et al., 2010, Otto, 2014).

Determining the relative numbers of core and noncore lineages in different skin habitats, their ariability, and the relationships between intrinsic physiological or consistent physical states (e.g. sex and handedness) and external environmental characteristics or behaviours (e.g. hand washing) are critical for establishing a healthy baseline for detecting and understanding microbial community differences associated with a wide variety of skin diseases (Fierer et al., 2008).

On human skin, *S. epidermidis* is the most common coagulase-negative *Staphylococci* to be encountered. Following colonization with *S. epidermidis*, previously germ-free mice (without resident skin microbiota) were observed to produce dysregulated cytokines in the skin and showed dysfunctional T helper type 2 (Th2) cell differentiation (Naik et al., 2012) (Chen and Tsao, 2013). Non-lesional skin from patients with Atopic dermatitis illustrates an increased number of TH2, TH22 and TH9 cells and, to a lesser degree, TH17 cells. In addition, an evidence of subclinical inflammation were demonstrated with a pro-inflammatory cytokine milieu (Gittler et al., 2012, Noda et al., 2015) (Esaki et al., 2016). Whilst *S. epidermidis* is generally considered a benign commensal in humans, it is able to act as a nosocomial pathogen, particularly in infections of indwelling medical devices. *S. epidermidis* survives through a passive mechanism and forms sticky agglomerations called biofilms to inhibit immunity responses by the host. *S. epidermidis* also produces protective surface polymers and exoenzymes and has the ability to secrete cytolytic members of the PSM family that are involved in the formation of biofilms. Some studies also provide evidence for a virulence gene reservoir function of *S. epidermidis,* since factors like immune evasion and antibiotic resistance seem to have transferred to *S. aureus*. In addition to its pathogenic properties, *S. epidermidis* also plays a beneficial role in balancing the microbiota on human epithelial surfaces. This is achieved by controlling the outgrowth of harmful bacteria like *S. aureus* (Otto, 2009).

The AMP mammalian such as LL-37 and the (PSMs) γ and δ produced by *S. epidermidis* both share similar features of which an α-helical character and a strong lipid membrane interaction. Based on the structure,

biophysical properties, and antimicrobial activity of PSMγ and PSMδ, (Cogen et al., 2010) suggested that *S. epidermidis* has a beneficial role in skin immune defence by producing innate AMPs (non-host-derived) on the skin surface. Similar to mammalian AMPs e.g. LL-37, (PSMs) γ and δ produced by S. epidermidis have an α-helical character and a strong lipid membrane interaction. LL-37 complies with both PSMs to reinforce the antimicrobial action by inducing directly lipid vesicle leakage and exerted selective antimicrobial action against skin pathogens such as *Staphylococcus aureus*.

1.3 Nitrogen cycle

Nitrogen (N) is a naturally occurring unreactive gas that forms approximately 78% of the Earth's atmosphere as N_2 (Hirsch and Mauchline, 2015). This element is essential for growth and reproduction in biological systems including plants and animals. Nitrogen is also found in amino acids, DNA and nitric oxide (NO). Despite the abundance of N_2 in the atmosphere, the strong triple bond between the N atoms in the molecule makes them relatively unreactive. However, living organisms require reactive nitrogen for incorporation into cells and are therefore dependent on the biogeochemical nitrogen cycle. Bacteria play a crucial role in the nitrogen cycle. In this cycle, N_2 is transformed via redox reactions including oxidation and reduction into bioavailable compounds as shown in Figure 1.5 (van de Leemput et al., 2011) (Hirsch and Mauchline, 2015, Stein and Klotz, 2016).

The nitrogen cycle can be broken down into three main processes including fixation, nitrification and denitrification (Hirsch and Mauchline, 2015, Stein and Klotz, 2016). During the fixation reaction, N_2 is reduced to ammonium (NH_4^+) and ammonia (NH_3) either by the direct action of nitrogen-fixing bacteria or via the dissimilation of nitrite and nitrate to NH_4^+ and NH_3. The nitrification process occurs when two consecutive processes including nitritation (oxidation of NH_3 to NO_2^-) and nitratation (oxidation of NO_2^- to NO_3^-) are carried out by two different groups of microorganisms, ammonia-oxidising bacteria (AOB) and ammonia-oxidising archaea (AOA). In the final dentirification step, N-containing molecules are reduced and N_2 is released back into the atmosphere. Recently, the anaerobic NH_4^+ oxidiation (anammox) pathway, in which NH_4^+ and NO_2^- molecules are converted to N_2 under complete anoxia, has been incorporated into the cycle (van de Leemput et al., 2011, Stein and Klotz, 2016).

1.4 Nitric oxide (NO)

NO plays an important role in biological processes as a signalling molecule between cells and is vital for the regulation of many physiological functions. NO is a free radical, small in size, soluble in water and lipids and diffuses rapidly. NO can also cross cell membranes without requiring specialised transporters. Although NO is free radical, it is kinetically extremely stable and has a low tendency to dimerise. NO also has low oxidant and reductant capacities and is a short-lived species in biological systems. This is due to its reactive nature towards other biomolecules and targets such as oxygen, free radicals (e.g. superoxide), metalloproteins (e.g. heme moieties and soluble guanylate cyclase) and thiols (Fukuto et al., 2012, Heinrich et al., 2013).

NO is formed by numerous types of cells in tissues, and in mammals permits relaxation of vascular muscle, has a neurotransmitter function and inhibits platelet aggregation. In addition to its physiological functions, NO is produced during inflammatory and immune responses. However, in these cases, its role is more complex and less clearly defined. NO can act as an antimicrobial agent through direct actions on the

pathogens or through stimulation of host immunity and/or by acting as a pro- or anti-inflammatory agent. NO contributes significantly to innate immunity as a toxic agent when faced with organisms carrying infections. It can also be responsible for inducing cell death and for the function of host immune cells to regulate specific immunity. Additionally, NO can provoke toxic reactions in conditions such as asthma, where it is thought to behave as a pro-inflammatory agent (Moncada et al., 1991, Lincoln et al., 1997). (Feelisch and Martin, 1995) (Schairer et al., 2012a).

As well as its immune function, NO is thought to modulate vascular tone. The role of NO in neurotransmission has also been widely reported since the earliest accounts of it as an effector molecule in humans in 1987. One of the NO synthase families, synthesise nitric arginine and numerous researchers have discovered NO synthases in the skin (Dippel et al., 1994, Goldsmith et al., 1996, Nameda et al., 1996). Furthermore, it has been demonstrated that, via the non-enzymatic route of acidification of nitrite, normal skin continuously releases gaseous NO (Weller et al., 1996).

1.4.1 Generation of NO through NOS dependent and independent pathways

NO is produced by numerous cells ranging from immune cells including dendritic cells, mast cells, monocytes and microglia to tissue supporting structures like keratinocytes, fibroblasts and endothelial cells (Schairer et al., 2012a). In humans, bioactive NO can be produced via two parallel pathways that are enzymatically and non-enzymatically mediated (Figure 1.5). NO is typically generated from L-arginine and oxygen by a family of enzymes known as NO synthases (NOSs). In more recent studies, a mechanism for the production of NO in mammals of a fundamentally different nature has been explained. Following this pathway, there is a reduction of the inorganic anions nitrate and nitrite to create bioactive NO within the blood and tissue through physiological hypoxia. Despite NO production by NOS being restricted when levels of oxygen decrease, the nitrate-nitrite-NO pathway undergoes an enhancement. The required NO production is supplied through the physiological and pathological oxygen and proton gradients due to the parallel action of the two pathways (Lundberg et al., 2008).

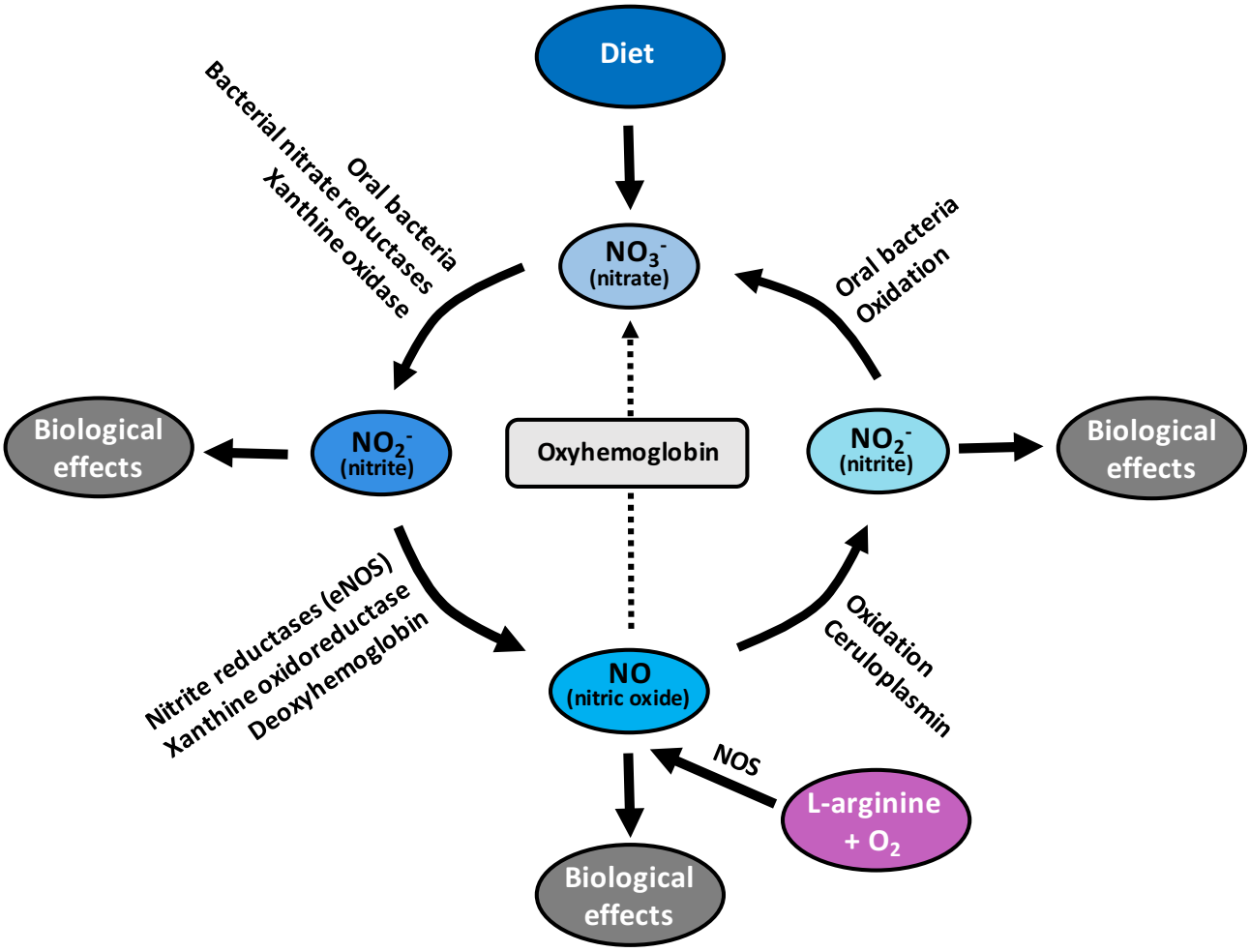


Figure 1.5 Nitrate-nitrite-nitric oxide pathway.

1.4.1.1 NOS dependent pathway (L-arginine-nitric oxide pathway)

The two-step enzymatic process via which NO is synthesised from L-arginine and molecular oxygen, uses electrons provided by NADPH. L-arginine is converted to NO and L-citrulline by the NOSs through the intermediate N-hydroxy-l-arginine. One molecule of NO is generated by one molecule of L-arginine (Figure 1.5) (Förstermann and Sessa, 2012).

Three different NOS isoforms exist including neuronal (nNOS, NOS1), inducible (iNOS, NOS2) and endothelial (eNOS, NOS3). These homodimers belong to a group of monooxygenases as they contain reductase and oxygenase domains. The nNOS and eNOS isoforms are constitutively expressed in neural and vascular systems, whereas iNOS can be synthesised by every cell type following cell activation (Szacilowski et al., 2005)Molecules involved in inflammation and infection are capable of triggering iNOS expression including bacterial lipopolysaccharide, interleukin-1β, interferon and tumour necrosis factor-α (Kiechle and Malinski, 1993). Neuronal NOS or nNOS was first characterised in neurons, hence its name, and produces NO in nervous tissue of both the central and peripheral system. On the other hand, endothelial NOS or eNOS was

originally characterised in endothelial cells (Nathan and Xie, 1994) (Stuehr and Marletta, 1985, Stuehr and Marletta, 1987). Low concentrations of NO (<1 µM) produced by the activity of cNOS require calcium ($Ca2^{+}$)-calmodulin. However, calmodulin is not required by iNOS for the production of high concentrations (1000 µM) of NO (Schairer et al., 2012a).

In the Skin, the keratinocytes, constitutively express the neuronal isoform of NO synthase (NOS1), whereas the endothelial isoform (NOS3) are express in the fibroblasts in the dermis and other cell types. Under certain conditions, all skin cells appear to be capable of expressing the inducible NOS isoform (NOS2).(Cals-Grierson and Ormerod, 2004).

1.4.1.2 NOS independent pathway (nitrate-nitrite-nitric oxide pathway)

Intake of dietary nitrate is substantial with a number of vegetables being an especially rich source of NO (Lundberg and Govoni, 2004). The total combined amount of this produced endogenously during a day by all three NOS isoforms is lower (Wennmalm, 1994). Some others food products also contain nitrites, particularly bacon and cured meat (Lundberg and Govoni, 2004, Vallance and Collier, 1994). Significant proportions of nitrites may also be found in potable water even though this is subject to strict regulation in many countries.

As demonstrated figure 1.5, activation of nitrate from endogenous or dietary sources firstly requires its reduction to nitrite. However, since mammals lack specific and effective nitrate reducatases, the conversion of nitrate to nitrite is mostly carried out by commensal bacteria on body surfaces and in the gastrointestinal tract (Lundberg and Govoni, 2004, Duncan et al., 1995). The redox position of nitrite between (i.e. oxidation and reduction reactions) signalling is unique to the nitrogen oxides carried out by microorganisms (van de Leemput et al., 2011, Hirsch and Mauchline, 2015, Stein and Klotz, 2016) . It is also unique in its relative stability in the blood and tissue (Gladwin et al., 2005). As soon as nitrite is produced, there are a number of ways in which it can be further reduced to NO in the body with the involvement of haemoglobin (Nagababu et al., 2003, Cosby et al., 2003), myoglobin (Shiva et al., 2007a, Rassaf et al., 2007), xanthine oxidoreductase (Godber et al., 2000, Cantu-Medellin and Kelley, 2013), ascorbate (Carlsson et al., 2001), polyphenols (Peri et al., 2005, Gago et al., 2007), and protons (Benjamin et al., 1994, Lundberg et al., 1994). When hypoxia and acidosis occur, the production of NO by these pathways is facilitated to a significant extent. Thus, NO generation can be ensured in situations that compromise oxygen-dependent NOS enzyme activity (Giraldez et al., 1997, Ostergaard et al., 2007). It appears that reduction of nitrite to NO and NO-modified proteins through physiological and pathological hypoxia, constitutes a significant factor in physiological hypoxic signalling, vasodilation, modulation of cellular respiration and how cells respond to ischaemic stress (Cosby et al., 2003, Shiva et al., 2007b, Shiva et al., 2007a).

1.4.2 Chemical biology of NO

NO molecules can either directly or indirectly mediate the effects of NO in the human system. (Figure 1.6). Constitutive configuration by constitutive NOS (cNOS) regulate the concentrations of NO contained in biological fluids. Small amounts of NO (pmoles) are responsible for its function as a signalling molecule and antioxidant through this constitutive production as a feature of the cardiovascular system (e.g. vasodilation, blood pressure control and neuronal physiologies (Shabeeh et al., 2013, Shabeeh et al., 2017). Signalling effects occur, for example, due to NO reactivity with metalloenzymes, such as soluble guanylyl cyclase (sGC)(Lundberg et al., 2015). sGC activation leads to signal transduction with increasing quantities of cyclic guanosine monophosphate (cGMP) in addition to the phosphorylation cascade being activated (Montfort et al., 2017, Sutton et al., 2018). In 2016, Patel emerged the cysteine/cystine (Cys/CysSS) redox couple, along with GSH/GSSG, as a powerful predictor of cardiovascular mortality (Patel et al., 2016). The detectors in the recent application replaced the employment of either high-pressure liquid chromatography (HPLC) or chromatographic separation technique based coupled to ultraviolet, fluorescence, or electrochemical detector with mass spectrometers, potentially offering more specific and sensitive measurements to determine these species (Weaving et al., 2006).

NO molecules may also bind to thiol groups inside the proteins and this leads to the production of S-nitrosothiols (RSNO) (Feelisch, 2008). This in turn results in a continuation in the biological effects as it operates as a NO-storage pool. Deserted by adequate upregulation of antioxidative defence mechanisms, the oxidative stress caused by the excess of ROS that leads to enhance the formation of reactive nitrogen oxide types (RNOS) production contributes to the development of myocardial and vascular dysfunction. Total free thiols (TFT) and membrane lipids play outstanding roles when the oxidation targets various cellular and tissue components through reactions Under oxidative stress conditions. (Munzel et al., 2015).

Both the increase in the lipid oxidation products' concentration and the shift in redox with reductions in reduced and increases in oxidized thiols are observed (Avery, 2011). Due to progressive changes in redox signalling at multiple levels, these processes are accompanied by perturbations of cardiac physiology at multiple levels (Burgoyne et al., 2012). The depletion of the TFT pool in serum can be measured as systemic oxidative stress. Comparing to the controls, patients with cardiovascular disease (CVD), including acute myocardial infarction has reported depletion in Serum TFT (Banne et al., 2003) (Kundi et al., 2015). Moreover, risk factors of CVD as well as aging, smoking, and obesity have been linked to thiol oxidation (Droge, 2002) (Go and Jones, 2011). The oxidative stress role in the pathogenesis of Heart failure (HF) has been supported by a great amount of evidence. Considering the relationship between total free thiols and oxidative stress, (Koning et al., 2016) study was aimed to address TFT role in chronic heart failure (CHF). The study suggested a promising strategy to improve disease outcomes in CHF by therapeutic modulation of free thiols to restore

redox status. In addition to illustrating favourable associations of serum-free thiols with markers of HF severity and prognosis

Serum LMW thiols have a small share and protein thiols predominate unlike the intracellular pool, which mainly consists of low molecular weight (LMW) thiols, (Turell et al., 2013). ROS and other reactive species readily oxidizing reduced thiols. Therefore, the level of thiols might be interpreted as a direct reflection of the overall redox status (Chung et al., 2013). compared to their intracellular counterparts, the thiols in serum are less readily reduced once oxidized. consequently, a relatively stable reflection of the systemic redox status is provided. More importantly, total free thiols are known to be receptive to therapeutic modulation are also active components of the antioxidant machinery, for example by cysteine derivatives such as N-actylcysteine (NAC) (Atkuri et al., 2007) Hence, they form a potential target for therapy.

Inducible NOS (iNOS) produced a significant amounts of NO (nmoles), during the immunological process, which frequently occurs in cases of host infection (Moncada and Higgs, 1993). With significantly high concentrations, NO produces deleterious effects on reactive oxygen species (ROS).

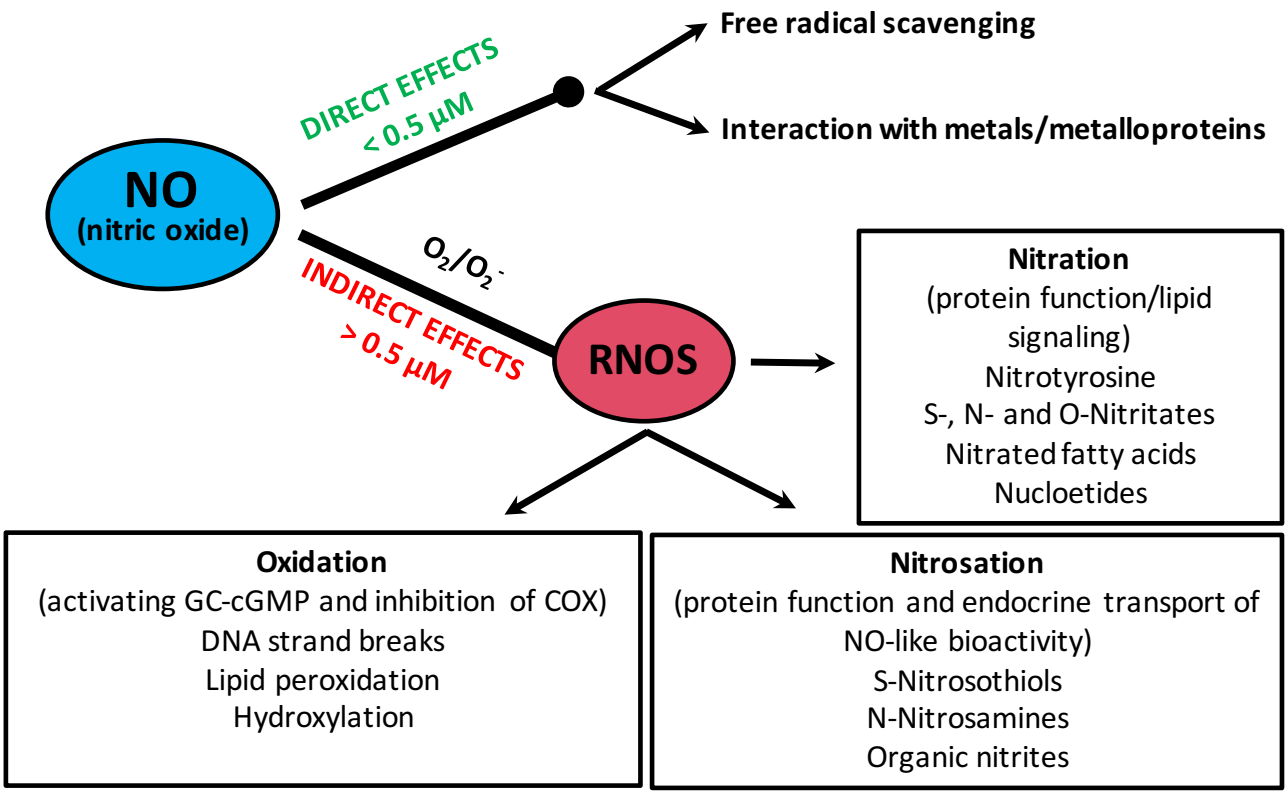


Figure 1.6 Direct and indirect effects of NO.

1.4.3 Applications of NO in health and disease

Since NO is involve in a wide range of biological functions ranging from protective to regulatory roles due to its antimicrobial and pro/anti-inflammatory properties, modifying the bioavailability of NO has led to the development and commercialisation of numerous pharmacological drugs for the treatment of various diseases (Moncada and Higgs, 1995) (Feelisch and Martin, 1995, Bogdan, 2001) (Szacilowski et al., 2005). In order to harness the potential benefits of NO as an antimicrobial agent, NO gas must be delivered to target cells at optimal concentrations for sufficient periods of time to elicit desired non-toxic effects. Numerous platforms have been evaluated to utilise the antimicrobial properties' of NO including NO generation from a probiotic patch, gaseous NO from a tank, NO released or donated from a pro-drug, and release from nanoparticles (Schairer et al., 2012b)

Direct administration of inhaled NO was tested for the treatment of pulmonary hypertension (Krasuski et al., 2000, Weinberger et al., 2001). However, administration of NO in this way can only be carried out by specialised staff for safety reasons and this may be overcome using NO-donor molecules such as metal nitrosyls, RSNO, organic NO_2^- and NO_3^- and diazeniumdiolates (NONOates) (Carpenter and Schoenfisch, 2012). NO is capable of mediating defence against infection either through the enhancement of host immunity or through direct action against the pathogen (Schairer et al., 2012a).

Some evidence has supported the use of NO-based therapies for the treatment of pulmonary infections. The efficacy of metal-nitrosyl sodium nitroprusside in dispersing *Pseudomonas aeruginosa* biofilms was demonstrated *in vitro*. P. aeruginosa is an opportunistic bacterium that colonises the epithelial cells in the lungs of cystic fibrosis patients. Anti-microbial therapies for these patients have proven ineffective in the past. These *in vitro* findings provided evidence that NO can be used to disperse bacterial biofilms, potentiate the effects of antibiotics and act synergistically with antimicrobial drugs. These findings were further extended and supported *in vivo* in a double-blind controlled clinical trial with cystic fibrosis patients. Patients were administered a low dose of gaseous NO without comprising their safety and the treatment resulted in a significant decrease in the level of *P. aeruginosa* aggregates (Howlin et al., 2017).

Human skin is a site for a wide-range of defence mechanisms mediated either at the level of cells (e.g. epithelial cells, lymphocytes, Langerhans cells) or molecules (e.g. free fatty acids, antimicrobial peptides, NO) (Scharschmidt and Fischbach, 2013, Belkaid and Segre, 2014, Polak et al., 2014). It is therefore a useful platform for percutaneous NO generation. Unlike other systems where direct application of gaseous NO may be practical, cutaneous generation of NO can be achieved via numerous methods. Such methods include: topical generation abiotic reduction of NO_2, topical generation of exogenous NO and stimulation of NO release from photolabile dermal stores (e.g. RSNO) (Weller, 2017, Monaghan et al., 2018).

Low level NO production in the skin is thought to play an important role as a barrier and in determining the rate of blood flow. NO liberated following UV irradiation or skin wounding can initiate a variety of coordinated responses involved in melanogenesis, immunosuppression and erythema. NO is also thought to protect keratinocytes against apopotosis induced by UV radiation. At macro-level, there are three different

rates of NO production taking place through the activity by NO synathases to play an important part in the skin's adaptility and function (Cals-Grierson and Ormerod, 2004). The commensal bacterium, S. aureus, can cause invasive infections, act as a virulent and transition from a commensal to a pathogenic organism and this is regulated by quorum sensing. A recent study demonstrated the affect of NO on interbacterial communication and its role in suppressing staphylococcal virulence by targeting the Agr quorum sensing system. NO directly modifies specific cysteine residues (C55, C123 and C199) of the AgrA transcription factor resulting in a reduction in AgrA promoter occupancy, a decrease in the transcription of agr operon and quorum sensing-activated toxin genes. In mice with iNOS knockout, which are used as a model of staphylococcal pneumonia, disease symptoms are more severe compared to wildtype controls and mortality rate, as well as proinflammatory cytokine responses are signficantly increased. This NO-mediated inhibition of S. aureusa virulence is an important indicator of the beneficial role of NO and its therpaeutic potential (Urbano et al., 2018).

1.4.4 Applications of NO on human skin

At the surface of the skin, in addition to the constitutive production of NO by dermal cells and immune cells, there is an additional inorganic source of NO coming from nitrogen oxides (NO_2^- and NO_3^-) (Bruch-Gerharz et al., 1998). Weller and colleagues demonstrated the generation of NO on the outer layer of the skin in healthy human volunteers via a NOS-independent mechanism (Weller et al., 1996). Basal levels of NO were measured on the surface of human skin (hand and arm) and these levels increased following acidification resulting from topical application of acidic buffer (citrate phosphate pH 3). A similar effect was observed following cutaneous application of NO_2^-, with levels of NO increasing in an NO_2^--concentration dependent manner, independently of the skin microflora. In addition to that the author demonstrated that sweat NO_3^- (mean concentration 39.7 ± 4.3 μmol L^{-1}) is the source of NO and is reduced to NO_2^- by cutaneous microbes located at the skin surface. These findings provided key evidence for the importance of NO_2^- and NO_3^- in sweat and a role for the resident microbial species at the skin surface in generating NO_2^-.

Since NO can behave as an antimicrobial agent and has wound healing effects, biomedical techniques for the generation and delivery of NO on the surface of skin have been explored extensively *in vivo* and in animals models in attempt to treat microbial infections(Weller and Finnen, 2006, Ghaffari et al., 2007) (Weller, 2009) (Orman and Brynildsen, 2016). Topical reagents containing NO_2^- for the generation of NO_2^- generation have been proposed for the treatment of fungal infections of the foot (Weller et al., 1998). The mechanism of

action is based on the production of NO on the acidic skin surface by abiotic reduction of the exogenously applied NO_2^-.

The acidification of NO_2^- to NO has also been reported to promote wound healing in healthy and diabetic mice (Weller and Finnen, 2006). Based on this mechanism of action, another study proposed an antibacterial and antifungal permeable adhesive patch for the generation of NO on the skin (Jones et al., 2010). The skin patch contains probiotic Lactobacillus, a carbon source and NO_2^-. The reduction of NO_2^- results in the formation of NO due to the acidic environments resulting from the formation of lactate by the probiotic bacteria in the patch. The concentration of NO formed by this process was sufficient to induce the death of clinically-relevant pathogens including *Staphylococcus aureus, Escherichia coli, Trichophyton mentagrophytes, Trichophyton rubrum, P. aeruginosa and Acinetobacter baumannii*. However, the limitation of this technology is the patch-to-patch variability in the concentrations of NO generated making it difficult to reproduce the release of controlled NO doses. Despite this, the in vivo feasibility of the patch was demonstrated using rabbit models with an infected wound exposed to *Staphylococcus aureus*. The patch reduced the microbial burden and promoted wound closure compare to untreated controls. There were no safety concerns as there was no significant change in the methaemoglobin (metHb) formation of NO metabolites in the serum of treated rabbits compared to control. These anti-microbial patches have not yet been tested in humans.

Whole-body exposure of healthy human volunteers to light was reported to lower blood pressure and was proposed to occur via the release of NO from skin stores (Oplander et al., 2009). This release was suggested to increase the levels of NO_2^- and total nitroso species (RXNO) circulating in the plasma. In a separate study, exposing healthy volunteers to two erythemal UVA doses correlated with a decrease in mean arterial blood pressure (Liu et al., 2014). Independent of UV-exposure, topical application of acidified NO_2^- containing liniments was also reported to decrease blood pressure (Oplander et al., 2012).

The therapeutic potential of NO-based technologies is quite promising since exogenous sources of NO can elicit similar biological processes to locally-generated NO and its metabolites. Modulating the bioavailability of NO on the surface of skin locally to elicit anti-microbial effects or systemically to induce changes in blood pressure not only can be achieved using abiotic approaches mentioned above, but it is also possible to generate NO on the skin surface using microorganisms. Topical application of ammonia-oxidising bacteria (AOB) have emerged as a cutaneous approach to restore the microbiota and as a biological tool to modify the bioavailability of NO and its metabolites on the surface of human skin.

1.5 Ammonia-oxidising bacteria (AOB)

AOB consist of a group of aerobic, gram-negative aerobes that belong to the *Proteobacteria* phylum (Koops et al., 1991). AOB can be classified as obligate chemolithoautotrophs, aerobically oxidising ammonia to nitrite as their unique means of energy and power reduction to alter carbon (e.g. from carbon dioxide, carbonates,

bicarbonates) by the Calvin cycle (Lees, 1952, Wallace and Nicholas, 1969, Koops et al., 1991, Arp and Stein, 2003, Chain et al., 2003). Having first been isolated by Winogradsky in the nineteenth century, biological ammonia oxidation has been acknowledged as largely being carried out by AOB species (Monteiro et al., 2014). It has been suggested that this forms part of the cutaneous flora during human development (Rook, 2009) (Rook, 2010). In addition, other environments have been noted, namely, anoxic, freshwater and marine habitats (Kowalchuk and Stephen, 2001, Lees, 1952, Dai et al., 2015, Güven and Schmidt, 2009).

The naturally-occurring, non-pathogenic AOB *Nitrosomonas eutropha* D23 (AO+ MistTM, MotherDirtTM) has been engineered by a United States company called AOBiome LLC as a cosmetic product (Mother Dirt) for topical administration in the form of a spray (Gryllos et al., 2014a, Gryllos et al., 2014b). The objective of this product is the restoration of natural microbial balance diminished by modern hygiene practises. Therapeutic interventions through continuing clinical trials have aided research into inflammatory skin diseases; for instance, atopic dermatitis, acne, rosacea and keratosis pilaris. By utilising such treatments, which contain ammonia-oxidising bacteria that release NO, it is possible to investigate the effect of NO on the microbial diversity of the skin.

1.5.1 Therapeutic use of live bacteria (host-microbiome interactions)

Antimicrobial therapies constitute a fundamental pillar of practices in dermatology. However, they involve risks, which are not fully understood. The latest research on gut microbial communities has indicated that treatment with ciprofloxacin led to a substantial and rapid decline of microbial diversity. Part of the microbial community started to regain strength within one week of finishing the treatment, while other microbes did not. After ten months, the gut had not been wholly restored to its baseline composition, which suggests that antibiotic perturbation can alter the community if these conditions persist (Dethlefsen and Relman, 2011). For instance, on pre-treating mice with antibiotics, it was clear that vancomycin-resistant enterococci (VRE) displaced the normal recovering commensal organisms. Likewise, in the gut of transplant patients, colonization by VRE came before bloodstream infection (Ubeda et al., 2010). These results have further implications for skin biology; namely, exploration of the effect of topical, repeated or continual usage of antibiotics on microbial community strength and the deleterious effects on useful microbes.

As antibiotic resistance increases, due to the wide range of antimicrobial treatments available to combat possible pathogens, it is crucial to investigate antimicrobial chemicals derived from microorganisms, as well as the potential of probiotics and probiotic microbial organisms as feasible alternative therapies (Kong and Segre, 2012). Contradictorily, society endeavours to promote healthy gut bacteria with probiotic yogurts, while at the same time using hand sanitizers to sterilize our skin. It may be necessary to develop emollients or other bacteria-related products to assist the growth of commensals. As evidence on skin microbiota

becomes more robust, such questions will lead future agendas for investigation into a greater understanding of the complex interactions, which regulate host-microorganism relationships (Myles et al., 2018).

1.6 Hypothesis

Skin microbiota dysbiosis is a significant factor associated with skin disorders like acne and atopic dermatitis with the presence and/or abundance of pathobionts such as Staphylococcus aureus linked to disease severity (Kong et al., 2012).

Modern living habits, more frequent washing, and cleaning, with more time spent indoors, have altered our microbiomes. It is possible that skin was colonized by different microbes and possibly ammonia-oxidizing bacteria (AOB)(Clemente et al., 2015) before the invention of modern soaps and detergents. AOB are ubiquitous in soil, but they are extremely sensitive to detergents (Whitlock and Feelisch, 2009). The high concentrations of ammonia found in human sweat can be converted into nitrite and nitric oxide by AOB.

Nitrite is a key component of the pathway to NO formation by further reduction at the epithelial surface. Nitric oxide (NO) has recently been shown to be a key regulator of biofilm function in respiratory mucosa, and has been shown to regulate the microbiome at epithelial surfaces. This source of nitrite may be biologically significant as these molecules are absorbed rapidly and efficiently via the skin. However, the precise influence on the established blood–saliva–stomach–blood cycle of nitrate/nitrite/NO is not known (Lundberg, 2012).

Recently Nitrosomonas eutropha, an AOB, has become commercially available as a spray for skin application which allows us to test these possibilities.

The main aim of my project is to study the impact on the skin of Nitrosomonas eutropha D23. We aimed to study this by applying Nitrosomonas eutropha, and measuring biological functions of the skin including barrier function, and the impact on the skin microbiome.

- NO metabolism at the epidermal surface of the skin regulates microbial diversity and influences quantitative measures of species making up the skin microbiome.

1.7 Aims and objectives

The aims of this project are to:

1. Characterise the microbial colonization in healthy skin pre and post application of AOB
2. Discover the role of NO production in variable regions of human skin.
3. To study the molecular mechanisms associated with inflammatory skin conditionsns and to categorise novel methods of intervention.
4. To study circulatory markers related to NO metabolism, oxidative stress and redox thiol metabolome. These markers are used as a proxy to evaluate the production of NO in a biological sample pre and post application of *N. eutropha* D23 on the skin.

Chapter 2 Materials and methods

2.1 Ethics

Initial design of the procedure was conducted prior to seeking ethical approval. Permission to proceed was then given by the Northern Ireland regional ethics committee (project ID 185688, reference 15/NI/0180) (Appendix A). Ethical approval was also granted by the Local University of Southampton (ERGO ID 17473)), University Hospital Southampton NHS Foundation Trust (R&D CRI 0320) (Appendix B) and Southampton Centre for Biomedical and Clinical Research. Certification of Good Clinical Practice (GCP) in addition to the relevant skills training, such as use of anthropometric devices, were obtained as required before commencing the data collection phase.

2.2 Enrolment of participants

For this study, healthy adult participants (n=23) from the British population were recruited. The exclusion criteria included (a) diagnosed and active/ongoing skin disease at the time of recruitment, (b) antibiotic course (topical or oral), (c) pregnancy, and (d) cardiovascular problems. Participants were enrolled upon receiving written and informed consent to participate willingly in this study (Appendix C and E), following guidelines of the Helsinki Declaration. During the experiment the room temperature was maintained at 21°C with Active ventilation system. The complete dataset was gained through the two visits made by each volunteer to the clinical research unit. The total visiting time was limited to three hours and both visits were exactly two weeks apart (14 days). Visit one, hereafter, will often be referred to as “baseline visit” and visit two as “the return visit”. During the baseline visit, height and weight were recorded and measurements were taken using calibrated devices (Appendix F). Volunteers completed an approved shortened version of the ‘skin phenotype’ questionnaire (Appendix G). A stethoscope and sphygmomanometer were also used to record blood pressure.

Skin samples were collected from four healthy volunteers, at their baseline visit to compare sampling methodology matrixed with two 16S rRNA hypervariable regions, V1-3 and V3-4 in chapter 3. Fifteen samples were taken from each participant (three samples per method from each volunteer). All samples were obtained from the same region (fossa inner elbow). Surface areas were disinfected with 70% ethanol prior to commencing. Sterile gloves were worn during sample collection.

2.3 Skin microbiome

2.3.1 Sample collection procedure

The diverse sampling methods are detailed below along with the sample processing techniques. Skin samples were obtained from inner-elbow of four healthy volunteers. Prior to proceeding with experiments, surface areas were disinfected with 70% ethanol, which kills bacteria via protein denaturation and dissolving the lipid membrane. Sterile gloves were worn during sample collection.

2.3.1.1 Swab

Tubed sterile dry swab tips soaked in 0.15 NaCl+0.1Tween20 were utilized. For 30 seconds, the swab was turned back and forth on the 4cm² surface. The extraction process was then started by placing the tips into PowerBead Tubes from a PowerSoil DNA Isolation Kit (#12888-100, Qiagen, UK).

2.3.1.2 Scrape

A 4cm2 area of skin adjacent to the swabbed region was scraped with a no. 15 scalpel blade gently with the blade horizontal. Materials were collected from the blade using tubed sterile dry swab tips, coated in 0.15 M NaCl+0.1 Tween20. The extraction process was started in the same way by placing the sample into PowerBead Tubes from the PowerSoil DNA Isolation Kit.

2.3.1.3 Swab over scrape

The swab technique process was repeated over the same area that was previously scraped with a scalpel as described above and for the same period of time (30 seconds). The extraction process was then initiated by placing the samples into PowerBead Tubes.

2.3.1.4 Tape Stripping

Skin sampling tape (#D-100, non-stick D-Squame, CuDerm Corporation) was sterilised with 70% ethanol. Only the non-adhesive white portion of the tape was handled by removing one 22mm section of the adhesive tape disc. Prior to sample collection, a minimum of one unused tape disc was collected as a negative control to account for any background contamination.

Tape stripping was completed by first disinfecting the gloves with 70% ethanol prior to contact with the subject. The sample was obtained by placing a tape disc on the surface of the skin and applying even pressure by pressing down with the thumb.

The disc was then peeled off the skin and the same single tape disc was reapplied to the selected skin sampling location and pressure was reapplied. These steps (from disinfecting the gloves to applying tape disc

with pressure to the selected skin region) were subject to repetition for approximately two minutes; this allowed for roughly 50 reapplications to the same skin surface area. Thus, the tape was saturated with matter from the skin surface, which includes the entire microbiome and skin corneocytes. The tape was sealed by folding the adhesive sides carefully to avoid adhesion to the PowerBead Tubes. Forceps sterilised with 70% ethanol were then used to place the tape into the PowerBead Tubes. The samples collected from participants in the PowerBead tubes were placed in processed immediately for genomic DNA extraction. All samples were prepared for multiplex PCR and 16s ribosomal RNA analysis to detect the complete range of bacteria found in a culture independent semi-quantitative manner as described below.

2.3.2 Genomic DNA extraction

Genomic DNA was extracted using the PowerSoil DNA Isolation kit (#12888-100, Qiagen, UK) by following the manufacturer's instructions. In a biosafety cabinet, the sampling discs were transferred into Lysing Matrix E tubes (MP Biomedicals, #116914050) using a pair of clean forceps. A volume of 500 µL of ATL lysis Buffer (Qiagen, #19076) was then added. Samples were bead-beat with a FastPrep-24 Instrument (MP Biomedicals) at a speed of 6.0m/s for 40 seconds. Next, samples were centrifuged at a maximum speed for 5 minutes. In the biosafety cabinet, 2ml sample tubes from the EZ1 DNA Tissue Kit (#953034, Qiagen) were labelled and 10µL of Proteinase K was added. A total of 200µL of the supernatant was transferred into the respective tubes. Samples were then vortexed for 10 seconds and incubated at 56°C for 15 minutes. During this time, the EZ1 Advanced XL instrument was setup according to manufacturer's instructions and 100µL of DNA was eluted. The DNA was later quantified (as described below) and a total of 50µL of the eluent was used for the library preparation.

2.3.3 16S ribosomal RNA gene PCR

PCR to amplify the 16S rRNA gene was completed using Illumina adapters, which are chemically synthesised, single or double-stranded oligonucleotides that can be ligated to the ends of DNA or RNA molecules. The construction of Illumina libraries is usually completed by ligating adapters to short fragments of DNA ranging from 100 to 1000 base pairs.

2.3.3.1 Amplification of 16S rRNA Hypervariable Regions

Amplification of the 16S rRNA gene was initiated using primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 534R (5'-GTGCCAGCAGCCGCGGTAA-3') for (V1-3) and 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and (5'-GGACTACCGGGGTATCT-3') for (V3-4) (Baker, Smith and Cowan 2003) targeting variable regions 1, 2 and 3 (V1–3) and 3, 4 (V3-4) (Sigma, UK).

2.3.3.2 16S rRNA PCR Cycling Conditions

Amplification of the 16S rRNA gene was completed using the primers mentioned above with a GeneAmp® PCR System 9700 (Applied Biosystems, UK). The following conditions were used: 3 minutes of denaturation at 98°C, 35 cycles at 98°C for 10 seconds, 55°C of annealing for 30 seconds, 30 seconds of extension at 72°C, followed by a final extension at 72°C for 5 minutes then 4°C holding temperature. Each sample was amplified in triplicate 25 µl reactions consisting of primers at final concentrations of 1X PCR reagent (Q5® Hot Start High-Fidelity 2X Master Mix, #M0492L, New England BioLabs, UK). Triplicate PCR reactions for each sample were then pooled and purified using the Agencourt AMPure XP beads (#A63881, Beckman Coulter, UK).

The purification was followed by PCR with 5µl Illumina sequencing adapters per sample (adapter 1 read ACTGTCTCTTATACACATCT and adapter 2 read AGATGTGTATAAGAGACAG). This step attaches dual indices and Illumina sequencing adapters using the Nextera XT Index Kit (#FC-131-1002, Illumina). The system was set to the following conditions: 3 minutes of denaturation at 98°C, 12 cycles at 98°C for 30 seconds, 55°C of annealing for 30 seconds, 30 seconds of extension at 72°C, followed by a final extension at 72°C for 5 minutes then 4°C holding temperature. This PCR amplification was followed by another purification using the same conditions. The number of PCR cycles was kept as low as possible to reduce amplification bias, and reduce the risk of amplifying contaminants present in kit reagents.

2.3.4 Amplicon purification with SPRI clean-up

The size of the fragments eluted from the bound beads can be determined by the concentration of polyethylene glycol (PEG). Solid Phase Reversible Immobilisation beads (SPRI) to DNA ratio of 1 would be produced with 50µl DNA added to 50µl of beads. The length of bound fragments and/or fragments remaining in solution is also altered as this ratio is adjusted. A lower ratio of SPRI:DNA results in a greater proportion of larger fragments when eluted. Different size ranges can be obtained from one sample with multiple purifications as we usually discard smaller fragments held in the buffer. Different size ranges can be obtained from a single sample with multiple purifications but smaller fragments retained in the buffer are usually discarded. This is partly due to the DNA fragment sizes affecting the total charge per molecule (i.e. large DNAs have larger chargers) and thus promoting their electrostatic interaction with the beads and resulting in the displacement of the smaller DNA fragments (Hadfield, 2012).

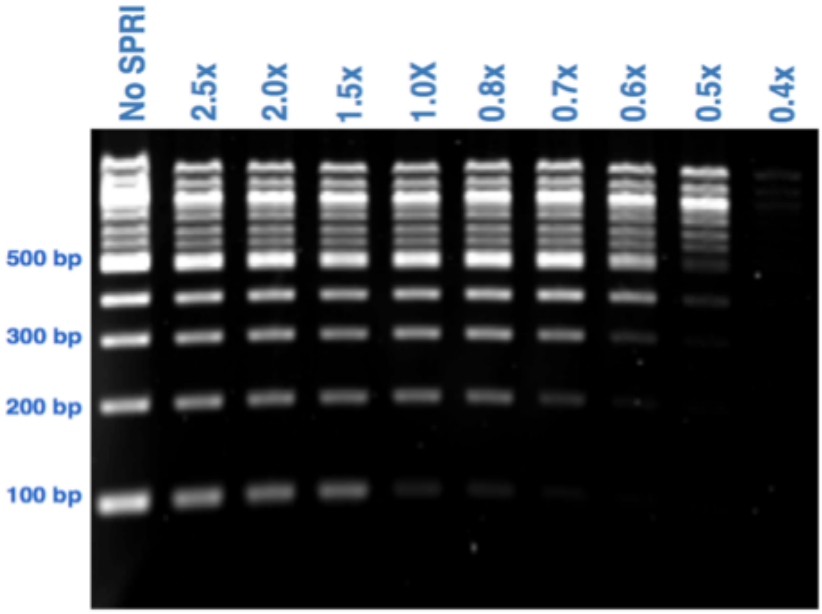


Figure 2.1 SPRI ratio impact on DNA size. The length of fragments that SPRI binds can be controlled by varying the amount that is added to the reaction. In this illustration 'x' refers to the volume to volume ration of SPRI to DNA (e.g. 1.0x SPRI is a 1:1 volume of SPRI:DNA). When x is lower, the size cut off becomes higher. Image taken from (Hadfield, 2012).

2.3.5 Library quantification

The DNA was quantified using a Qubit double stranded DNA (High Sensitivity) Assay Kit (#Q32866, Life Technologies) with the Qubit 2.0 Fluorometer (Life Technologies Ltd). Room temperature was controlled at 22°C as the Qubit® dsDNA HS assays are designed to be performed at room temperature between 22 to 28°C. A total of 1 µl DNA was added to the reagents provided in the kit and the concentration was measured using the Qubit fluorometer at 485/530 nm as indicated by the manufacturer's protocol. This assay tolerates common contaminants, such as salts, free nucleotides, solvents, detergents and proteins well.

2.3.6 Library preparation

The DNA sample pool (2nM) was denatured using 0.2N NaOH, diluted to 10pM, and combined with 25% denatured 4nM PhiX as outlined in the Illumina protocol. Samples were sequenced on the MiSeq sequencing platform in the Wessex Investigational Sciences Hub, Genomics Facility at the University Hospital Southampton NHS Trust using a 2 x 300 cycle V3 kit, following standard Illumina sequencing protocols.

2.3.7 Bioinformatics analysis

2.3.7.1 Quality filtering, OTU picking and taxonomic assignment

Amplicons were initially processed using the Quantitative Insights in Microbial Ecology (QIIME2, v2019.1) pipeline (Bolyen et al., 2019). Following trimming of forward reads to Xbp and reverse reads to Xbp, reads were dereplicated, amplicon sequence variants (ASVs) identified and chimeras removed using dada2 v1.13.0. Taxonomy was assigned using the Greengenes database v.13.8. The R package qiime2R (https://github.com/jbisanz/qiime2R) was used to merge QIIME2 artefacts that could then be processed in Phyloseq v1.29.0 in R v3.6.0 using RStudio. Figures were produced using the package ggplot2.

2.3.7.2 Alpha and beta diversity

Observed species, Chao1, Shannon index and Simpsons diversity were computed in Phyloseq. Beta Diversity was determined using the weighted and unweighted UniFrac distance metric (Lozupone and Knight, 2005), and visualised using principal co-ordinates analysis (PCoA).

2.4 Nitric oxide

2.4.1 Measuring skin surface area

The surface area of participants' hand (n = 16) was calculated by tracing their hand onto a piece of paper and inserting the measurements in the formula below. The formula for hand surface area (HSA) described previously by other researchers (Hsu and Yu, 2010) based on three-dimensional (3D) scan data.

HAS = 2.48 * (hand length) * (hand breadth)

2.4.2 Determination of skin pH

A skin pH meter (pH 905, Courage – Khazaka Electronic, Köln, Germany) was used to determine skin pH in four areas of interest including: the foot, hand, head and inner elbow. Three recordings were taken per area. The pH electrode was stored in a potassium chloride maintenance buffer and was rinsed with 0.9% sodium chloride and carefully dried using a KimWipe in preparation for each visit.

2.4.3 Trans-epidermal water loss (TEWL)

Trans-epidermal water loss recordings were taken using Tewameter® TM 300 (Courage – Khazaka Electronic, Köln, Germany) by following the operating conditions (RT: 10-40° C and RH: 30-70 % R) in the manufacturer's instructions. The reading accuracy of the probes has been verified using probe calibration function 30 mins

prior to each visit. This was repeated in all four regions of interest including: the head, hand, inner elbow and foot. 10-12 recordings were taken at each location.

2.4.4 Saliva samples

Saliva samples from volunteers were collected using SalivaBio (Salimetrics Europe, Newmarket UK) oral swabs (10 x 30mm) by following manufacturer's instructions. Samples were first centrifuged at 800 x g for 15 minutes. Next, samples were aliquoted into rinsed Eppendorf™ vials and stored at -20°C until further analysis.

2.4.5 Detection of NO emanations

The release of nitric oxide (NO) from the skin of study subjects was recorded by a procedure first established in the Feelisch laboratory as part of an MMedSc thesis in 2016 (Fiona Hares; MEDI6104, University of Southampton). A gas-phase chemiluminescence detector (CLD 88sp; ECO MEDICS AG CLD 88, Duernten, Switzerland) was used along with recording frequency set at 4 Hz for EDAQ PowerChrom™ (ADInstruments GmBH, Spechbach, Germany) software in order to obtain graphical recordings of NO emanations from the four areas sampled. CLD 88 110ml/min sampling tube was joined via a 0.22 □m filter to an adapted transparent 50-micron Mylar™ bag, which was 25m x 38cm in size. An additional bag was utilized for arm, hand and foot areas (Bacofoil Flavour Seal™ Roasting Bag Medium). Prior to the participant's arrival, filters were secured manually to cover the bag surface. In order to limit internal air volume, it was protected with Bacofoil Flavour Seal™ Roasting Bag (in medium) and secured with micropore tape. The head region was contained with a transparent plastic elasticated shower cap covered with the same plastic bag used for the other region. The DENOX 88 (ECO MEDICS AG, Duernten, Germany) facilitated NO-free room air, which was generated in the distal side of the bag by means of a filter tube. A summary of this has been provided in figure 4.1.

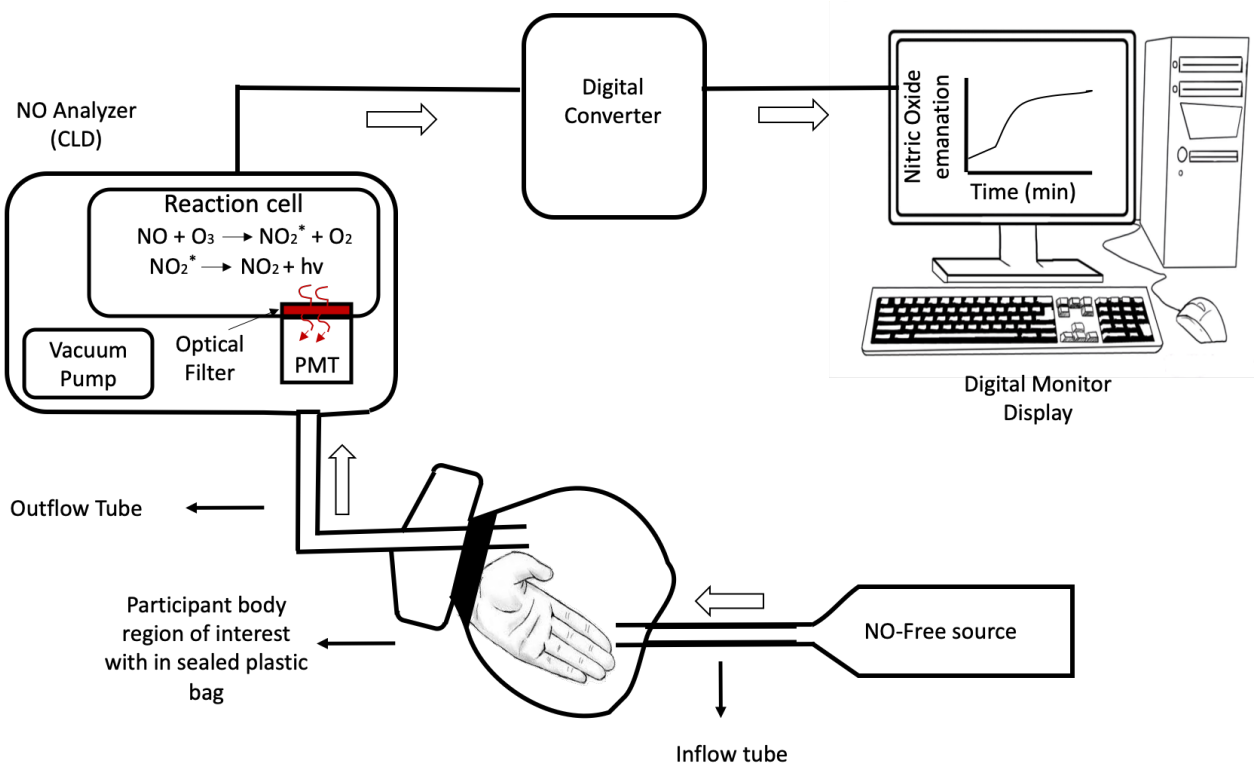


Figure 2.2 Schematic diagram of NO emanations in situ recording device and chemiluminescence detection (CLD). The participant's limb or other region of interest was enclosed with a plastic bag. A gaseous NO-free source was attached via a filter from one end, and a chemiluminescence detector from another. The CLD was attached to an analogue/digital converter, which was in turn connected to a digital monitor display.

2.4.6 Recording NO emanations

A 30-second sampling of room NO was conducted prior to the attachment of an in-built bag filter. In the first established procedure by Hares, 2016, The duration of the total NO detected was for 2 - 20 minutes per region. When the graph showed a plateau, the mean maximum voltage (V MM) were calculated. the baseline room value (V room) subtracted from over the two-minute period prior to the end point. In this study the duration of the NO recordings was controlled as a part of methodological optimisation, After the plateau graph indicated that twenty minutes had elapsed, the recorded data for all body areas examined was complete. Undercurve area of 16 minutes NO recording were integrated using PowerChrom® (eDAQ Pty Ltd, Australia) software and concentrations extracted from a NO calibration curve, where known concentrations of NO were generated by nitrite reduction (Tri-iodide mixture solution, under acidic conditions)(Feelisch et al., 2002). In the case of no rise being observed after fifteen minutes, it was considered that the NO emanations were lower than the ECO MEDICS ANALYZER CLD 88 sensitivity threshold and were consequently attributed with 0.05nmol.min^{-1} as an arbitrary value.

2.4.7 Chemiluminescence detection (CLD)

Chemiluminescence detection (CLD) is a technique that allows the detection of gaseous NO and is based on the reaction between ozone (O_3) and NO. As shown in the formulas below, nitrogen dioxide (NO_2^-) is primarily and temporarily in an excited state when generated; its relaxation to the ground state is accompanied by an emission of light, which can be measured by a photomultiplier tube within the detector. The intensity of the light emitted is proportional to the concentration of NO.

$$NO + O_3 \longrightarrow NO_2^* + O_2$$

$$NO_2^* \longrightarrow NO_2 + hv$$

2.4.8 Sweat collection and processing

It was requested that participants engage in ten minutes of moderate cardiovascular activity. This involved the use of a 19cm exercise step (Ultra Sport Europe, Derby, UK). A nylon sweat top (SWEATSUITS, Bristol, UK) was also worn by participants whilst exercising. Monitoring of the participants was ongoing during the exercise and medical aid was on-hand as well as telephone access if necessary. After the exercise was completed, participants removed their nylon sweat suit and a post-exercise value was obtained by taking a second recording of NO emanations from their hand. SalivaBio oral swabs (10 x 30mm) Salimetrics Europe, Newmarket UK), which had been pre-weighed, were used to collect sweat from participants' wrists. Using scissors sterilised with 70% ethanol, the swabs were cut in half lengthwise and securely placed onto both wrists with Parafilm® M. These were immediately removed after the exercise to be stored in airtight SalivaBio tubes (Salimetrics Europe, Newmarket, UK). Prior to 15 minutes of centrifugation at 800 x g, the swabs were reweighed *in situ* and rinsed using phosphate buffered saline with a volume to weight ratio of 3:1. Subsequently, centrifugation was completed with one hour of collection. The liquid from the bottom half of the tube was removed and aliquoted into rinsed vials (Eppendorf™). These vials were then stored at -20°C.

2.4.9 Blood collection and processing

A total of 10ml of venous blood was collected using EDTA containing BD vacutainer blood collection tubes (BD Vacutainer®, BD). One half of the samples were immediately treated with (v/v) N-ethylmaleimide (NEM) in buffer and the other half remained untreated prior to being centrifuged at 800 x g for 15 minutes before being aliquoted into rinsed Eppendorf cryo-vials and snap frozen with liquid nitrogen and stored at -20 °C until further analysis.

2.4.10 Biomarker analysis

2.4.10.1 NO metabolism markers

2.4.10.1.1 NOx

NOx (i.e. NO_2^- and NO_3^-) were quantified in non-NEM treated plasma samples, using HPLC for its measurement according to local standard operating procedures, and as essentially described in sections 2.4.2.2 and 2.4.3, with minor modifications. Aliquots of plasma samples were pre-treated with methanol (1:1 ratio), immediately vortexed and allowed to stand for 15 min at room temperature, in order to precipitate plasma protein. Samples were then centrifuged at 16,100 x g, for 20 min, at 4 °C and placed in plastic vials for measurement through HPLC (Eicom, Japan). NOx concentrations were calculated by interpolation of a calibration curve using $NaNO_2$ and $NaNO_3$ as standards.

2.4.10.1.2 Plasma total nitroso species

The levels of nitroso species (RXNO) were quantified in NEM-treated plasma using a methodology based on the CLD of NO released upon reductive cleavage of nitroso species, in an acidic environment, and in the presence of a mixture of KI/I2, as previously described (Feelisch et al., 2002)

2.4.10.1.3 cyclic Guanosine 3, 5-monophosphate

Plasma levels of cGMP were quantified using an enzyme-linked immunosorbent assay (ELISA, cGMP Parameter Assay Kit, R&D Systems®, Biotechne®), according to manufacturer's instructions for the analysis of human plasma samples.

In 100 ul of plasma, cGMP is present competes with a fixed amount of cGMP called horseradish peroxidase (HRP) found on a rabbit polyclonal antibody during the incubation of 3 hours. Basing on the competitive binding technique

Prior to determine the bound enzyme activity by adding a 200 µL substrate solution to the wells incubated room temperature for 30 mins, the excess conjugate and unbound sample must be washed. The colour absorbance read is 450 nm. The concentration of cGMP in plasma samples was obtained from interpolation of the calibration curve using cGMP as standard, and a four-parameter logistic curve-fit (SoftMax® Pro, Molecular Devices).

2.4.10.2 Anti-oxidant capacity

The ferric-reducing ability of plasma (FRAP) assay was used to evaluate the total antioxidant capacity of the human samples (Benzie and Strain, 1996). This assay entails the reduction of ferric Fe^{3+} to ferrous Fe^{2+} ions at low PH 3.6 and is based on the colorimetric detection (λ = 593 nm) of a Fe^{2+}- tripyridyltriazine blue complex.

The antioxidant capacity of plasma samples was calculated by interpolation of a calibration curve, using $FeSO_4 \cdot 7H_2O$ as standard.

2.4.10.3 Lipid peroxidation

Two distinct parameters were measured in plasma for evaluating oxidative damage of lipid components, and used as markers of lipid peroxidation, which include the determination of malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE). The levels of 4-HNE in plasma samples were quantified using the 4-HNE ELISA Kit (Elabscience). The ELISA was completed following manufacturer's instructions.

Using the Competitive-ELISA principle, the kit provides micro ELISA plate that has been pre-coated with 4-HNE.A fixed amount of 4-HNE on the solid phase supporter located on the Biotinylated Detection Ab specific to 4-HNE competes with 50µL of 4-HNE in the sample or standard Incubated for 45 min at 37°C, during the reaction. Prior to adding 100 µL Avidin conjugated to Horseradish Peroxidase (HRP) to each microplate well and incubated for 30 min at 37°C, the excess conjugate and unbound sample or standard are washed from the plate. 50 µL stop solution were added to terminate the enzyme-substrate reaction. Measuring the colour change spectrophotometrically at a wavelength of 450 nm. Calibration curves were obtained using standards provided in the kits and sample concentrations were interpolated from a four-parameter logistic curve.

Plasma samples were subjected to protein precipitation and centrifuged to obtain the supernatant, which was mixed with thiobarbituric acid reactive species (TBARS) colour reagent in a 2:1 sample-to-reagent ratio. TBARS detects and quantifies a marker for oxidative stress (MDA) and polyunsaturated fatty acids. Tubes were placed in a pre-heated block, and incubated for 30 minutes at 90 °C. Tubes were then cooled down for 10 minutes at room temperature, and a 50-µL aliquot was placed in a 96-well plate to measure absorbance at 532 and 750 nm. The concentration of MDA in samples was calculated by interpolation of a calibration curve using MDA solution as a standard.

2.4.11 Thiol analysis

2.4.11.1 Total free thiols

The concentration of plasma total free thiols (TFT) was determined using Ellman's reagent by following a previously published protocol (Koning et al., 2016). The concentrations were determined using a calibration curve constructed with L-cysteine as a standard.

DTNB is compound used for quantitating free sulfhydryl groups in plasma also known as Ellman's reagent; 5,5'-dithio-bis-(2-nitrobenzoic acid) which has a highly oxidizing disulfide bond. This compound is stoichiometrically reduced by free thiols in an exchange reaction, to form a mixed disulfide and releasing one

molecule of 5-thio-2-nitrobenzoic acid. A yellow-coloured product produced as results of the interaction between a solution of this compound and sulfhydryl groups. Using a spectrophotometer, the yellow-coloured product can be quantified based on its strong absorbance at 412 nm. The standard concentrations were determined using a calibration curve constructed with L-cysteine.

2.4.11.2 Redox thiol metabolome

To analyse redox thiol metabolomes, numerous circulating metabolites were considered including cysteine (Cys), cysteine (CysSS), homocysteine (HCys), homocystine (HCysSS), N-acetylcysteine (NAC), oxidised (GSSG) and reduced glutathione (GSH), glutamylcysteine (GluCys), cysteinylglycine (CysGly), and sulfide. These were measured using ultrahigh-performance liquid chromatography in combination with electrospray-ionisation tandem mass spectrometry (UPLC-ESI-MS/MS) as previously described (Sutton et al., 2018).

The detection of these analytes was measured by electrospray-ionisation tandem mass spectrometry (LC-MS/MS). To find suitable separation conditions by ultrahigh-performance liquid chromatography (UPLC), a reaction between authentic standards of reduced thiols and excess NEM in buffered solution at pH 7.4 and mixed with disulfide standards.

2.4.12 AO+ Mist^{TM} intervention

Application of AO+ Mist^{TM} (Mother Dirt Cambridge, MA) subsequent to bathing/showering, for the two weeks between baseline and return visits, was requested of participants. All participants were given a sealed AO+ Mist^{TM}, which is a live probiotic spray for the skin formulated with a patented, live “peacekeeper” bacteria (Figure 4.2). Given that no participant had ever used AO+ Mist^{TM} before, a test application was carried out for all participants on the dorsal surface of their hands. Participants were kept for observation for 15 minutes as a safety measure in case of a systematic or local reaction. Participants were additionally given Mother Dirt Cambridge, MA cleanser and shampoo for personal use. Participants were instructed on how to apply the mist on their own and all were encouraged to use it twice a day (first thing in the morning, after showering and before sleeping to allow the bacteria maximum exposure on the skin), although manufacturer’s recommendation was to use it on the sweaty areas of the body. A log was kept with the case report form of batch numbers and date of product expiry.

Figure 2.3 AO+ Mist™, cleanser and shampoo. Image of Mother Dirt products used by participants. The products claim to result in the following changes: generate naturally healthy-looking hair (shampoo), restore good bacteria to the skin (AO+ Mist) and clean skin without removing good bacteria (cleanser). Image taken from (DIRT, 2017).

2.4.13 Return Visit and statistical analysis

Upon the return visit, each recording was repeated. Samples of plasma, red blood cell count, saliva and sweat were then subject to analysis within one month for total nitrite/nitrate (NOx) with the use of high-performance liquid chromatography (HPLC). Procedures for analysis of samples and standard methods in the laboratory are complex and have been described thoroughly elsewhere (Bryan et al., 2004). Statistical analysis was conducted using GraphPAD Prism® software (v8, GraphPad Software, Inc., La Jolla, CA, USA) A paired two-tailed t-test was used to determine any statistically significant differences between groups and results were considered significant when $p<0.05$.

Chapter 3 Comparison of sampling and 16S rRNA sequencing methods for the analysis of skin microbiomes

3.1 Introduction

The skin is the largest organ in the human body, and it is populated by a large diversity of microbes (Ferretti et al., 2017). There is increasing evidence that the skin microbiome plays a crucial role in the defence against pathogens, educating and assisting the immune system and homoeostasis (Ferretti et al., 2017, Chen and Tsao, 2013). It is also widely acknowledged that skin microbiota dysbiosis is a significant factor associated with skin disorders like acne, atopic dermatitis, psoriasis and dandruff (Kong and Segre, 2012), with the presence and/or abundance of pathobionts such as Staphylococcus aureus linked to disease severity (Kong et al., 2012). Atopic eczema in particular is a common skin condition, which may start at any age, but onset is most often in childhood (Ziyab et al., 2017). It affects ~20% of children in the UK at some stage and incidence is increasing (Taylor-Robinson et al., 2016).

Studying the skin microbiome is thus essential to better understand the microbiome-host crosstalk and to associate its specific configurations with cutaneous diseases. Several community profiling approaches have proved successful in unravelling the composition of the skin microbiome and overcoming the limitations of cultivation-based assays, but these tools remain largely inaccessible to the clinical and medical dermatology communities. The study of the skin microbiome is also characterized by specific technical challenges, such as the low amount of microbial biomass with an extensive human DNA background. In addition, the choice of sampling method has implications for accurate reconstruction of microbial community composition.

To date, pre-moistened swabbing is the most established skin sampling technique (Aagaard et al., 2013, HMP, 2012a, Huttenhower et al., 2012). Tape stripping provides a high biomass recovery but is not suitable for all body sites due to its dimensions and sample acquisition time (Chng et al., 2016, Zeeuwen et al., 2012). The biomass collected is also greater with the skin scrape sampling method. Therefore, skin scrape samples are thought to be useful for studies of lower abundance microorganisms like fungi (Grice et al., 2008, Findley et al., 2013). A further sampling technique that enables both host and microbiota DNA collection through tissue homogenization is a skin punch biopsy, which can be further modified by concomitant use of tissue selection, for example using laser-capture microscopy. However, this is an invasive procedure and reduces the ability to sample from multiple body regions in the same individual (Grice et al., 2008, Nakatsuji et al., 2013). Cup scrubbing is another sampling technique that was pioneered for culture-based studies but can also be used for microbiome studies. Previous studies have shown that swabs, tape strips and cup scrubs were highly concordant for relative abundance of bacterial taxa when analysed using 16S rRNA sequencing (Chng et al., 2016).

In addition to the choice of sample method, consideration needs to be given to the choice of molecular target. The 16S rRNA gene has been used extensively for microbiome studies (Kim and Chun, 2014) including those of the human skin (Kong, 2011). Of the nine "hypervariable regions" (V1–V9), V4 is the region used most widely in human microbiome studies. However, some authors have suggested that V1 offers the most suitable region for investigating the skin microbiome since it has been shown to better distinguish between different species of bacteria. This is particularly important in the skin with particular reference to the genus Staphylococcus, which includes known commensals (S. epidermidis) and potentially pathogenic (S. aureus) species. Distinguishing between S. epidermidis and S. aureus, and other species is important, yet there is no consensus in skin microbiome studies of which hypervariable region to study (Kong, 2016a, Meisel et al., 2016, Castelino et al., 2017).

3.1.1 Aims

The aim of this study was to compare sampling methodology matrixed with two 16S rRNA hypervariable regions, V1-3 and V3-4.

3.2 Results

Skin samples were collected from 4 male healthy volunteers using swab, scrape, post-scrape swab and tape. Samples were collected from the arm (inner-elbow) in triplicate.

After filtering, 16S rRNA amplicon sequences for v1-3 and v3-4, total frequency for both regions were calculated (2,221,627 and 771,470, respectively).

Table 3.1 Frequency per sample table (V1-3). List showing minimum frequency, 1st quartile, median frequency, 3rd quartile, maximum frequency and mean frequency.

Frequency per sample	
Minimum frequency	3
1st quartile	20,110.25
Median frequency	28,495.50
3rd quartile	37,050.00

Maximum frequency	121,731.00
Mean frequency	34,712.92

Table 3.2 Frequency per sample table (V3-4). List showing minimum frequency, 1st quartile, median frequency, 3rd quartile, maximum frequency and mean frequency.

Frequency per sample	
Minimum frequency	1
1st quartile	6,852.75
Median frequency	10,468.50
3rd quartile	16,678.75
Maximum frequency	31,786.00
Mean frequency	12,054.22

3.2.1 Alpha diversity of the skin microbiota using different sampling methods and 16S rRNA variable regions

The alpha diversity of the skin microbial community was measured using four different estimators to compare the microbial communities of different skin sampling methodology (swab, scrape, post-scrape swab and tape) matrixed with two 16S rRNA hypervariable regions, V1-3 and V3-4.

To calculate richness, Observed OTU (total number of species, amplicon sequence variants (ASVs) in this case) and Chao1 (a richness estimator for low abundance OTUs) were used. In addition, Shannon (a quantitative measure of community evenness) and the Simpson index (a measure of number and diversity of species) were used to explore the structure of the microbial community.

Overall, analysis of the V1-3 region suggested greater richness and diversity in the bacterial community than when using V3-4 (Fig 3.1).

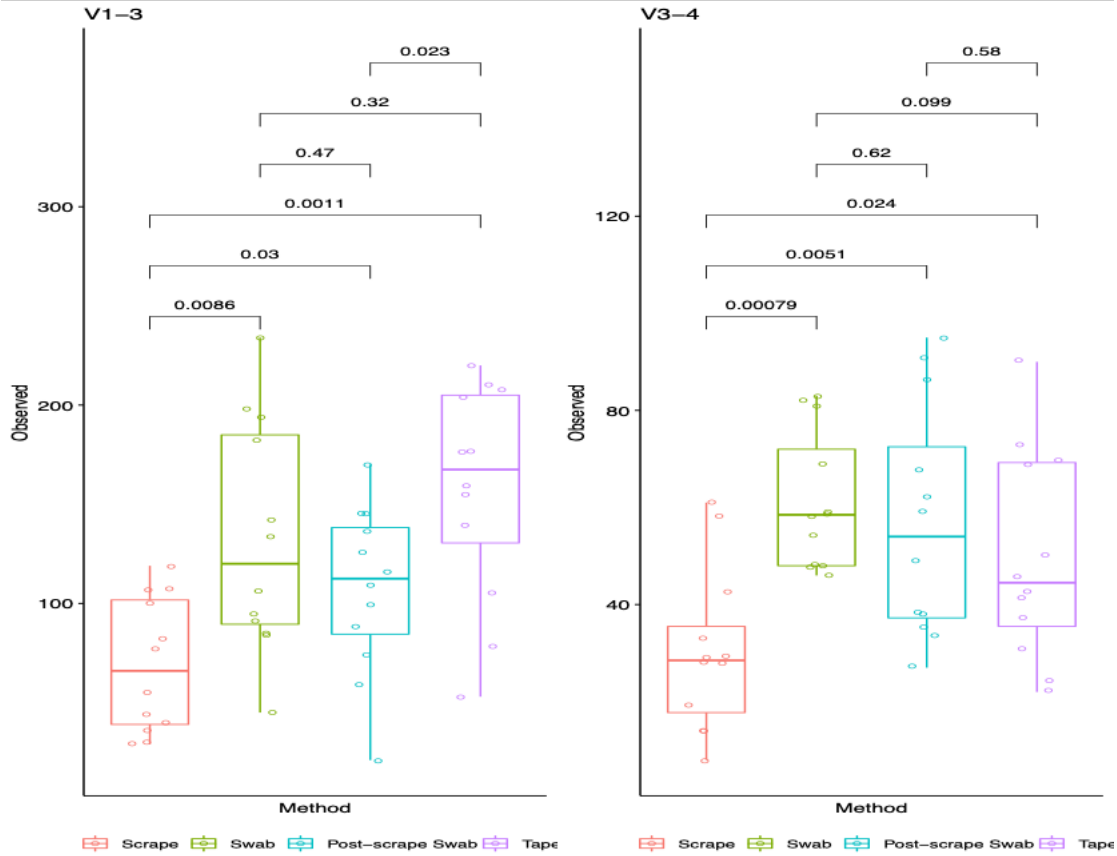
Observed OTU and Chao1 analysis of V1-3 revealed statistically significant differences in species richness or diversity. Examining the different sampling methods showed that scrape sampling gave lower estimates of

species richness compared to the other sampling methods (scrape vs. swab p= 0.0086, scrape vs. post-scrape swab p=0.03 and scrape vs. tape p= 0.0011, Kruskall-Wallis ANOVA). Similarly, using V3-4, scrape sampling also showed significantly lower richness compared to the other sampling methods (scrape vs. swab p= 0.00079, scrape vs. post-scrape swab p=0.0051 and scrape vs. tape p= 0.024, Kruskall-Wallis ANOVA). In addition, the post-scrape swab method showed a statistically significantly lower richness when compared to tape sampling method when using V1-3, however this was not observed for V3-4.

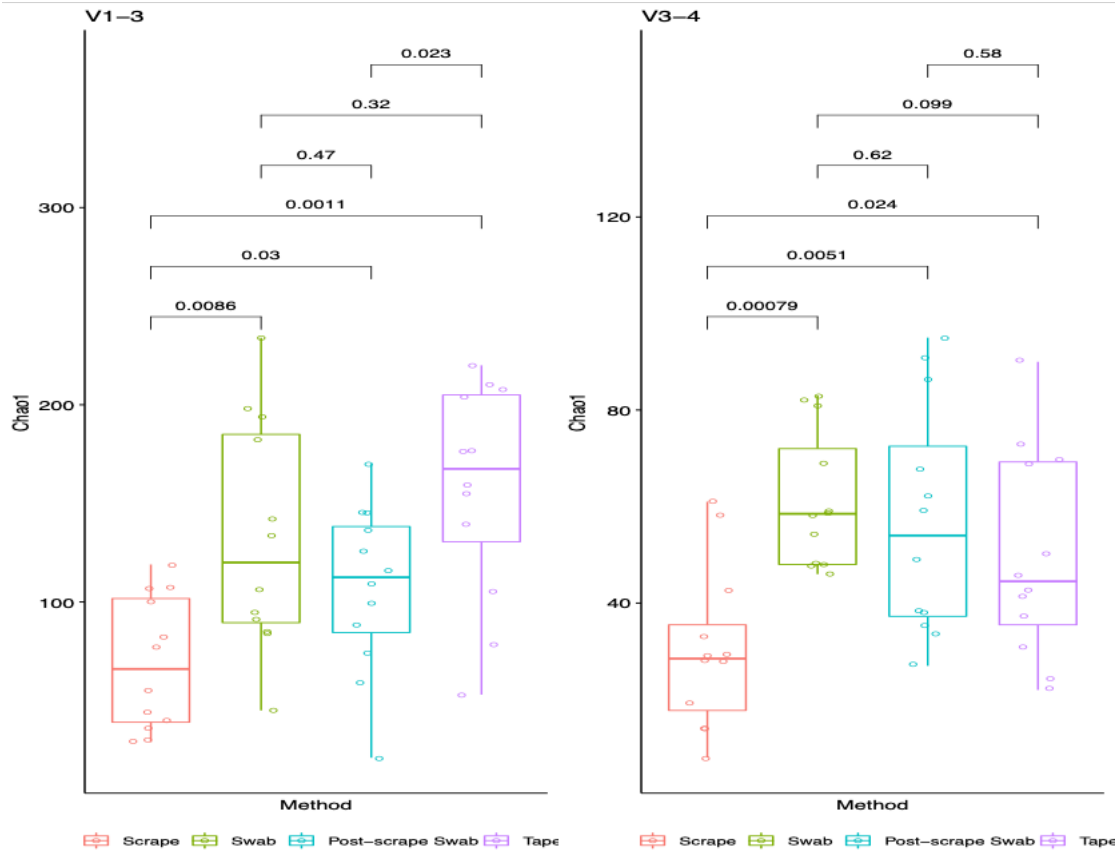
Simpson's index showed no statistically significant differences between the sampling methods using V1-3. However, the scrape sampling method was statistically significantly lower compared to both the swab and post-scrape swab methods when V3-4 was used (p= 0.0036 and 0.045, respectively).

Statistically significant differences were seen using Shannon for V1-3. The scrape method showed a less even community composition compared to swab p= 0.024. However, as was the same with Simpson and V3-4, the Shannon results from scrape samples were significantly lower than both the swab and post-scrape swab methods (p = 0.0018 and p=0.028, respectively), again suggesting a less even community composition was being recovered.

A


V1–3
0.023
0.32
0.47
0.0011
0.03
0.0086
300
200
100
Observed
Method
Scrape
Swab
Post–scrape Swab
V3–4
0.58
0.099
0.62
0.024
0.0051
0.00079
120
80
40
Observed
Method
Scrape
Swab
Post–scrape Swab

B


V1–3
0.023
0.32
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0.03
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Chao1
Method
Scrape
Swab
Post–scrape Swab
Tape
V3–4
0.58
0.099
0.62
0.024
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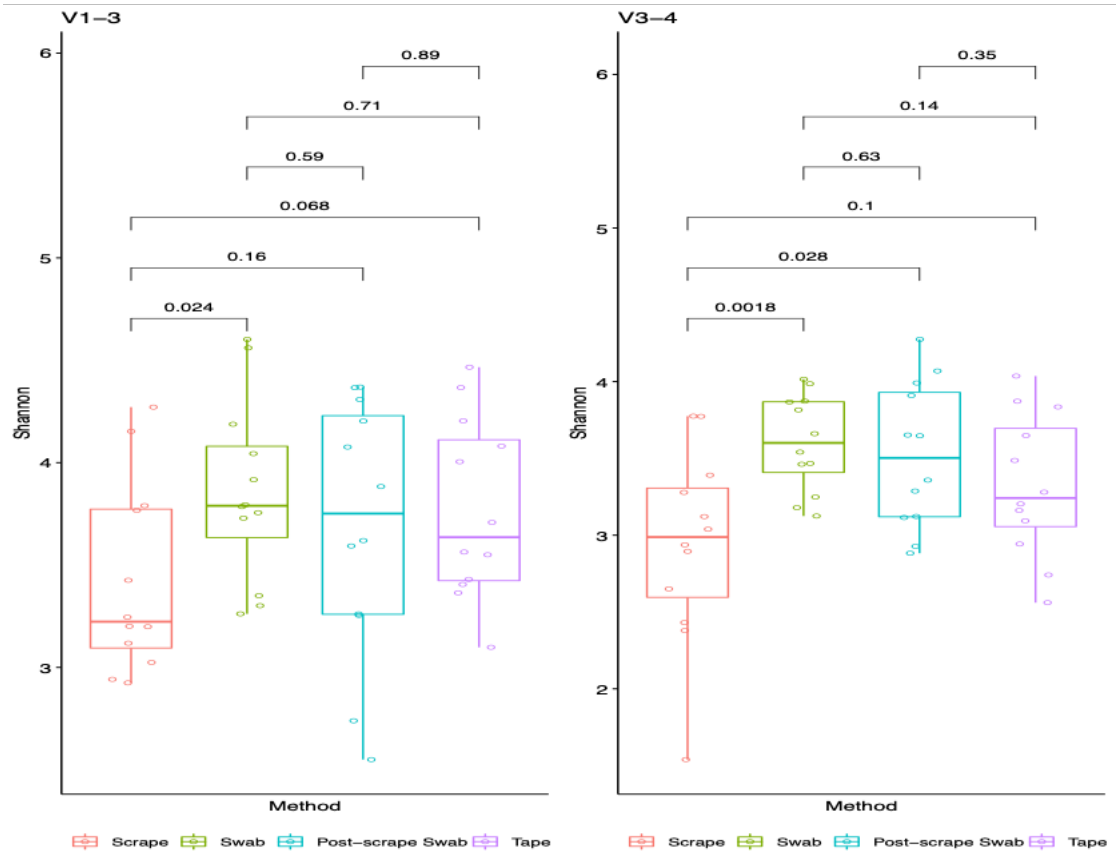
120
80
40
Chao1
Method
Scrape
Swab
Post–scrape Swab
Tape

C


V1–3
V3–4
Shannon
Method
0.89
0.71
0.59
0.068
0.16
0.024
0.35
0.14
0.63
0.1
0.028
0.0018
Scrape
Swab
Post–scrape Swab
Tape

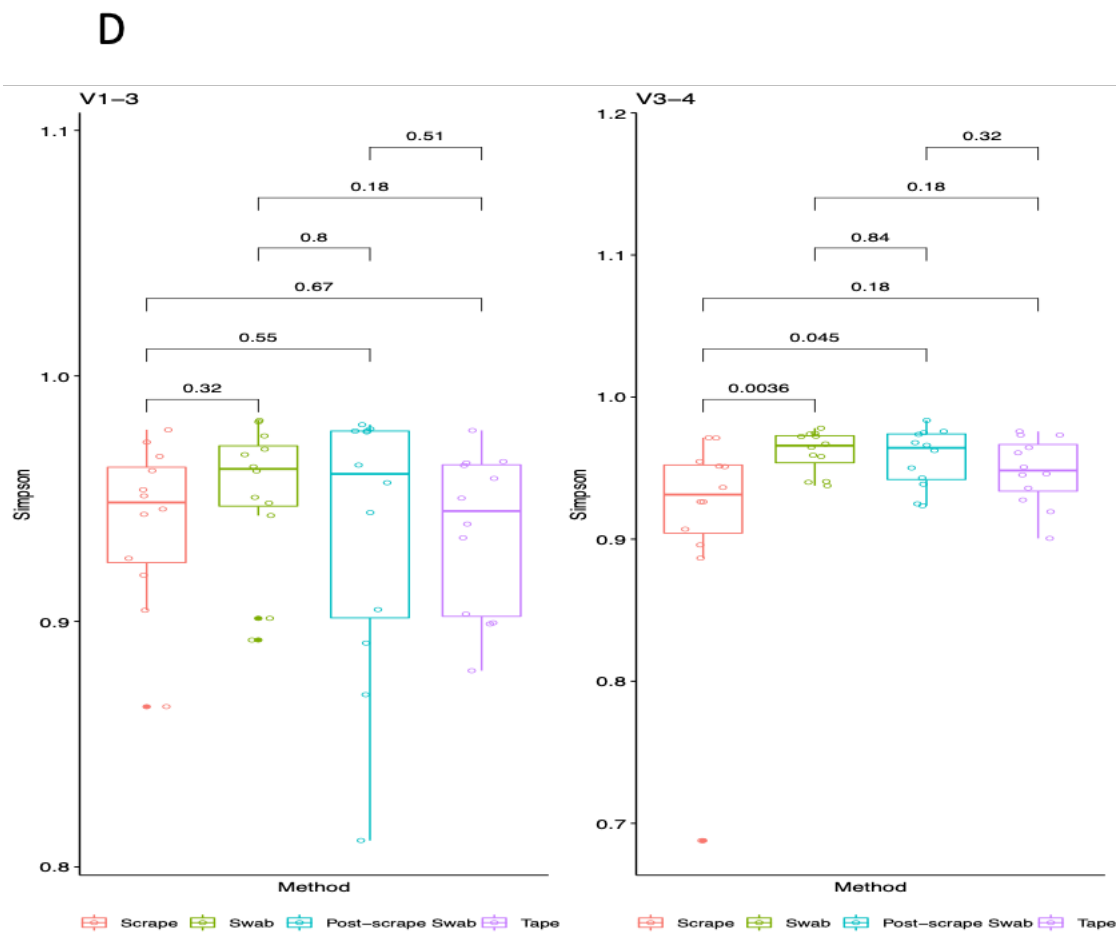


Figure 3.1 Alpha diversity comparions of four skin sampling methods. Four measures of alpha diversity were examined. These were A) Observed OTU (richness), B) Chao1 (richness), C) Simpson (diversity) and D) Shannon (evenness) and where compared using both V1-3 (left panels) and V3-4 (right panels) regions of the 16S rRNA gene. Data points are presented as each sample analysed of the triplicate independently, grouped by sampling method for four volunteers (total n=12).

3.2.2 Sample ordination of beta diversity

Bray-Curtis analysis (a quantitative measure of community dissimilarity) was used to compare the microbiota composition of skin samples (Figure 3.2). Here, tape sampling showed a community composition that was statistically significantly different to the scrape method for both V1-3 and V3-4, p=0.026 and p=0.002 respectively. No other differences were observed.

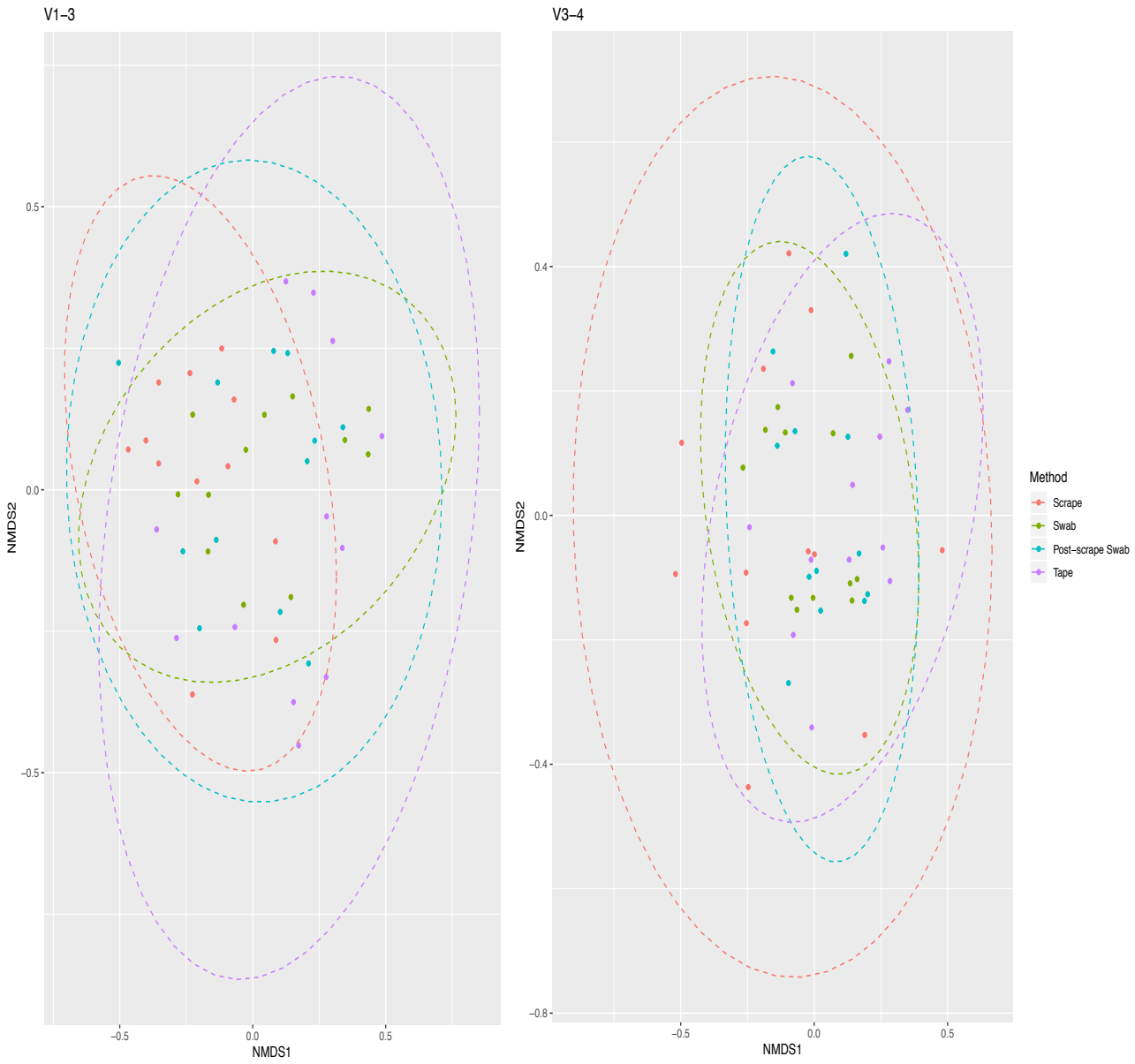


Figure 3.2 Ordination analysis, using Nonmetric Multidimensional scaling (NMDS) of Bray-Curtis distance. The effect of different sampling methods (coloured dots) are compared for each of V1-3 (left panel) and V3-4 (right panel). Ellipses show 95% CI. Data points are presented as each sample analysed of the triplicate independently, grouped by sampling method for four volunteers (total n=12).

3.2.3 Taxonomy identification of V1-3 and V3-4.

Class-level taxonomy classifications for each of V1-3 and V3-4 are shown in Figures 3.3 and 3.4 respectively. Clear differences in the increased abundance of ASVs classified as Actinobacteria and those ASVs classified as Bacteria can be seen with the V3-4 analysis. Examining Phylum-level classification, once the unassigned ASVs had been removed from further analysis (Figure 3.5) highlighted this increased relative abundance of Actinobacteria. The main Phyla identified were similar for both hypervariable regions and included Actinobacteria, Proteobacteria, Firmicutes, Bacteroidetes and Fusobacteria. The mean relative abundance and ranges for each are shown in (Table 3.3).

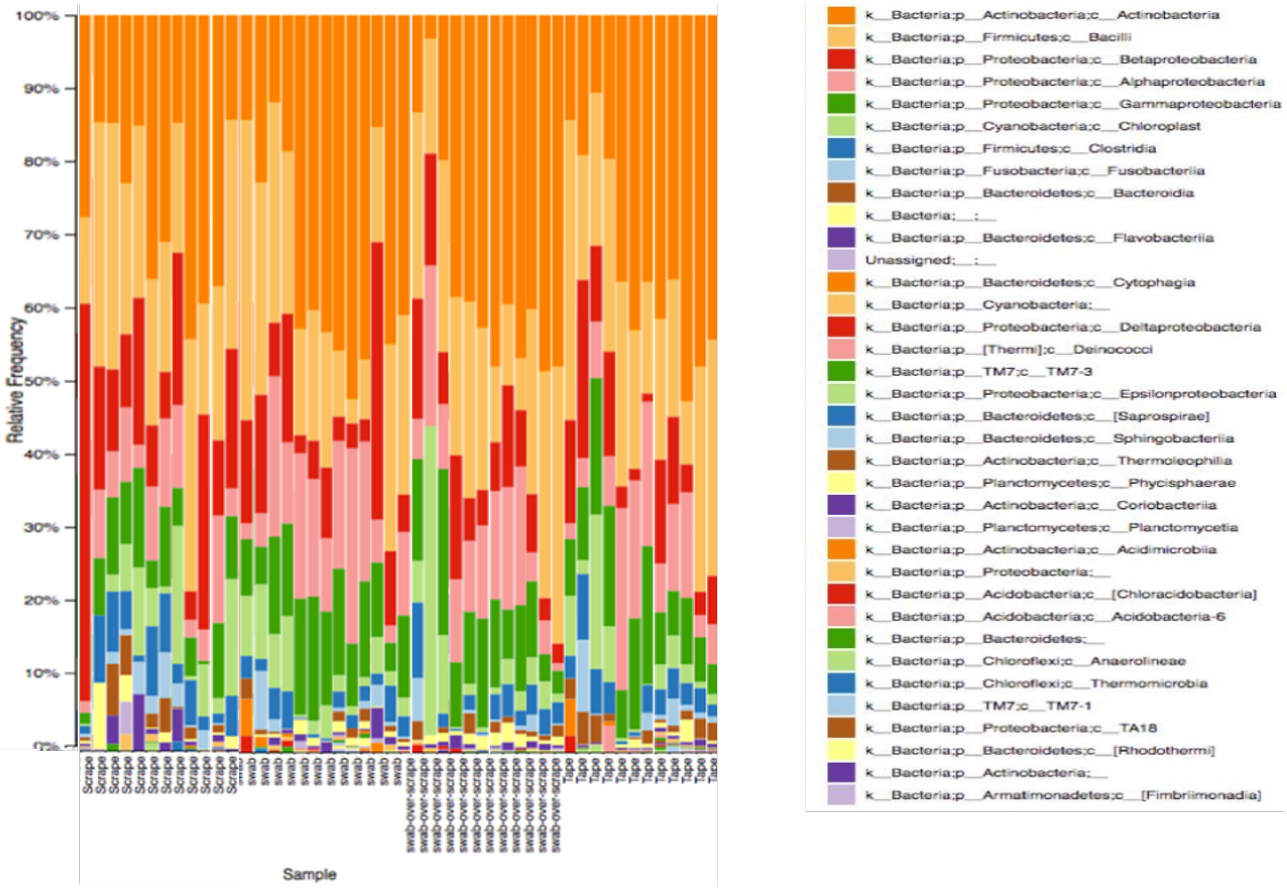


Figure 3.3 Taxonomic identification at Class-level for V1-3. Proportional bar plot showing the relative frequency of class-level groups of bacteria for each sample analysed, grouped by sampling method.

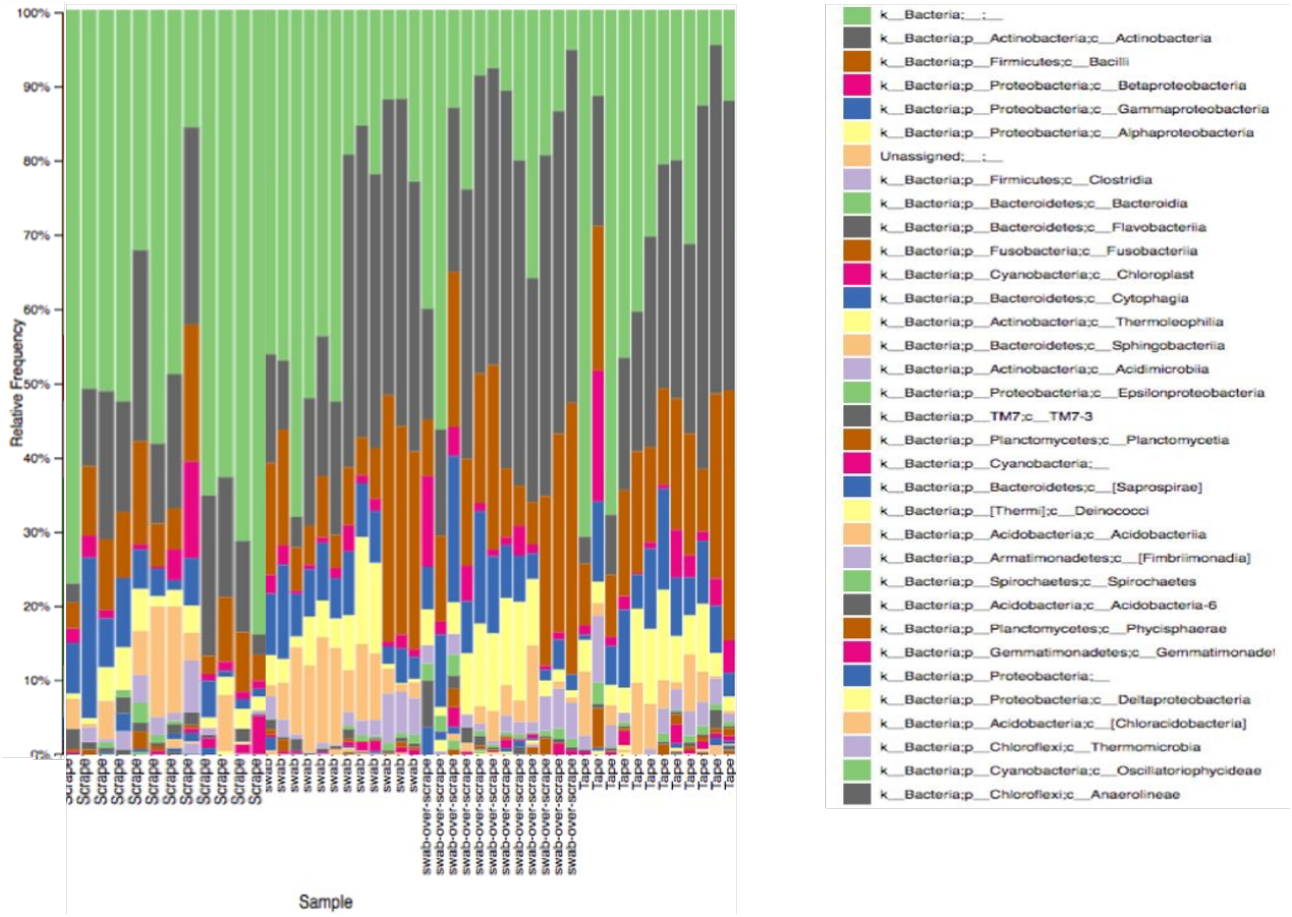


Figure 3.4 Taxonomic identification at Class-level for V3-4. Proportional bar plot showing the relative frequency of class-level groups of bacteria for each sample analysed, grouped by sampling method.

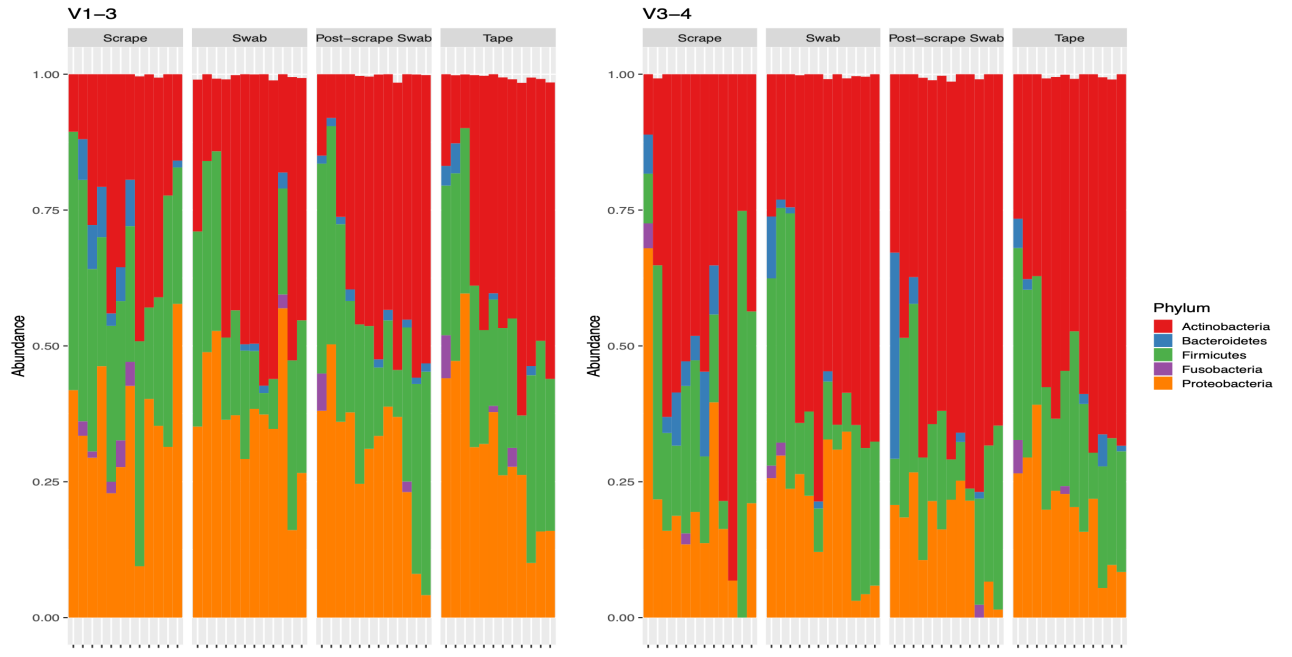


Figure 3.5 Bar plot showing the relative abundance of the dominant bacterial phyla using V1-3 (left panels) and V3-4 (right panels) across sampling methods.

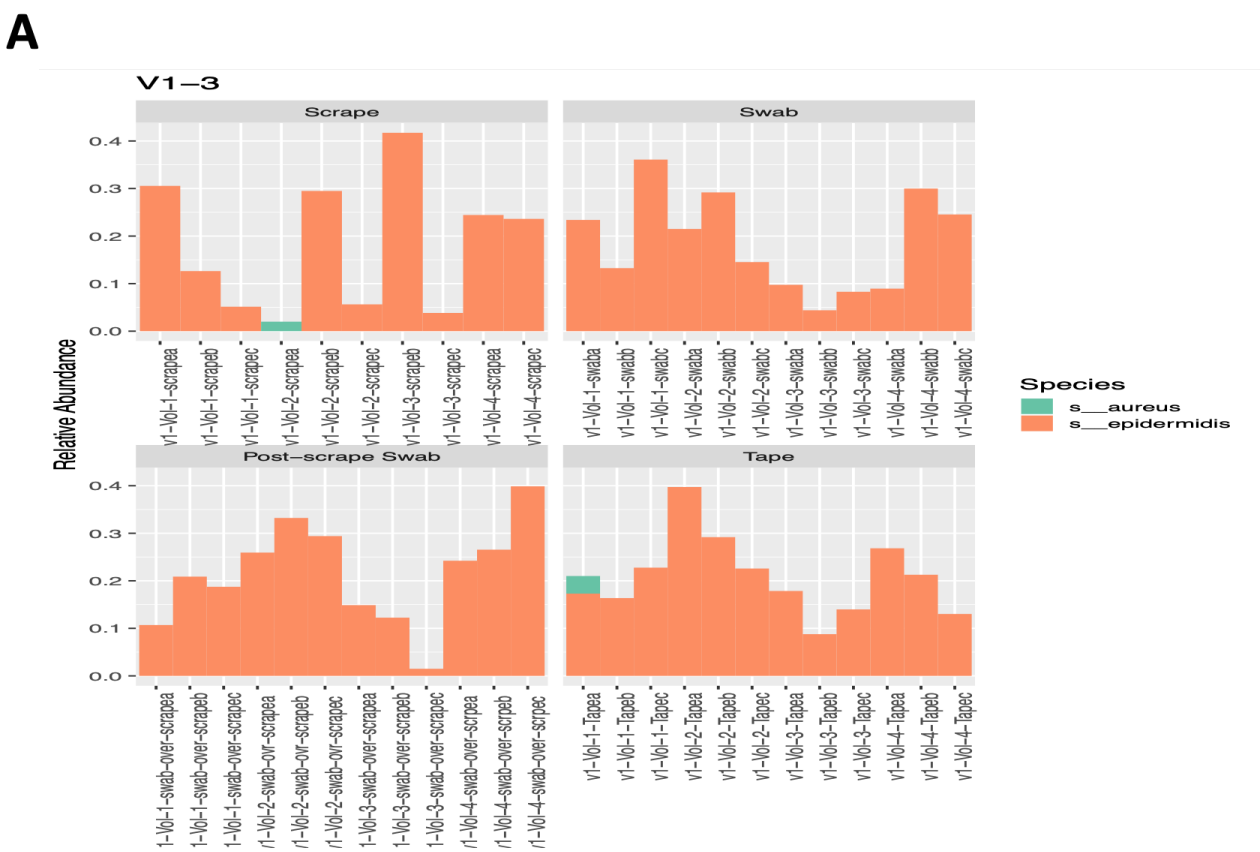
Table 3.3 Taxonomy phylum levels using different sampling methods and V1 and V4 16s rRNA regions.

	Abundance mean (Range)				
	Actinobacteria	**Bacteroidetes**	**Firmicutes**	**Proteobacteria**	**Fusobacteria**
Sampling method	**V1-3**				
Scrape	28.4% (10.6% 48.7%)	6.2% (1.3% 9.3%)	31.8% (16.8% 47.6%)	34.9% (9.5% 57.7%)	3.1% (1.2% 4.9%)
Swab	39.5% (13.4% 57.3%)	1.7% (1.2% 2.9%)	21.8% (3.9% 35.9%)	37.5% (16.1 56.9%)	2.5%
Post-scrape swab	40.3% (8.1% 55.8%)	1.6% (1.2% 2.1%)	27.4% (8.6% 41.1%)	30.2% (4.1% 50.3%)	4.4% (1.9% 6.9%)
Tape	39.4% (9.9% 61.2%)	3.0% (1.1% 5.5%)	26.8% (10.9% 35.1%)	31.2% (10.1% 37.8%)	4.2% (1.2% 7.9%)
	V3-4				
Scrape	49.9% (11.1% 93.2%)	7.7% (15.6% 3.0%)	26.0% (5.2% 74.9%)	23.2% (6.8% 68.0%)	3.3% (2.0% 4.6%)
Swab	68.3% (23.1% 78.6%)	3.4% (1.1% 11.4%)	22.4% (4.6% 50.7%)	20.9% (3.1% 26.4%)	2.3% (2.3% 2.4%)
Post-scrape swab	61.2% (32.8% 76.2%)	11.4% (1.2% 37.9%)	18.6% (2.2% 33.9%)	17.3% (1.5% 26.7%)	2.4%
Tape	54.2% (26.6% 69.7%)	3.2% (1.0% 5.9%)	23.3% (8.5% 35.3%)	20.2% (5.5% 39.2%)	3.8% (1.4% 6.1%)

3.2.4 Identification of Staphylococcus species.

The percentage of ASVs with a species-level designation of Staphylococcus were compared between 16S rRNA hypervariable regions and sampling methods (Figure 3.6). Clear differences can be seen with a much higher number of ASVs identified as Staphylococcus using the V1-3 assay. The raw data in Table 3.4 reinforces this finding. The mean relative abundance of *S. epidermidis* for V1-3 (tape = 19.5%, scrape = 19.7%, swab = 24.1% and post-scrape swab = 21.5%) were compared using Kruskal-Wallis ANOVA test and showed no significant differences between the sampling methods. No S. epidermidis was identified using V3-4 but both *S. equorum* (scrape = 8.0%, swab = 5.5% and post-scrape swab = 4.2%) and *S. haemolyticus* were detected (tape = 24.4%, scrape = 10.6%, swab = 21.2% and post-scrape swab = 18.9%).

Staphylococcus aureus was found in two volunteers out of four. The mean relative abundance was 3.7% in the tape samples from volunteer 1 and 2.0% using the scrape samples of volunteer 2. Tape samples from volunteer 1 also yielded S. aureus using V3-4 with a relative abundance of 5.5%.



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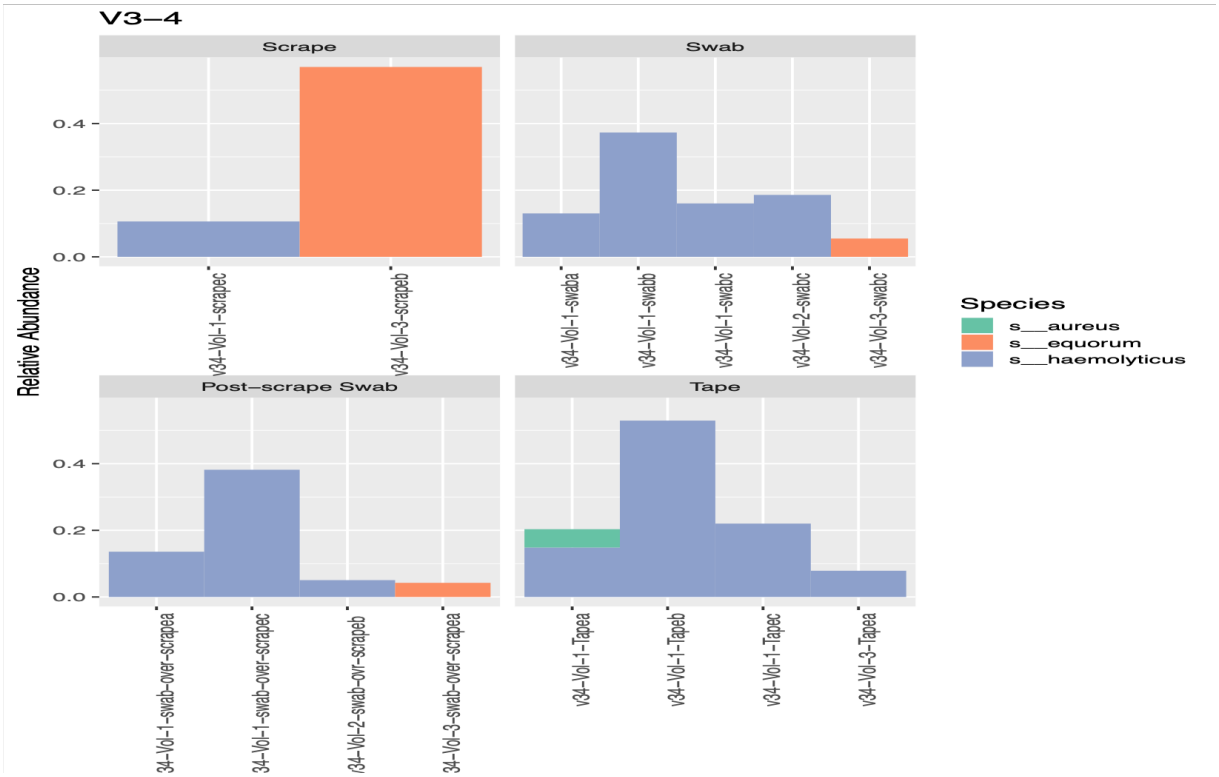


Figure 3.6 Relative abundance of ASVs classified as *Staphylococcus* for V1-3 (A) and V3-4 (B) across sampling methods. Bar plot showing the relative frequency of species-level groups of *Staphylococcus* bacteria (coloured) for each sample analysed of the triplicate, grouped by sampling method (total n= 12).

Table 3.4 Table showing relative abundance (mean and ranges) of ASVs classified of the triplicate as Staphylococcus for V1-3 and V3-4 across sampling methods.

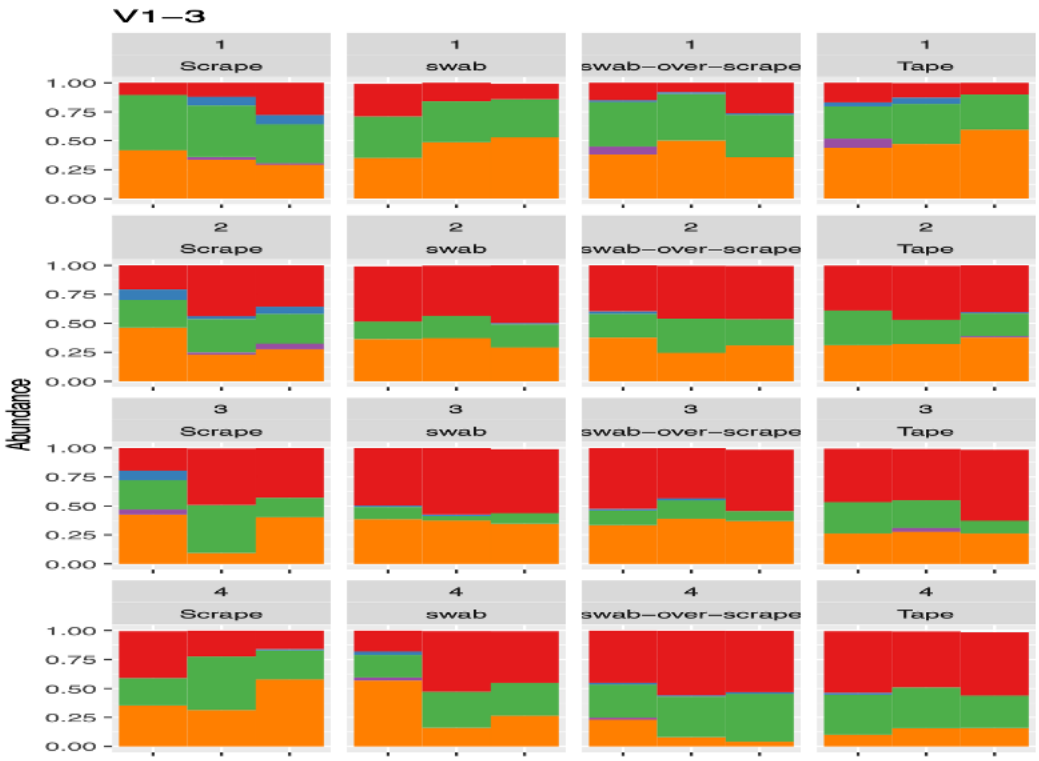
V1-3	**"Staphylococcus aureus"**	**"Staphylococcus epidermidis"**
Tape	3.7%	19.5% (3.7% - 39.7%)
Scrape	2.0%	19.7% (3.8%- 41.7%)
Swab	0%	24.1% (4.4%- 36.1%)
Post-scrape swab	0%	21.5% (1.5%- 39.9%)

V3-4	"Staphylococcus aureus"	"Staphylococcus equorum"	"Staphylococcus haemolyticus"
Tape	5.5%	0%	24.4% (7.9% -52.9%)
Scrape	0%	8.0%	10.6%
Swab	0%	5.5%	21.2% (13.0%- 37.3%)
Post-scrape swab	0%	4.2%	18.9% (5.1%- 38.2%)

3.2.5 Comparisons of bacterial phyla relative frequency for four healthy volunteers across sampling methods.

Triplicate samples were collected from four volunteers for each sampling method (Scrape, swab, post scrape swab and tape). Each bar plot represents a single sample from these triplicate samples (total n=12/per volunteer). As shown in Figure 3.7 Scrape showed less frequency comparing to the other sampling methods for both V1-3 and V3-4 16SrRNA region.

A



B

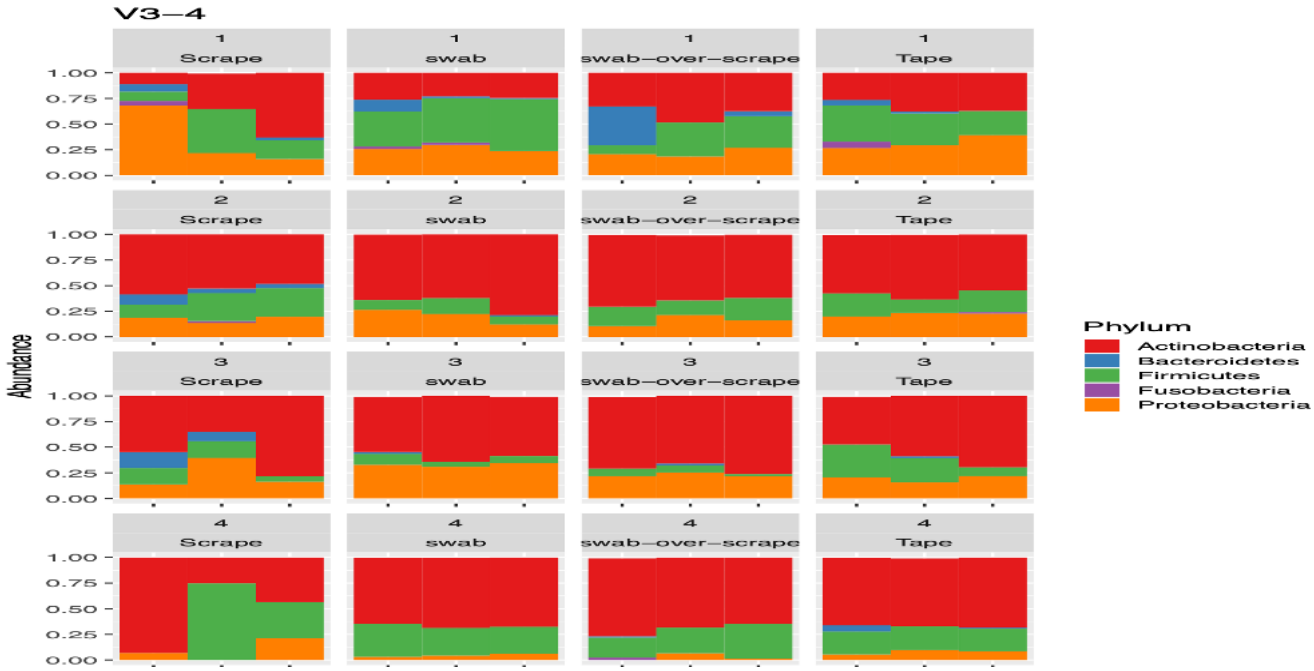


Figure 3.7 The relative abundance of the dominant bacterial phyla using V1-3 (A panels) and V3-4 (B panels). Proportional bar plot showing the relative frequency of phylum-level groups of bacteria for each sample analysed of the triplicate, grouped by sampling method and volunteers' number (1,2,3 and 4 represented by row).

3.3 Discussion

There remains ongoing discussion as to the most appropriate combination of sampling method and sequencing of 16S rRNA hypervariable region in order to characterise the microbiota of the skin. Whilst pre-moistened swabbing is the most established sampling technique (Aagaard et al., 2013, HMP, 2012a) (Paulino et al., 2006) tape stripping is considered a potentially better method that provides a high amount of biomass, however it is not suitable for all body sites due to its dimensions and sample acquisition time (Chng et al., 2016, Zeeuwen et al., 2012). Alternative methods of skin scraping and dry swabbing, despite the latter yielding reduced biomass (Schowalter et al., 2010), have also been proposed. To address these questions, we sampled the skin of four healthy volunteers using triplicate samples acquired using four different sampling methods and subjected each to 16S rRNA analysis using either hypervariable regions 1-3 or 3-4.

In terms of sampling method, Skin samples were collected from 4 male healthy volunteers using swab, scrape, post-scrape swab and tape. Samples were collected from the arm (inner-elbow) in triplicate. Scrape sampling consistently gave both lower richness and evenness estimates of alpha diversity – suggesting this method was failing to capture a representative fraction of the skin microbiome compared to the other methods. Post-scrape swab also gave some inconsistent results with the V1-3 assay, but only for measures of richness (observed OTU and Chao1). When the compositions of the samples were compared using Bray-Curtis distance measures, again only the scrape samples were shown to be compositionally distinct from the others. In addition to that, the comparison of the relative frequency of phylum-level groups of bacteria for each sample analysed of the triplicate for four volunteers revealed that Scrape showed less frequency comparing to the other sampling methods for both V1-3 and V3-4 16SrRNA region.

The greatest differences were observed for the comparison of 16S rRNA hypervariable regions. Examining the higher levels of taxonomic classification revealed differences in the proportion of ASVs only classified as Bacteria, and those of Actinobacteria that were both higher in the V3-4 analysis. One hypothesis would be that there is preferential amplification of Actinobacteria using the V3-4 primers, however there is little data to support this at present. The explanation of the former (greater abundance of poorly classified sequences) is harder to explain. The reverse reads for both assays were excluded from the analysis on the grounds of poor quality, and separate classifiers were trained for each of the hypervariable regions as recommended in QIIME2 workflows and detailed in section 2.3.7 of the methodology chapter. In addition, despite the low biomass of skin microbiomes and the inherent risks of contamination (Salter et al., 2014) when generating 16S amplicons a negligible amount of contamination, as indicated by the sequence counts from the negative control samples (tape or swabs that were processed in an identical fashion to those that had sampled skin surfaces), was observed for V1-3 (sequence count = 3) and V3-4 (sequence count =1). Once these ASVs were excluded however the composition of the microbiome was broadly as expected for both V1-3 and V3-4 based

on previous work showing that Actinobacteria dominated (51.8%), followed by Firmicutes (24.4%), Proteobacteria (16.5%) and Bacteroidetes (6.3%) (Grice et al., 2009).

Looking at *Staphylococcus* sp. in particular, a greater number of ASVs classified to this genus were identified in V1-3 analysis. Whether this is a feature of the known better taxonomic classification of this region for Staphylococci (Chakravorty et al., 2007) or the issue with potential biases in amplification noted above for V3-4 are hard to determine. Nevertheless, Kruskal-Wallis ANOVA test showed no significant differences between the methods. The absence of *S. epidermidis* from V3-4 is a concern given the prevalence found with V1-3 and the known dominance of this species as a skin commensal (Otto, 2009)It is also interesting to note that *S. aureus* was also found using V1-3. Taken together this strongly suggests V3-4 is not suitable if the goal is to detect and distinguish species of Staphylococci and performs worse than V1-3 when measures of community diversity are examined, a finding that mirrors previous research (Meisel et al., 2016).

There are limitations to the approach presented here. The use of a small number of volunteers (n=4) and only one body site for sampling makes extrapolation to different skin types difficult. In addition, a lack of data on bacterial biomass recovered, which could have been achieved through use of a 16S qPCR assay for example, would have added quantitative data and made between sampling method comparisons more informative. A similar approach (qPCR assay) to determine colonization density of *Staphylococcus* sp. would also have yielded useful data. Lastly, there were a lack of positive control which could have been achieved through use of mock microbial community. Also, we did not examine other skin commensals which may have shed light on the issue of Actinobacteria abundance in the V3-4 dataset as my main bioinformatics analysis focus were on *Staphylococcus* sp, that associated with eczema.

Overall, these data provide useful information for investigators of the skin microbiome. We suggest that the optimal approach to characterising diversity, with potential advantages for the recovery of *S. aureus*, incorporates tape sampling followed by sequencing analysis of the V1-3 hypervariable region of 16S rRNA.

Chapter 4 Evaluation of skin site-specific nitric oxide in a disease-free cohort of healthy adults

4.1 Introduction

In this chapter, we adapted Weller and colleagues (Weller et al., 1996) method to measure NO emanations using Chemiluminescence detection (CLD) from four isolated anatomical regions for 16 minutes.

Although NO synthesis in the skin has been presumed in large part to be mediated by keratinocyte reduction of nitrite, it remains possible that at low levels, commensal bacteria may contribute to these processes. Assessment of the effect of sweat generation (via mild exercise), moisturising the skin and the acute application of N. eutropha D23 (AO+ Mist™, MotherDirt™, AOBiome LLC on NO emanation was carried out as part of the hypothesis development and protocol optimization.

4.1.1 Aims

Aim 1 - To evaluate human skin site-specific nitric oxide in healthy volunteers

Aim 2 - To test our hypothesis that the acidic composition of sweat generation (via mild exercise) governs NO levels in the skin by the simple chemical reduction of nitrite.

Aim 3 - Make the novel discovery that sweat production brings about an increase in NO emanations from the skin.

4.2 Results

4.2.1 Volunteers and responses

Volunteers were excluded from the study if they had an active diagnosis of skin disease, were taking topical or oral antibiotics, known to be pregnant, or if there was any known medical contraindication to performing moderate cardiovascular exercise. Seven healthy adult (>18 years) volunteers were enrolled between March to June 2017. Only one was lost to follow up. This female could not attend due to prior holiday commitments and was excluded from the analysis. Three volunteers reported a history of previous skin disease. Four volunteers reported an allergy condition. The data are illustrated in table 4.1.

Table 4.1 Demographic and anthropometric subject data.

VOLUNTEER CODE	1	2	3	4	6	7	8
AGE	36	25	24	29	25	27	28
GENDER	M	F	F	F	F	F	F
HEIGHT	171.6	168.6	155	162.5	173.2	172.2	154.8
WEIGHT	74.7	72.4	65.7	93.4	58.2	81.8	54.4
BLOOD PRESSURE	105/63	114/61	131/78	109/74	113/67	126/76	104/56
ETHNICITY	Asian	White	White	White	White	White	Mixed
FITZPATRICK SKIN TYPE	4	2	1	2	1	2	1
HISTORY OF SKIN DISEASE	Yes	NO	NO	NO	NO	Yes	Yes
IF YES WHAT?	Acne					Eczema	Eczema
ALLERGY	NO	NO	Hay fever	NO	Hay fever	Hay fever, Asthma	Hay fever, Food allergy

4.2.2 NO emanations can be sampled from healthy humans

We adapted Weller and colleagues' method (Weller et al., 1996) to measure NO emanations using CLD from four isolated anatomical regions (Arm, hand, head and foot) for 16 minutes to allow regions to achieve a steady state as shown in Figure 4.1. Additionally, assessment of the effect of acute application of AO+ mist (Fig 4.5) and MQ water (Fig4.4) was carried out as part of the hypothesis development and protocol optimization. NO emanations were measured from different body regions arm, hand, head and foot as described in methodology section 2.4.5 for 16 mins during the first visit across the entire cohort Fig 4.2.

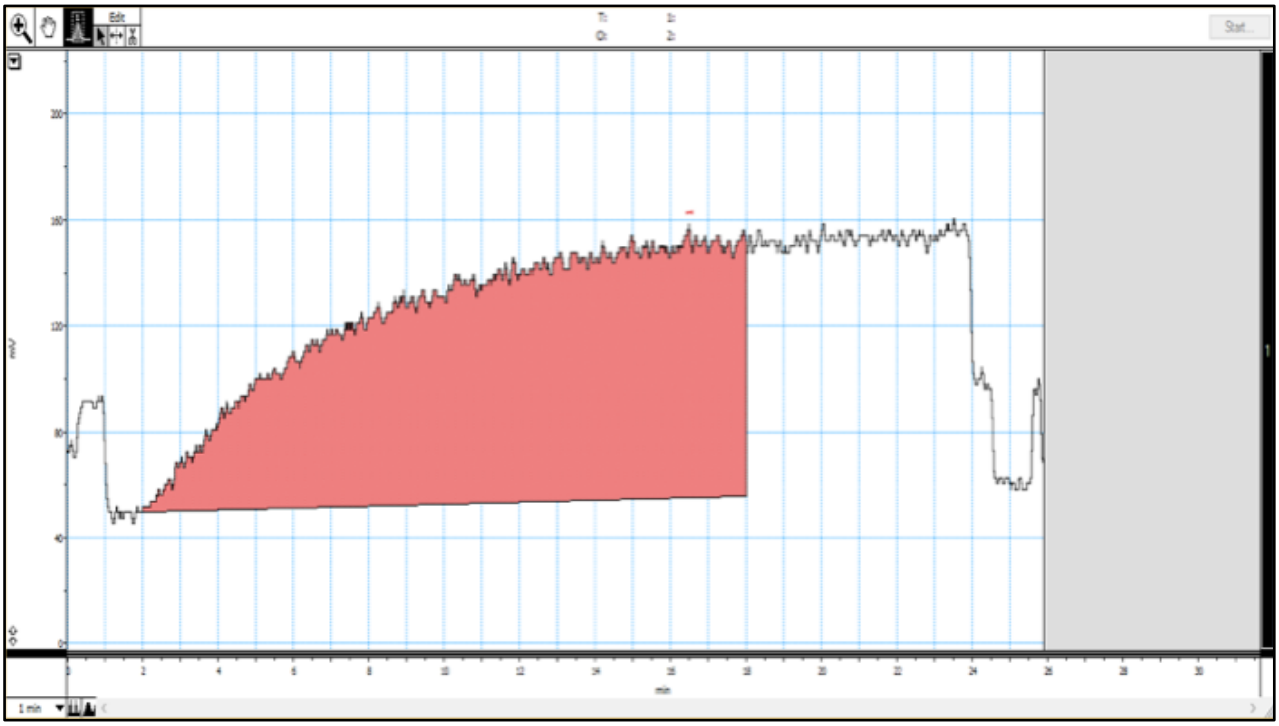


Figure 4.1 An example of real time recording of NO emanation from isolated anatomical regions to reach a steady state. Calculations of total NO identified per minute. The area under the curve was integrated and NO readings were recorded using a PowerChrom data acquisition system (eDAQ).

4.2.3 NO emanations from the skin

4.2.3.1 Skin NO emanation is dependent upon body site

To determine whether or not there was a statistically significant difference between NO emanations across distinct body sites, NO was measured from various body sites and ANOVA was used to compare the results. Although there were variations between the different body regions, no statistically significant difference was identified ($p = 0.13$). An analysis of the mean value for NO emanation suggest that foot is the region with the

highest value = 0.17 ± 0.10 µmoles, followed by Hand = 0.09± 0.06 µmol, Head = 0.08 ± 0.06 µmol and Arm = 0.07± 0.05 µmoles, respectively. Figure 4.2 illustrates a tukey plot of average basal NO emanations from Arm n= 20, hand n= 20, head n= 5 and foot n =5 at the first visit.

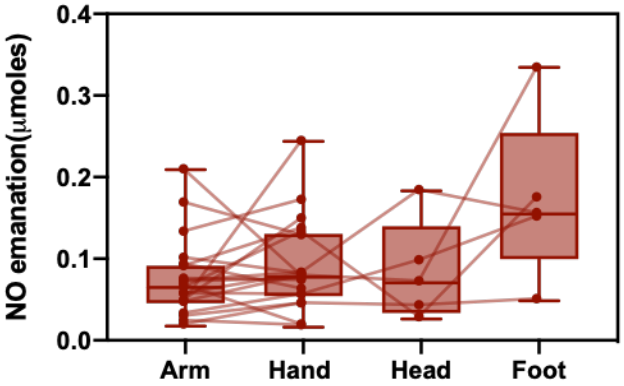


Figure 4.2 Basal NO emanations from all participants at the first visit. ANOVA measurements revealed variations between the different body regions but this difference was not statistically significant (p = 0.13). Data for each individual is superimposed on top of the box-plot (Tukey) Error bars show ± standard error of the mean (SEM).

4.2.3.2 Hand NO emanations before and after moderate intensity exercise

In order to test the hypothesis that sweat rather than acidity of the skin (as Weller proposed) interacts with skin to increase NO emanation, volunteers underwent moderate exercise while a body part (hand) was placed in a plastic bag. Whilst exercise stimulated NO emanation in all individuals, across the cohort a Two tailed paired t-test evaluation of hand NO emanations before and after moderate intensity exercise did not reach statistical significance (n = 5; p = 0.062). An analysis of the mean value of volunteers for NO emanation

before and after moderate intensity exercise suggest an increase in NO emanation (Baseline = 0.06± 0.06 and Post exercise =0.21 ± 0.13) Fig 4.3.

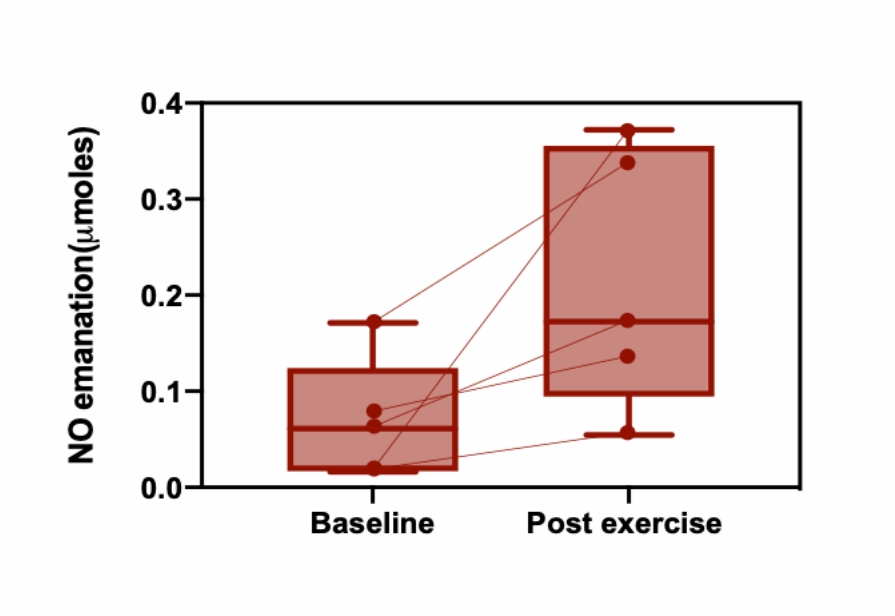


Figure 4.3 Hand NO emanations before and after moderate intensity exercise. Graph illustrates the effect of sweating on NO emanations from the hand. There was a non-statistically significant difference in NO emanation following exercise (n = 5, p = 0.062). Data for each individual is superimposed on top of the box-plot (Tukey). Error bars show ± standard error of the mean (SEM).

4.2.3.3 Effect of wetting the hand with MilliQ-water and acute application of AO+ Mist on hand NO emanation.

To address whether sweat components (including ammonia) or moisture alone could generate an increase in NO emanation, control experiments were performed on a subset of individuals with similar profiles as the main cohort. MQ was sprayed on the skin after taking baseline measurements and suggest no change in NO emanation (Baseline = 0.14 ± 0.08 and Post moisture = 0.17 ± 0.09). Results demonstrate that moisture in the form of MQ water did not significantly alter baseline NO emanations p = 012 (Figure 4.4).

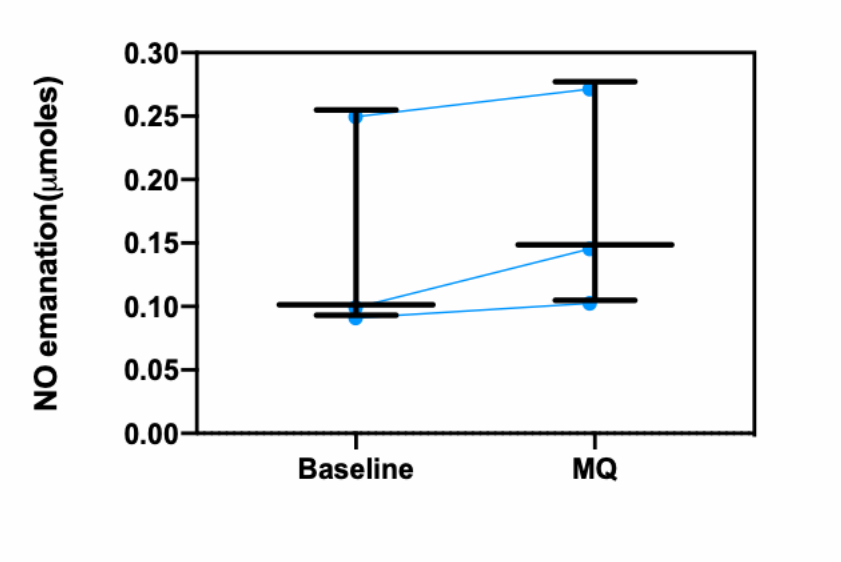


Figure 4.4 Effect of moisture the hand with MilliQ-water on NO emanation. Tukey plot illustrates the effect of MQ, on NO emanations in the hand. There was a non- Data for each individual is superimposed on top of the box-plot (Tukey). Error bars show ± standard deviation of the mean (SD).

With topically applied water excluded as one of the possibilities modifiers of NO regulation, the effect of acute AO+ Mist application was then examined more closely. Application of five uniform puffs of AO+ Mist on both sides of the hand applying approximately 1 x 10^6 cells each puff. Results demonstrated that NO emanation increases more robustly with application of AO+ Mist (Figure 4.5).

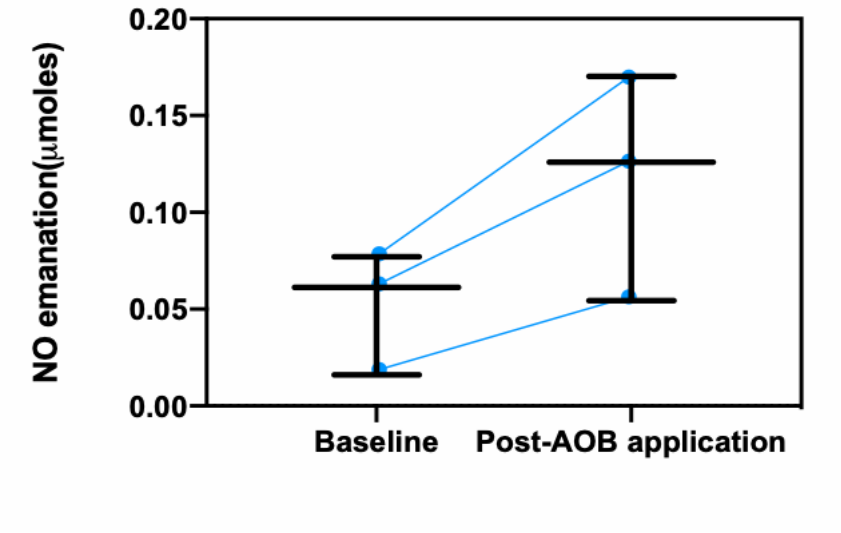


Figure 4.5 Effect of acute application of AO+ Mist on the hand NO emanation. Tukey plot illustrates the effect of acute application of AO+ Mist, on NO emanations in the hand. There was no significant change in the NO emanation after moisturising the hands of three individuals p = 0.053. Data for each individual is superimposed on top of the box-plot (Tukey). Error bars show ± standard deviation of the mean (SD) = 0.05 ± 0.03 to 0.11 ± 0.05.

4.2.4 Regional skin pH and TEWL varies in healthy adults.

Skin pH was successfully sampled in the arm n= 21, hand n= 21, head n= 5 and foot n= 5 of healthy volunteers. To determine whether or not there was a statistically significant difference between pH readings across distinct body sites, pH was measured from various body sites and paired t-test was used to compare the results. Although there were variations between the different body regions, no significant difference was identified (arm vs hand p = 0.34, arm vs head p = 0.30, arm vs foot p = 0.44, hand vs head p = 0.27, hand vs foot p = 0.98 and head vs foot p = 0.40). The mean value for pH were calculated for arm = 5.66 ± 0.66, hand = 5.57± 0.66, head = 6.87 ± 2.65 and foot = 5.91 ±0.93, respectively. Figure 4.6 (Left panel) illustrates a tukey plot of average basal pH from the healthy participants at their first visit.

Additionally, TEWL was measured from various body sites and paired t-test was used to compare the results. Although there were variations between the different body regions, no significant difference was identified (arm vs hand p = 0.16, arm vs head p = 0.09, arm vs foot p = 0.06, hand vs head p = 0.70, hand vs foot p = 0.14 and head vs foot p = 0.10). An analysis of the mean value for TEWL suggest that foot is the region with the highest value = 21.64 ± 14.64 g m^{-2} h^{-1}, head = 13.11 ± 5.39 g m^{-2} h^{-1}, head = 8.64 ± 4.82 g m^{-2} h^{-1} and arm = 7.61 ± 4.12 g m^{-2} h^{-1}, respectively. Figure 4.6 (Right panel) illustrates a tukey plot of average basal TEWL from the healthy participants at their first visit.

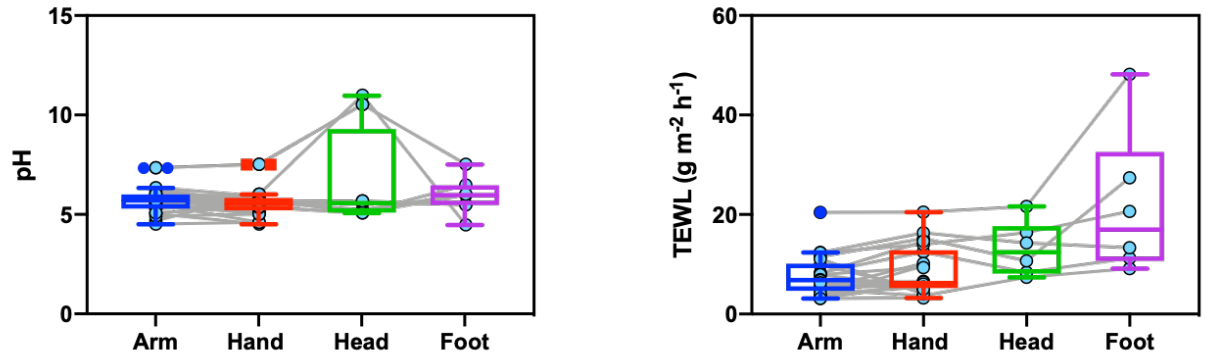


Figure 4.6 Basal pH and TEWL from all participants at the first visit. Paired t- test measurements revealed pH (Left panel, each data point represents the mean values of 3 pH measurements) and TEWL (Right panel, each data point presents as median values of 10-12 TEWL measurement) variations between the different body regions but this difference was not statistically significant. Error bars show ± standard error of the mean (SEM).

4.3 Discussion

4.3.1 NO emanation can be sampled from healthy humans

In this study, I first refine the method designed originally by Weller *et al.*, (1996) and further modified by Hares (2016) for measuring NO emanation from human skin. I determined the rates of NO emanation using a refined version of a mechanism originally designed by Weller et al.(Weller et al., 1996) A methodological discrepancy potentially accounting for this is that Weller *et al.*, (Weller et al., 1996) used a rigid glass cylinder to make recordings, with the subject's hands sealed using a diving cuff and with low inflow of room air. By comparison, we used a higher inflow rate of NO-free air and replaced the cylinder with a more versatile 50 µm MylarTM bag. This minimized the volume of 'dead space' within the recording chamber; reducing the delay between NO production and detection improving sensitivity. In addition, subjects were more comfortable and we could analyse body regions regardless of shape e.g. hand versus foot/ankle. Prior to the recruitment of volunteers, the capacity of our adapted version of the Weller et al. (Weller, 1996) method to measure NO emanations from isolated anatomical regions was tested to demonstrate proof of principle in all four regions.

Compared to the study carried out by Weller et al. (Weller et al., 1996), this study was of a larger scale as it recruited more participants and benefited from having both male and female volunteers from different ethnic backgrounds. Dietary nitrate intake and room temperature were controlled. The age range was narrow and thus our results can only be interpreted within the context of a young-adult population.

After the methodology was optimised, it was then necessary to determine the baseline NO emanation from different body regions before investigating the effect of exercise (sweat component) and AO+ Mist on NO emanation from the skin. Findings from this investigation suggest that NO is released from multiple skin regions even in the absence of observable sweating. Overall, I determined that the relative regional differences in NO emanation were qualitatively the same for all four body compartments (arm, hand, head and foot). There were no clear trends between demographic parameters, NO emanations and the profiles of the cohort, but further investigation using a larger cohort would be needed to validate these findings.

4.3.2 Exercise increases NO emanation

In this study, I have shown that exercise increases the rate of NO production from the skin. This was assessed by comparing basal and post-exercise NO levels under the same conditions on the same day. The effect of exercise was only examined in one body site (hand) and this region was selected because it is well-vascularized tissue, has a diverse microflora and has a high density of sweat glands.

Emanation rates varied widely across the cohort (see Figure 4.2), suggesting a large range in healthy individuals. NO emanates in larger quantities from the foot (mean = 0.17 ± 0.10 µmoles NO), where a high sweat gland density is reported. This is in contrast to the rest of the body regions where NO emanations were roughly the same (arm mean = 0.10± 0.07 µmoles; hand mean = 0.06 ± 0.06 µmoles; head mean = 0.08 ± 0.06 µmoles). These regions have varying amounts of sweat gland density (head>hand>arm) and although the correlation does not clearly correlate with sweat gland density, the possibility is not completely excluded by these data. To fully address this question, it wold be necessary to control for sweat gland density, activity and volume flow, but this was beyond the scope of this study.

The involvement of sweat in NO emanations was also considered. Compared to MilliQ -water, which served as the control, the results suggest that sweat does promote NO emanation. Although moisture does increase NO emanation, it does not do this to the same extent as sweat (see Figure 4.5). The effect of acute application of AO+ Mist also appears to increase NO emanations from baseline to a similar degree as sweat alone. Indeed, the data presented here suggest that the acute application of AO+ Mist did increase overall NO emanation in an equivalent amount to the presence of sweat.

4.3.3 Regional skin pH and TEWL varies in healthy adults.

Skin pH was successfully sampled in the arm, hand, head and foot of healthy volunteers. There were differences in body site pH, but these were minimal (Fig 4.6, Left panel).

These observations and the variability across participants could be the result of endogenous and exogenous factors such as anatomical site, hydration, use of cosmetics, washing regimens, ethnicity and age (Schmid-Wendtner and Korting, 2006; Bíró et al, 2018; Farage et al, 2018).

Skin trans-epidermal water loss (TEWL) samples were repeated in the arm, hand head and foot of healthy volunteers from Table 4.1 and Table 5.1. Measurements were made using the Tewameter probe. The results showed no statistical evidence of difference in TEWL between body regions during the first visit in the (arm (arm vs hand p = 0.16, arm vs head p = 0.09, arm vs foot p = 0.06, hand vs head p = 0.70, hand vs foot p = 0.14 and head vs foot p = 0.10). Fig 4.6(Right panel). However, the data suggest a strong trend that TEWL readings are higher in the foot. It seems probable that the study was too small to detect the statistical difference. However, the sole of the foot incorporates the thickest epidermis and stratum corneum on the body. Therefore, it would be expected that epidermal barrier would be most effective in this region. Clearly, the fact that the foot contains a high density of sweat glands, is likely to modify the measurement of water evaporation from the skin. To undertake TEWL measurements volunteers were rested for 20 minutes in a temperature and humidity-controlled environment. Whilst this is likely to ‘normalise’ sweating in the same body site compared at different time points it will not normalise sweating between body sites. Therefore, it seems likely that the differences observed (albeit non-significant) in sweating between body sites may

account for the differences in TEWL measurements and may have a bearing on other measurements between body sites.

Chapter 5 Signature profile of *Nitrosomonas eutropha* D23 at a human systemic level

5.1 Introduction

The following chapter considers the results obtained from a short and timed application of *N. eutropha* D23 (AO+ Mist™, MotherDirt™, AOBiome LLC) on the skin of disease-free volunteers. Both local and systemic changes in NO metabolism were assayed by means of a panel of readouts for *in vivo* bioactivity.

In more detail, exposure of the skin to AOB was enhanced by requiring volunteers to undertake application to a large surface area of skin twice per day for 14 day. Additionally, highly sensitive biochemical assays of systemic NO pathway metabolism were measured in addition to skin measurements.

This work was conducted in parallel to another PhD research project by Diogo Silva (Feelisch group). In order to illustrate a more complete profile of the systemic effect of cutaneous application of *N. eutropha* D23 over 14 days, clinical parameters and circulatory biomarkers used herein have been combined from both PhD research projects. Data are presented to clearly indicate which results were generated by work from this PhD. Further analysis of plasma samples collected from the volunteers enrolled in the aforementioned clinical study was conducted independently.

5.1.1 Aims

Overall, this chapter aims to explore potential local and systemic effects following application of *N. eutropha* D23 on the skin of healthy adult volunteers. The specific aims and objectives are as follows:

Aim 1 - To observe the effect on NO emanation before and after application of *N. eutropha* D23 for 14 days, in specific body areas, hand, and arm (inner elbow).

Aim 2 - To evaluate changes in local skin physiology before and after intervention with *N. eutropha* D23 by measurement of changes in skin pH and trans-epidermal water loss

Aim 3 - To evaluate systemic hemodynamic changes mediated by *N. eutropha* D23 by measurement of systolic and diastolic blood pressure.

Aim 4 - To explore a potential metabolic biosignature of the application of *N. eutropha* D23 on the skin by measurement of a panel of circulatory biomarkers related to NO status and metabolism for Saliva, sweat RBC, as well as oxidative stress and redox thiol metabolome, using the plasma samples collected from the volunteers.

5.2 Results

5.2.1 Participant profile

A total of 16 adults (4 males and 12 females) volunteers over the age of 18 (25 – 41 years old) were recruited for this study (demographic information summarised in Table 5.1). Blood pressure was presented as mean of three measurements, for both systolic and diastolic pressures. Participants were predominantly white (total 14) and two were of Asian origin. The participants' skin types were mainly 1 – 3 according to the Fitzpatrick skin type chart. Seven volunteers reported previous skin inflammatory conditions and four had a history of asthma and/or hay fever allergies.

After the initial assessment, peripheral venous blood samples were processed on site (as described in 2.4.9) and stored alongside the blood samples collected earlier from the small subset of volunteers listed in Table 4.1 for later analysis. Volunteers were given a bottle of commercial AO+ Mist™, (MotherDirt™, AOBiome LLC) containing a live, axenic suspension of N. eutropha D23. Sample collection and measurements were repeated after 14 days of using these products daily.

Table 5.1 Demographic and anthropometric subject data.

Volunteer Code	Date Consent	Age	Gender	Height	Weight	Blood pressure	Ethnicity	Fitzpatrick skin type	History of skin disease	If yes what?	Allergy
9	20/06/2019	38	F	178.3	63.4	124/88	White	2	Yes	Viral Warts (left hand)	No
10	09/07/2019	39	M	163.4	66.9	111/81	White	2	No		No
11	31/07/2019	28	M	180	66.75	120/73	White	3	Yes	Acne and Viral Warts (left knee and left foot)	No
12	01/08/2019	25	F	159.5	85.4	123/75	White	2	Yes	Viral Warts (Fingers)	Asthma
13	02/08/2019	32	F	158.5	77.5	122/79	White	1	Yes	Viral Warts (Fingers +Feet)	No
14	08/08/2019	27	M	178.5	67.2	125/80	White	3	No		No
15	08/08/2019	41	F	175.7	83.55	108/60	White	2	No		No
16	13/08/2019	26	F	180.9	74.9	118/75	White	1	Yes	Eczema, acne and viral warts (hands)	No
17	15/08/2019	33	M	172	73.55	114/67	Asian	3	No		No
18	16/08/2019	26	F	163.3	55.4	111/65	White	2	Yes	Eczema as a child and viral warts (hands and arms)	No
19	21/08/2019	40	F	164.7	84.75	108/68	White	3	No		No
20	23/08/2019	31	F	170.6	57.75	117/73	Asian	3	No		No
21	03/09/2019	29	F	164.4	47.95	105/58	White	1	No		Asthma
22	11/09/2019	31	F	169.9	57.45	107/59	White	1	Yes	Acne and Viral Warts (Thumbs)	Hay fever
23	18/09/2019	39	F	161.5	93.2	126/65	White	1	No		Hay fever, Asthma
24	20/09/2019	39	F	173.1	62.75	121/76	White	3	No		No

5.2.2 NO emanations from the skin

5.2.2.1 NO emanations after 14 days of daily AO+ Mist application

In order to examine the chronic effect of AO+ Mist, NO emanations were measured in each of the four regions 14 days after initial application. Participants were instructed on how to apply the mist on their own and all were encouraged to use it twice a day (first thing in the morning, after showering and before sleeping to allow the bacteria maximum exposure on the skin). Even though no statistical differences, NO emanations were higher post AOB application in the arm (0.09 ± 0.12 to 0.10 ± 0.06 µmoles), hand (0.09 ± 0.05 to 0.12 ± 0.09 µmoles) and head (0.08 ± 0.06 to 0.13 ± 0.04 µmoles). In the foot, a similar increase was not detected. Two-tailed paired t-test showed no evidence that the differences were statistically significant (arm, $p = 0.83$; hand, $p = 0.49$; head, $p = 0.11$; foot, $p = 0.17$) Fig 5.1.

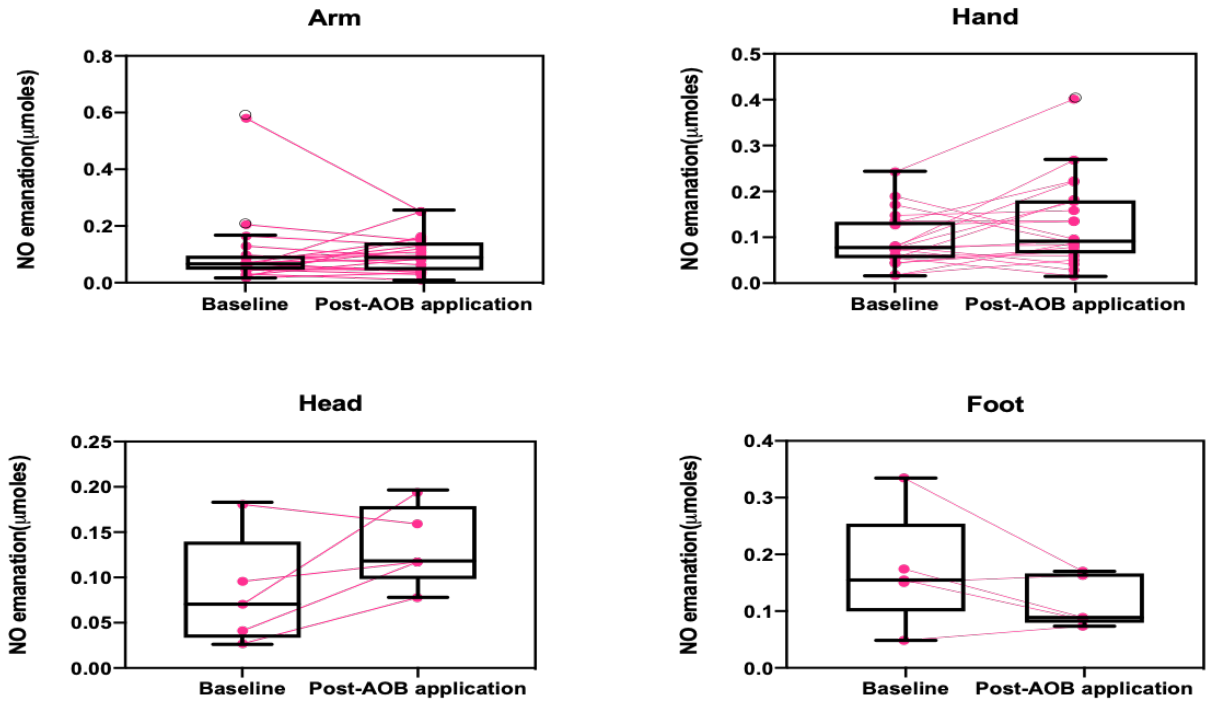


Figure 5.1 NO emanations after 14 days of daily AO+ Mist application. Data for each individual is superimposed on top of the box-plot (Tukey) and show the effect of AO+ Mist application on NO emanations in different body regions (arm and hand) of healthy participants. The measurements were recorded for 16 minutes. The application of AO+ Mist over 14 days resulted in a non-statistically significant difference in the in the NO emanations across any of the body parts assessed using paired t-test including arm (n= 21,p = 0.83), hand (n=21,p = 0.49), head (n=5,p = 0.11) and foot (n=5,p = 0.17).

5.2.3 Regional skin pH varies in healthy adults

The pH measurements were all repeated in the same way as the first visit in the follow-up visit (second visit) after the participants had applied AO+ Mist for a period of 14 days. Skin pH was successfully sampled in the arm, hand, head and foot with three measurements taken per area. Fig 5.2 illustrated the pH readings of healthy volunteers before and after 14 days of daily AO+ Mist application. As evaluated by paired t-test, there was a statistically significant increase in both hand and arm skin pH between visits for both arm (from 5.66 ± 0.58 to 6.04 ± 0.48, p = 0.011) and hand (from 5.44 ± 0.64 to 5.94 ± 0.56, p =0.004) suggesting that chronic AO+ Mist application had an effect on skin pH. However, the head (6.38 ± 2.31 to 5.63 ± 0.34 p=0.49) and foot (6.18 ± 0.84 to 6.10 ± 0.40 p=0.82) showed lesser effects and these were not significant.

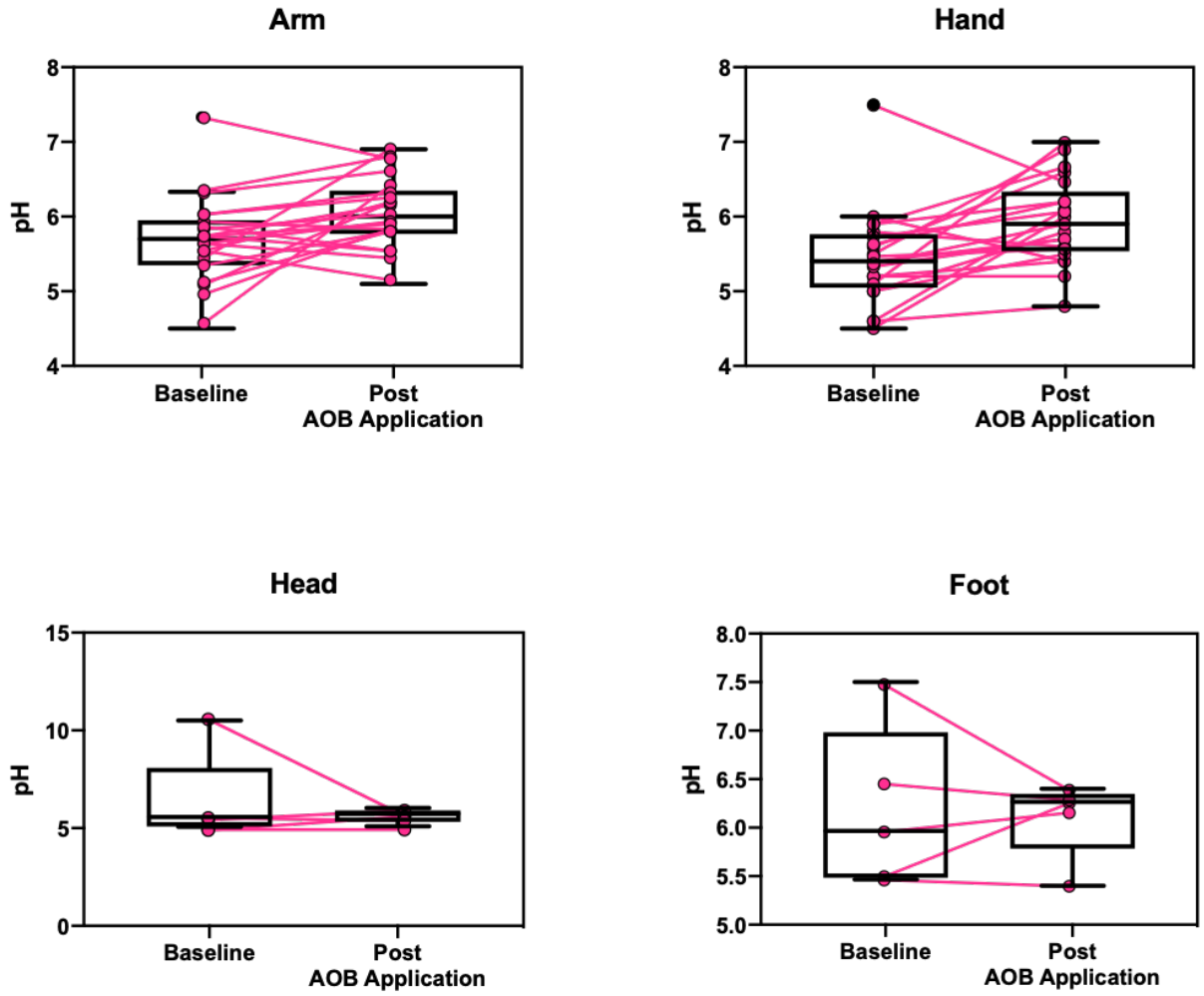


Figure 5.2 The effect of chronic AO+ Mist application for 14 days on skin pH of arm, hand, head and foot. Data for each individual is superimposed on top of the box-plot representation (Tukey). Values were measured at days 0 (baseline) and 14 (post-AOB application) for healthy participants. Each data point represents the mean values of 3 pH measurements (y-axis). There were significant differences in the skin pH between the first and second visit in regions: arm (n=21, p = 0.011) and hand (n=21, p = 0.004). There were no statistically significant differences in the skin pH between the first and second visit in regions: head (n=5, p = 0.49) and foot (n=5, p = 0.82).

5.2.4 Regional skin trans-epidermal water loss (TEWL) varies in healthy adults

Skin trans-epidermal water loss (TEWL) was successfully sampled in the body regions (Arm n= 21, hand n=21, head n=5 and foot n=5) of healthy volunteers. Similar to the process for skin pH measurements, TEWL readings were repeated for all participants during a second visit after 14 days of daily AO+ Mist application. Measurements were made using the Tewameter probe with 10-12 recordings per body area. statistical significance was assessed using paired t-test for all body regions. Overall, the highest changes in TEWL after 14 days of daily AO+ Mist application were recorded for the head, with a slight decrease in median value from 12.88 ± 6.00 to 11.15 ± 2.75 g m^{-2} h^{-1}. For the remaining body regions evaluated, 14 days of daily AO+ Mist application led to an increase in the median values of TEWL from 7.38 ± 4.38 to 7.59 ± 4.59 g m^{-2} h^{-1} for arm, from 8.28 ± 4.61 to 9.06 ± 4.63 g m^{-2} h^{-1} for hand and from 23.31 ± 15.72 to 25.33 ± 16.96 g m^{-2} h^{-1} for foot. The results in Fig 5.3 showed an overall statistically non-significant difference in TEWL between visits in the arm (p = 0.88), hand (p = 0.49), head (p = 0.59) and foot (p = 0.86).

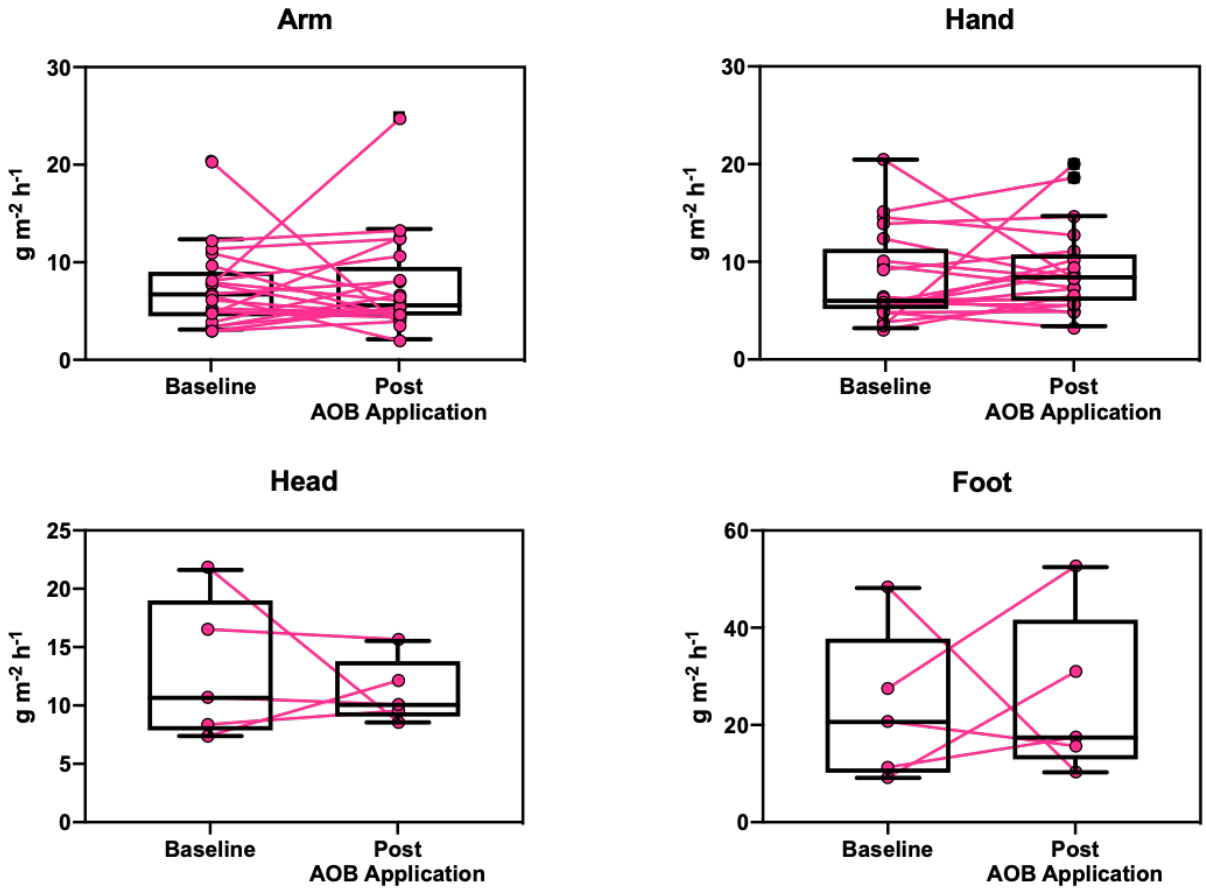


Figure 5.3 Effect of chronic AO+ Mist application over a period of 14 days on TEWL of arm, hand, head and foot. Data for each individual is superimposed on top of the box-plot representation (Tukey). Values were measured at days 0 (baseline) and 14 (post-AOB application) for all participants. A Two-tailed paired t-test revealed no statistically

significant difference between the two visits in any of the all body regions arm (n=21, p = 0.88), hand (n=21, p = 0.49), head (n=5, p = 0.59) and foot (n=5, p = 0.86).

5.2.5 Skin surface area

In this study of NO emanation, body sites were enclosed in plastic and the NO sampled from the enclosed air. To control for the effect of surface area on the measurement of NO emanation, hand skin surface area was measured for 16 healthy participants (Table 5.1) as described previously (Chi-Yuang Yu 2009, Yao-Wen Hsu & Chi-Yuang Yu 2010). To assess the relationship between hand surface size and NO emanations the range of hand surface size values were plotted against their respective NO emanations in hand body regions analysed (figure 5.4). These data indicate no correlation between hand surface size and NO emanation in the hand (R squared = 0.12, p = 0.18).

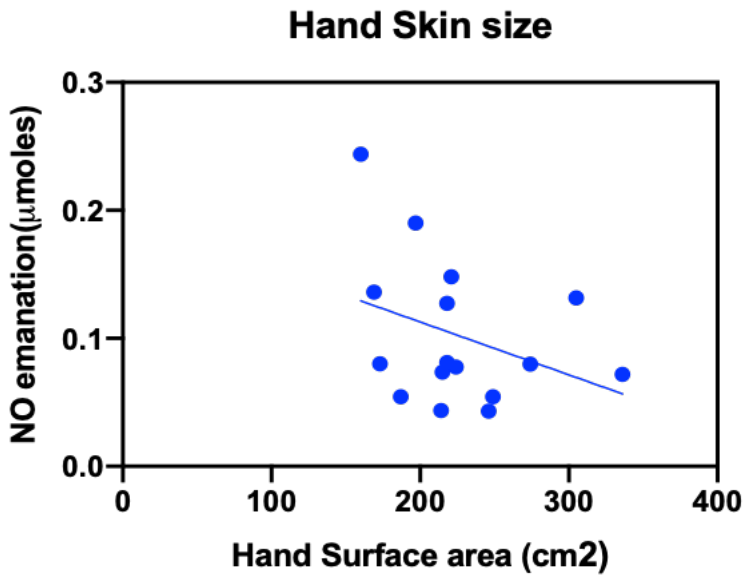


Figure 5.4 Correlation between NO emanation and hand surface size for 16 participants. The plot illustrates the relationship between hand surface size and NO emanations in the hand. Each data point represents the combined measurement for an individual. There was a non-statistically significant correlation between hand surface size and NO emanation (R squared = 0.12, p = 0.18).

5.2.6 Correlation between NO emanation and pH and TEWL

To determine relationship between pH and NO emanations, the range of pH values were correlated with their respective NO emanations that were observed across arm and hand body regions of individuals. Both regions remained relatively unchanged in pH between both visits and therefore showed no correlation (R^2: arm = 0.001 and hand = 0.007). Figure 5.5-A illustrates arm (Left panel) and hand (Right panel) data in Tukey's plots.

Data presented in chapter 4 suggested a possible link between sweating and NO emanation. TEWL can be a surrogate measure of sweating (Harmsze et al., 2008) and therefore to assess the relationship between sweating and NO emanations the range of TEWL values were plotted against their respective NO emanations in all the body regions (Fig 5.5B). The results showed no correlation between TEWL and NO emanation in the arm (R^2= 0.005) and hand (R^2= 0.008) (Tukey's plots). However, it is important to recognise that whilst TEWL may offer a surrogate measure of sweating, other factors may also be important in modifying NO emanation.

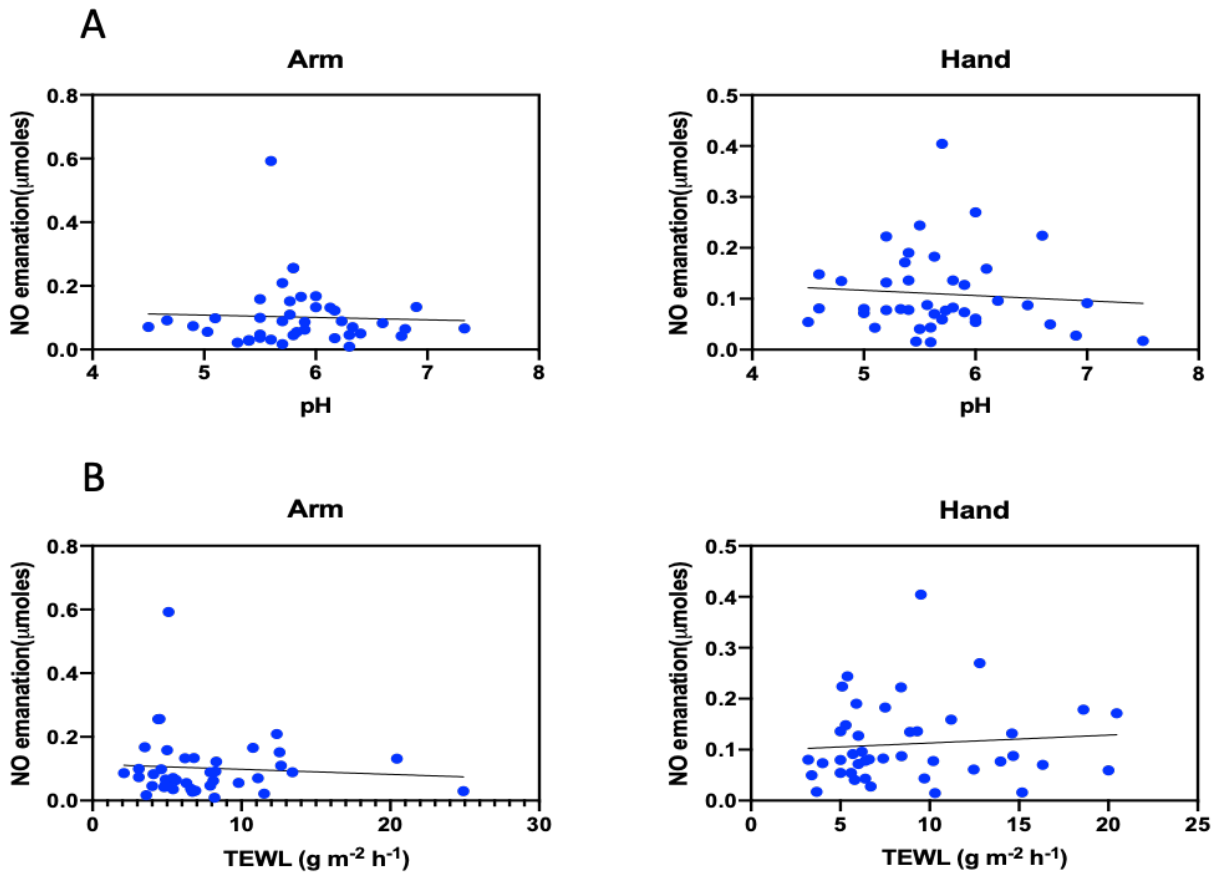


Figure 5.5 Correlation between NO emanation and hand pH and TEWL for 41 participants. The Tukey's plot (A) illustrates the relationship between NO emanations and pH in the arm (A-left panel and hand A-right panel). There was a non-statistically significant correlation between hand pH and NO emanation (R squared arm = 0.001 and hand = 0.007). Data points represent both pre and post AOB challenge combined. Additionally, The Tukey's plot (B) illustrates the relationship between NO emanations and TEWL in the arm (B-left panel and hand B-right panel). There was a non-statistically significant correlation between hand surface size and NO emanation (R squared arm = 0.005 and hand = 0.008). data points represent both pre and post AOB challenge combined.

5.2.7 Effect of Skin AOB application on blood pressure

The systolic (A) and diastolic (B) blood pressure of 21 participants was measured at baseline (day 0), within a homeostatic range and after 14 days of daily AO+ Mist application. Data for each individual is superimposed on top of the box-plot (Tukey) in Figure 5.6. Blood pressure is presented as mean systolic and diastolic pressure (n = 3). There was an increase in systolic median from 114 to 116.6 mmHg. However, diastolic blood pressure slightly decreased after 14 days of AO+ Mist application, from 73.33 to 71.66 mmHg. However, these changes were not statistically significant (Systolic p = 0.81 and Diastolic p= 0.91).

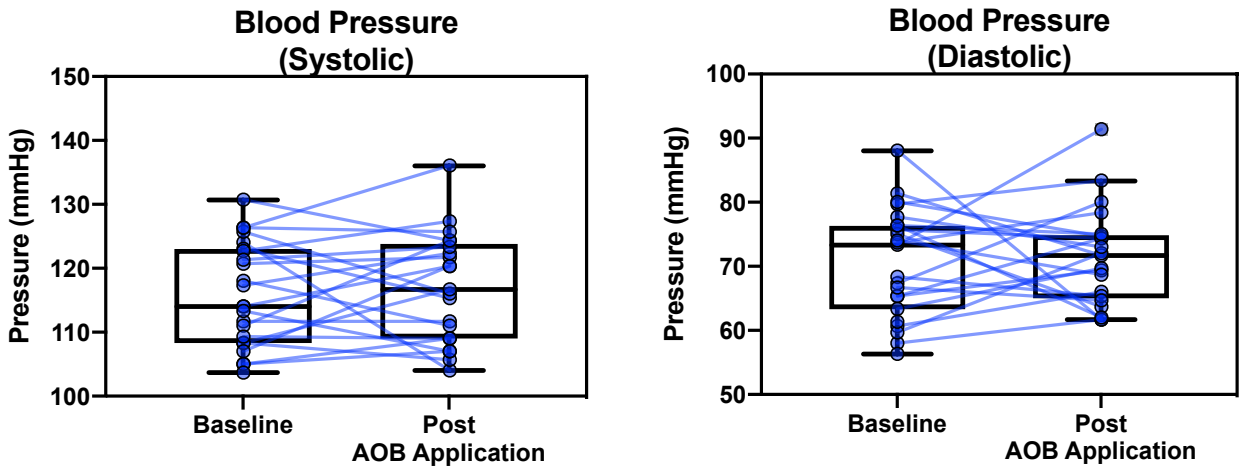


Figure 5.6 Measurement of the blood pressure of the volunteers, separated in (left) systolic and (right) diastolic blood pressures, at days 0 (baseline) and 14 (post-AOB application) for 21 participants. Data for each individual is superimposed on top of the box-plot (Tukey) representation (panels A and B). Blood pressure is presented as mean systolic and diastolic (n=3 measurements) pressure measurements.

5.2.8 Systemic markers of NO physiology

To fully characterise whether skin NO regulation might be important for systemic inflammatory responses a comprehensive set of systemic measures of NO physiology were undertaken before and after AOB application to the skin.

5.2.8.1 Nitric oxide metabolites (NO_2^- and NO_3^-) in sweat and saliva

Since NO is short-lived in biological media (e.g. sweat, saliva, red blood cells and plasma), the oxidative breakdown products of NO (nitrite and nitrate) were used as a proxy for NO. Nitrite and nitrate were measured by HPLC as noted in the methodology section. In the following analysis of sweat and saliva samples were collected from the small subset of five volunteers listed in Table 4.1.

Sweat was collected from both visits (first and second) and analysed in all volunteers. Neither nitrite nor nitrate changed significantly after chronic application of AO+ Mist for a period of 14 days. Data for each

individual is superimposed on top of the box-plot (Tukey) representation of the nitrite and nitrate levels in the first and second visit from all participants is illustrated in figure 5.7. Although there was a general decrease in the levels of nitrite and nitrate in the second visit compared to the first, there was no statistically significant difference (two tailed paired t-test, p = 0.39 (nitrite); p = 0.34 (nitrate).

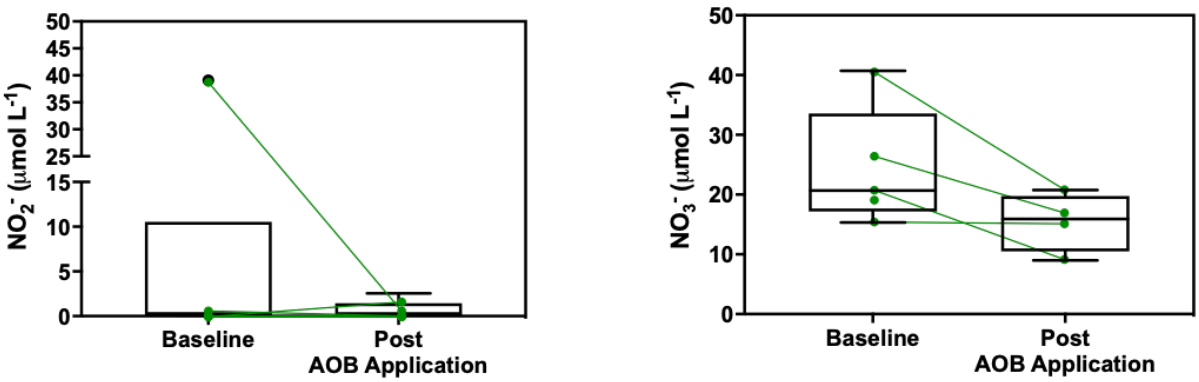


Figure 5.7 Effect of chronic application of AO+ Mist on sweat nitrite and nitrate levels. Data for each individual is superimposed on top of the box-plot representation (Tukey). Values were measured at days 0 (baseline) and 14 (post-AOB application) for 5 participants showing nitrite (left) and nitrate (right) levels in the sweat in the first and second visit. Nitrite and nitrate measurement did not significantly change using Two tailed t-test p = 0.39 (nitrite); p = 0.34 (nitrate). Sampling and data analysis by R. Alyami, HPLC was run by Feelisch group.

The same process was repeated for measuring the levels of nitrite and nitrate from saliva. Saliva was collected using Salimetrics swabs, which were centrifuged and analysed using HPLC for nitrite and nitrate. The results revealed no statistically significant difference between the levels of saliva nitrite p = 0.84 and nitrate p = 0.42 in the second visit compared to the first. Data for each individual is superimposed on top of the box-plot representation (Tukey) as illustrated in figure 5.8 and suggest that chronic application of AO+ Mist has no effect on the saliva nitrite and nitrate levels.

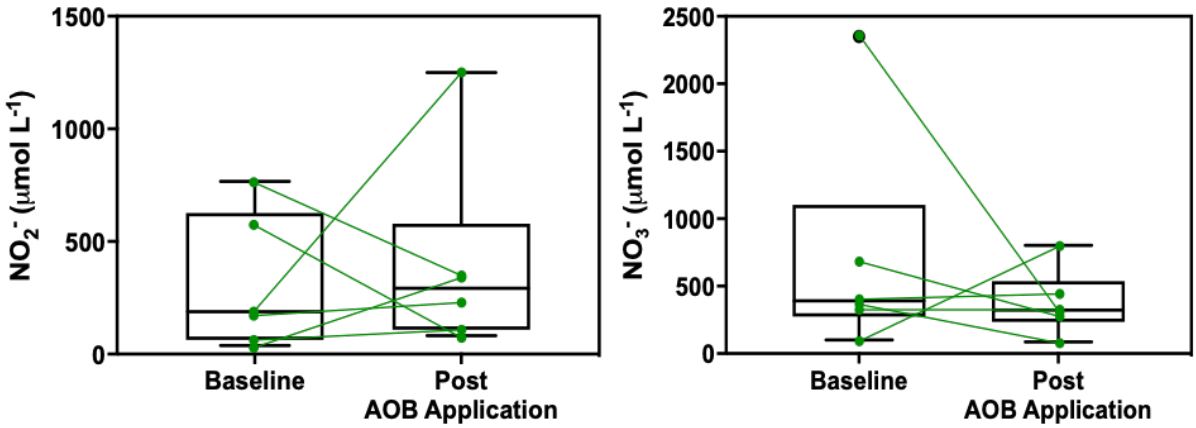


Figure 5.8 Effect of chronic application of AO+ Mist on saliva nitrite and nitrate levels. Data for each individual is superimposed on top of the box-plot representation (Tukey). Values were measured at days 0 (baseline) and 14 (post-AOB application) for 5 participants showing nitrite (left) and nitrate (right) levels in the sweat in the first and second visit. Nitrite and nitrate measurement did not significantly change using Two tailed t-test: p = 0.84 (nitrite); p = 0.42 (nitrate). Sampling and data analysis by R. Alyami, HPLC was run by R. Alyami with Feelisch group assistant.

5.2.9 Circulatory levels of nitrite and nitrate in erythrocytes are unaffected by AO+ Mist application

Similar to the previous biological samples analysed (sweat and saliva), blood was collected as described in section 2.4.9 of the methodology chapter. The same process was repeated for measuring the levels of nitrite and nitrate from red blood cells (RBC). The results revealed no statistically significant difference between the levels of red blood cell nitrite p = 0.84 and nitrate p = 0.25 in the second visit compared to the first. Data for each individual is superimposed on top of the box-plot representation (Tukey) as illustrated in figure 5.9 and suggest that chronic application of AO+ Mist has no effect on the RBC nitrite and nitrate levels.

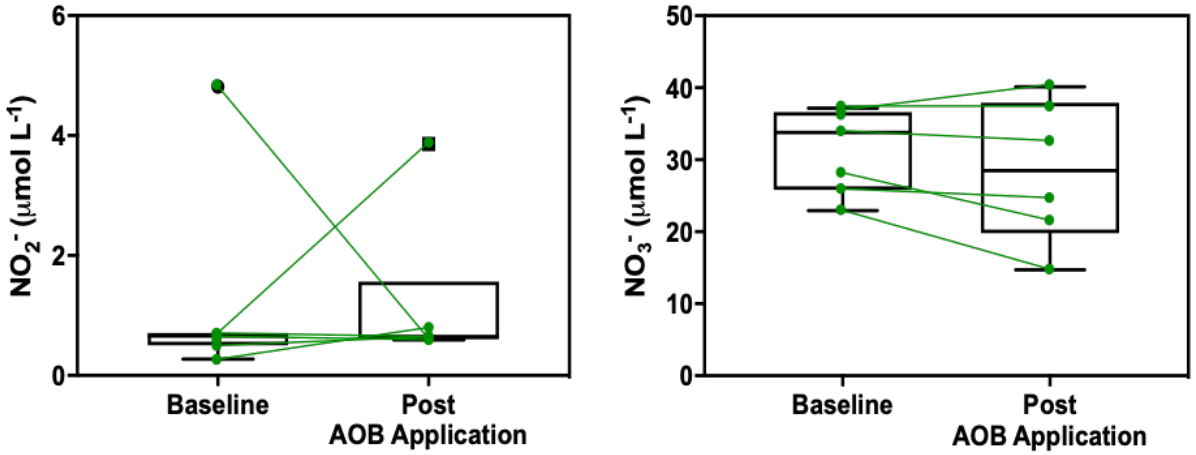


Figure 5.9 Effect of chronic application of AO+ Mist on RBC. Data for each individual is superimposed on top of the box-plot representation (Tukey). Values were measured at days 0 (baseline) and 14 (post-AOB application) for 5 participants

the levels of red blood cell (RBC) nitrite (left) and nitrate (right) in the first and second visit. Nitrite and nitrate measurement did not significantly change using Two tailed t-test p = 0.84 (nitrite); p = 0.25 (nitrate). Sampling and data analysis by R. Alyami, HPLC was run by Feelisch group.

Similar to the previous biological samples analysed (RBC), HPLC was used to measure nitrite and nitrate levels in plasma. The plasma nitrite and nitrite levels are illustrated in a box-plot (Tukey) in figure 5.10. A two-tailed paired t-test revealed no statistically significant change in the nitrite (p = 0.52) and nitrate (p = 0.70), cGMP (p = 0.14 and RXNO (p = 0.18) levels between visits.

NOx is a marker used as a proxy to evaluate the production and metabolism of NO in a biological sample. At individual levels, some decreases in the median concentration value of circulating NOx are observed (figure 5.10 top graphs). Even though no statistical differences were found for NOx concentration between visits for this cohort 14 days of daily AO+ Mist application led to a decrease in the median concentration of NO_2- from 0.13 to 0.09 µmol L-1 and a decrease in the median concentration of NO_3-, with a drop in the median baseline concentration of 27.55 to 25.80 µmol L-1 NO3-.

The cGMP levels (figure 5.10 bottom left graph) show a non-significant increase in the median concentration from baseline 124.35 to 130.7pmol L-1. No major changes in the concentration of RXNO levels (figure 5.10 bottom right graph) for this cohort were observed, as indicated by no significant change the median concentration 28.78 to 30.9 µmol L-1 RXNO.

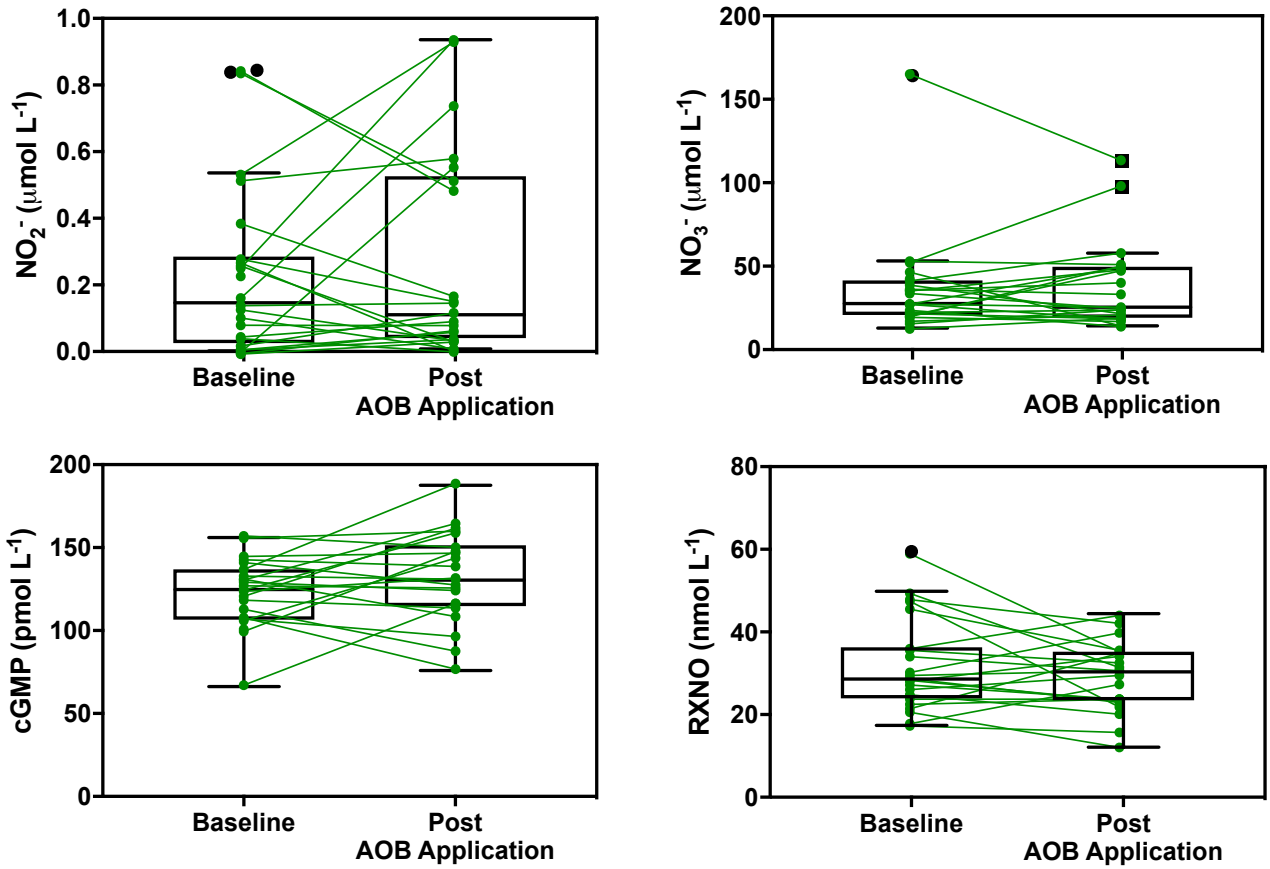


Figure 5.10 Four plasmatic biomarkers NOx (NO2- and NO3-), total nitroso species (RXNO), and guanosine 3, 5-cyclic monophosphate (cGMP). Values were measured at days 0 (baseline) and 14 (post-AOB application) for 21 participants. P value (NO2- = 0.52, NO3- = 0.70, cGMP = 0.14 and RXNO =0.18). Sampling and data analysis by R. Alyami, HPLC and CLD was run by Feelisch group. cGMP assay was performed by Feelisch group.

5.2.10 Oxidative stress

Oxidative stress markers (4-HNE, TBARS and FRAP) and lipid peroxidation markers in the plasma of all participants were analysed. During oxidative and inflammatory conditions, enhanced reactive oxygen species production results in the oxidative breakdown of polyunsaturated fatty acids and this can damage cell membranes. The end products from such a process were measured in this cohort including 4-HNE and malondialdehyde. The levels of 4-HNE that slight decreased in the median from 9.55 to 8.47 ng mL-1. However, there was no change in the median of TBARS following the application of AO+ Mist from 4.3 to 4.4 µmol.

To measure the total antioxidant capacity of samples, the levels of FRAP were measured in the plasma of all participants A slight decrease in the median from 990.15 to 960.2 µmol. The individual trends for 4-HNE, TBARS and FRAP were a generally variable following AO+ Mist application to varying extents. Statistical

significance was assessed using paired t-test. There was no significant change following 14 days of AO+ Mist application p values: 4-HNE= 0.38; TBARS = 0.63; FRAP= 0.77, respectively) Fig 5.11.

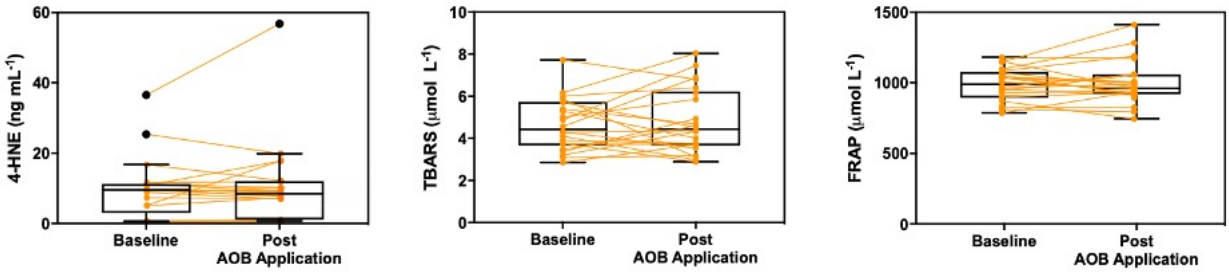


Figure 5.11 Oxidative stress biomarkers: 4-HNE, TBARS and FRAP. Only paired plasma samples were considered (21 volunteers). Data for each individual is superimposed on top of the box-plot (Tukey) representation. p values: 4-HNE= 0.38; TBARS = 0.63; FRAP= 0.77). Sampling and data analysis by R. Alyami, Oxidative stress biomarkers 4-HNE, TBARS and FRAP assays were performed by Feelisch group.

5.2.11 Redox thiol metabolome

The concentration of plasma total free thiols (TFT) were normalised to the plasma protein content from 21 volunteers as described in section 2.4.11 of the methodology chapter. Data for each individual was superimposed on top of the box-plot (Tukey) representation as shown in figure 5.12. After 14 days of AO+ Mist application, there was a statistically significant increase in the concentration of TFT of participants (p= 0.0060) as indicated by an overall increase in the median concentration from baseline 2.97 to 3.95 µmol g protein-1.

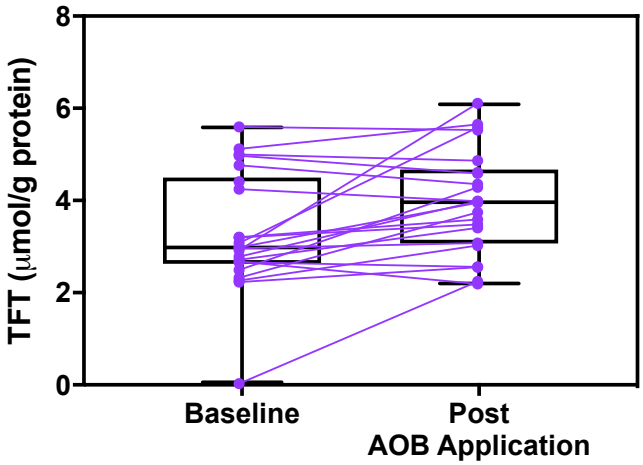
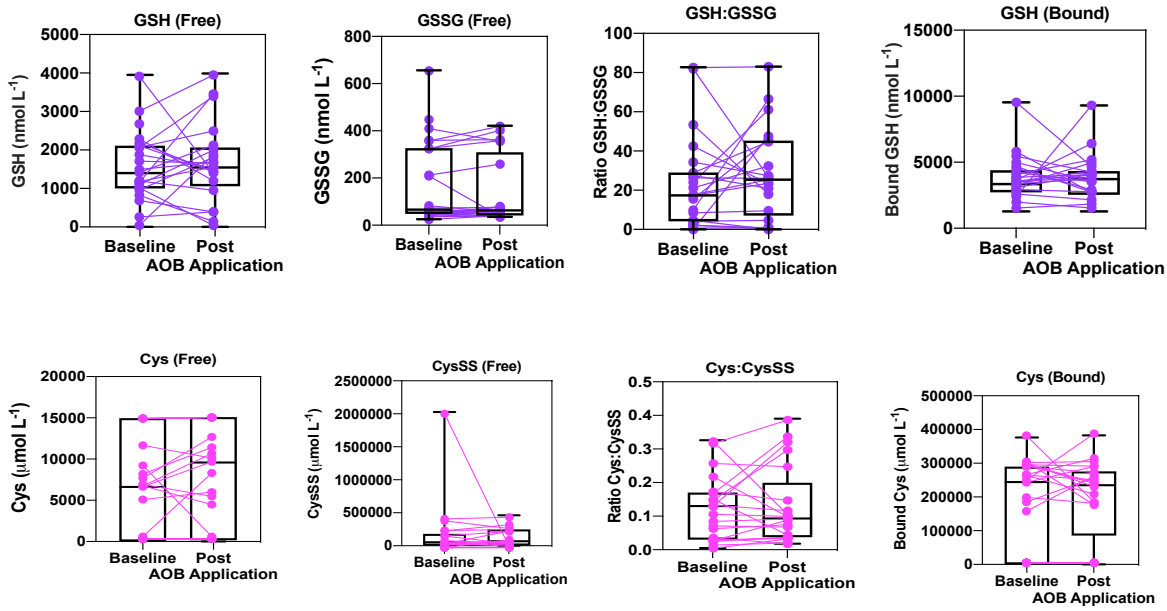


Figure 5.12 Plasma total free thiols (TFT). Only paired plasma samples were considered (21 volunteers). Data for each individual is superimposed on top of the box-plot (Tukey) representation. There was a statistically significant increase

in the concentration of TFT of participants (p value = 0.0060). Sampling and data analysis by R. Alyami, mass spectrometry was performed by Feelisch group.

The remaining thiol-related metabolites (total of 19) were analysed as part of the targeted redox thiol metabolome in these individuals, using mass spectrometry-based methods. This analysis included the measurement of free and bound thiol-related metabolites, namely cysteine (Cys), cystine (CysSS), homocysteine (HCys), homocystine (HCysSS), N-acetylcysteine (NAC), oxidised (GSSG) and reduced glutathione (GSH), glutamylcysteine (GluCys), cysteinylglycine (CysGly) and sulfide. The initial assessment of the metabolite changes within the redox thiol metabolome was done by evaluating overall trends (individual trends for each metabolite are presented in Fig 5.13). Results revealed considerable heterogeneity between individual study participants but no significant changes in thiol-related metabolites (neither in the free nor in the bound forms) between visits in the cohort analysed.



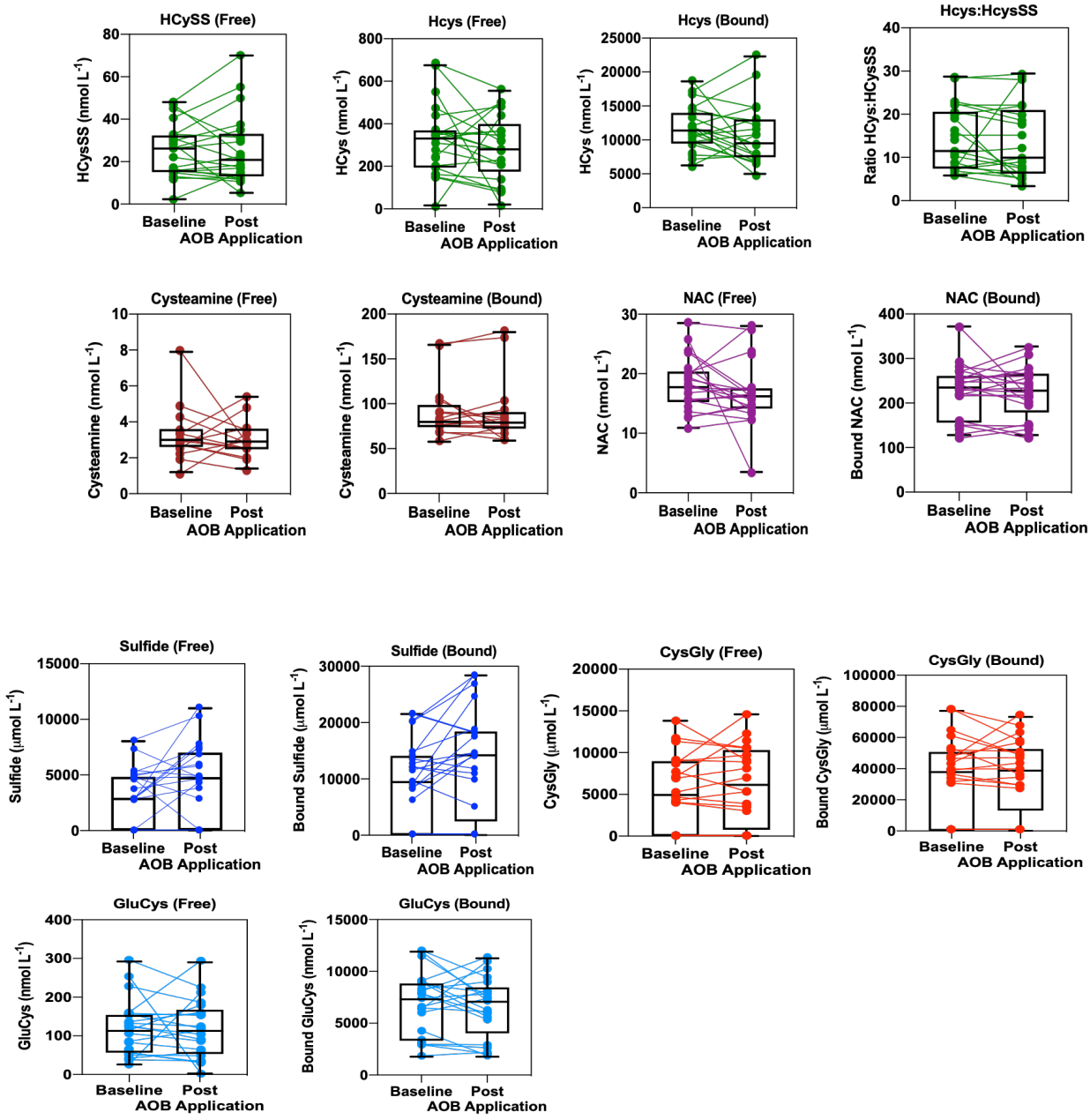


Figure 5.13 Levels of free and bound thiol-related metabolites after 14 days of AO+ Mist application. Data for each individual is superimposed on top of the box-plot (Tukey) representation. Analysis of 21 participants included the measurement of free and bound thiol-related the metabolites were measured include: cysteine (Cys), cystine (CysSS), homocysteine (HCys), homocystine (HCysSS), N-acetylcysteine (NAC), oxidised (GSSG) and reduced glutathione (GSH), glutamylcysteine (GluCys), cysteinylglycine (CysGly) and sulfide. Sampling and data analysis by R. Alyami, mass spectrometry was performed by Feelisch group.

The results are shown in figures 5.14 below indicate a visual overall fold change in the concentration of these metabolites After 14 days of AO+ Mist application.

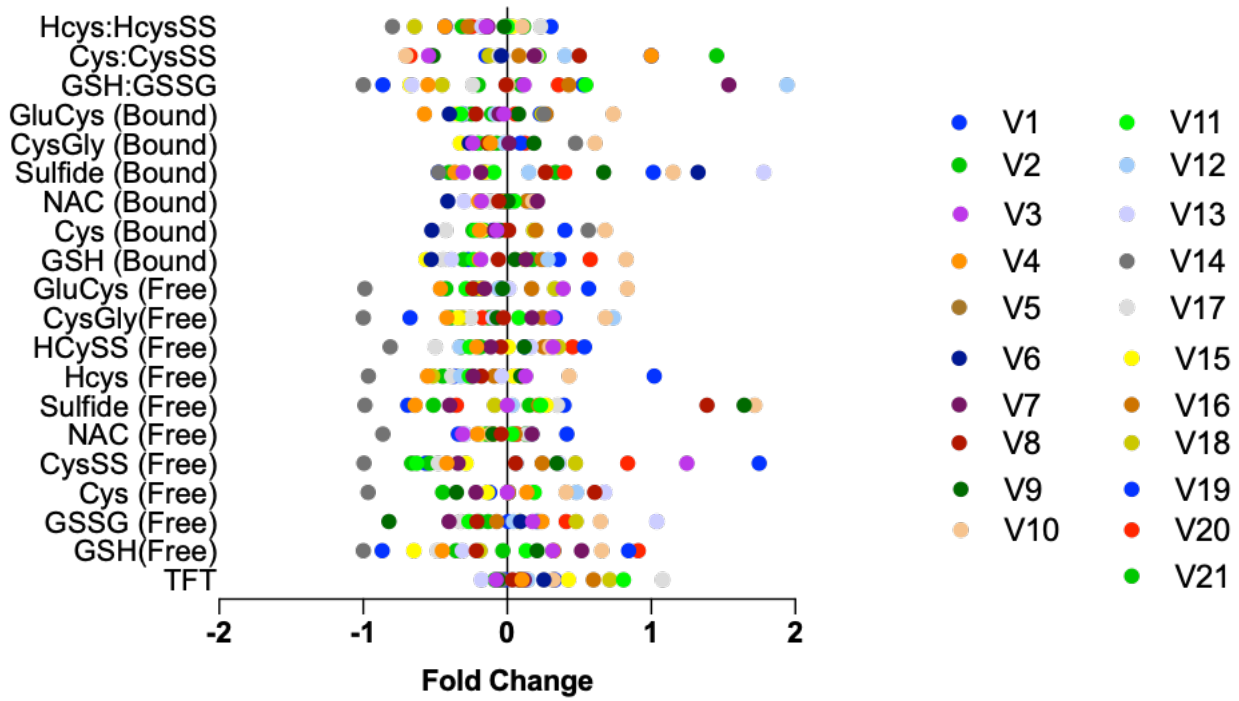


Figure 5.14 Fold change analysis (compared to baseline levels) of 20 thiol-related metabolites measured in plasma samples of 21 individuals, namely, free and bound GSH, free GSSG, free and bound Cys, free CysSS, free and bound HCys, free HCysSS, free and bound NAC, free and bound sulfide, free and bound CysGly, free and bound GluCys, and TFT.

5.3 Discussion

5.3.1 Effect of AO+ Mist application on NO emanation

Significant differences between body sites and NO emanation was noted with the overall order of NO release rates as follows: foot > hand > arm > head. After controlling for surface area of the sampled area in one site (hand), there appeared to be no evidence that surface area of measures site could account for the differences seen (Fig 5.4). The effects of AO+ Mist application over 14 days on the arm, hand and head did not show a significant increase in NO emanation (arm, $p = 0.83$; hand, $p = 0.49$; head, $p = 0.11$;). Interestingly, the foot showed non-significant decrease p = $p = 0.17$.(Fig 5.1). Whilst it seems possible that the volunteers may have less reliably applied the AOB spray to the foot due to the anatomical location, thus leading to differing results from other body sites, I have no objective evidence to support this. However, as these data were not statistically significant, further participants would be required to determine whether or not the data trends showing increased NO with AOB application would be born out. The numbers analysed to date are too low to confirm this. the power required to confirm a difference in NO emanation would require us to test 38 individuals. As a cautious approach, I have obtained an ethical approval to undertake further testing of 60 individuals. In addition, more prolonged application of AO+ Mist may be required to modify NO emanation from healthy skin.

Of the sites that were within easy reach, the hand shows the highest density of sweat glands and is a site of significant sweating activity, it could be postulated that this provides some evidence for a direct interaction between sweating and Nitrosomonas (Fig 4.3).

5.3.2 Effect of the intervention on the pH of the human skin

To investigate whether or not changes in NO emanation were dependent on skin pH, the skin pH measurements were taken from all participants and analysed in relation to NO emanations. Previous investigations (Miyamoto et al., 2011) (Weller et al., 1996, Finnen et al., 2007) have supported that the behaviour of NO species in the skin is pH dependent. Previous literature considered that detectable NO levels are regulated by simple acid/base chemistry, whereby NO production would increase in acidic environments, via the protonation of NO_2- in sweat. While skin pH values were variable across this cohort (as shown in Fig 4.6), values were well within the limits of normal skin pH (Dreno et al., 2016).

Weller et al. (Weller et al., 1996) submerged the hand of 12 participants in an acidic buffer (citrate, pH 3) and observed an increase in NO levels, while NO levels decreased in a basic tris(hydroxymethyl)aminomethane buffer (pH 9). Unexpectedly, our data strongly suggest that NO emanations from the human skin are independent of pH (R^2: arm = 0.001 and hand = 0.007; Fig 5.5).

However, the pH measurements pre and post 14 days of AO+ Mist application (Fig 5.2) showed a statistically significant increase for both arm (n =21, p = 0.011) and hand (n 21, p =0.004) suggesting that chronic AO+ Mist application had a strong effect on skin pH.

Skin pH changes in the head and foot showed no significant different after 14 days of daily AO+ Mist application (head n=5, p = 0.49) and foot (n=5, p = 0.82) but the numbers analysed in these sites were fewer.

Whilst these data are not consistent with Weller and colleagues' findings and did not replicate that skin NO is tightly regulated by skin pH, the two studies utilised different approaches for measurement of NO and therefore, it is important to note that Weller examined a smaller cohort (n=12) and that the protocol involved immersing the hand in an acidified or alkaline buffer, whereas here, I report 'real-world' measurement from skin, following 14 days of application of AO+ Mist. (Weller et al., 1996) (Weller et al., 1996).

5.3.3 Chronic application of AO+ Mist and trans-epidermal water loss (TEWL)

As TEWL levels vary between individuals, limited literature exists regarding the expected normal ranges in healthy individuals. In this study, there was no statistically significant change in the arm (p = 0.84), hand (p = 0.86), head (p = 0.59) and foot (p = 0.86) TEWL levels between the first and second visit of healthy volunteers, which took place after 14 days of daily AO+ Mist application. In light of the fact that NO emanation appeared to increase upon acute application of AOB, this suggests that NO emanations from human skin are independent of TEWL (R^2: arm = 0.005 and hand = 0.008) Fig 5.5.

5.3.4 Blood pressure changes after 14 days of AO+ Mist application

The systolic (A) and diastolic (B) blood pressure of 21 participants was measured at baseline (day 0), within a homeostatic range and after 14 days of daily AO+ Mist application (Figure 5.10). There was an increase in systolic median 114 to 116.6 mmHg. In contrary, diastolic blood pressure slightly decrease after 14 days of AO+ Mist application, as indicated by a decrease in the median value 73.33 to 71.66 mmHg. However, these changes were not statistically significant (Systolic p = 0.81 and Diastolic p= 0.91).

5.3.5 Systemic and circulatory levels of NOx, cGMP and RXNO and oxidative stress are unaffected by AO+ Mist application

Systemic and circulatory levels of nitrite, nitrate, cGMP and RXNO were unaffected by AO+ Mist application as measured in saliva, sweat, RBC and plasma. Additionally, evidence of systemic changes was provided by plasma biomarker analysis. Although no significant changes were found in oxidative mechanism, as indicated by the concentration of biomarkers (e.g. 4-HNE, TBARS) related to oxidative stress upon topical administration of N. eutropha D23. Anti-oxidative marker (FRAP) also showed no change in the concentration upon topical administration of N. eutropha D23.

5.3.6 Redox thiol metabolome

Following AOB application to the skin, a statistically significant increase in plasma TFT concentration was shown. Such a marked and consistent response suggests a tightly regulated response to *N. eutropha* application on human skin. Studies have suggested increased TFT concentrations are associated with improved cardiovascular outcomes and patient survival (Frenay et al., 2016, Koning et al., 2016) possibly by acting as antioxidant 'buffer' to ROS and reducing harmful oxidative stress (Bryan et al., 2004).

The redox thiol metabolome in the plasma of all participants was analysed and a statistically significant increase in TFT was observed in plasma samples of 21 individual (Fig 5.16). By contrast, there was no change in the concentration of numerous small thiol-containing metabolites (Fig 5.18) after 14 days of AO+ Mist application. The reason for this divergence in thiol-dependent responses requires further investigation. Nevertheless, the observed increase in total free thiol concentrations in the majority of study participants would seem to warrant further study.

Chapter 6 The effect of perturbation of the skin microbiome with *N. eutropha*

6.1 Introduction

The regulation of the skin microbiome is important in many inflammatory conditions and limited evidence exists for their benefit to atopic dermatitis, acne and rosacea (Lee et al., 2019). Previous work has shown that loss of microbial diversity arises in conjunction with disease flares of atopic eczema (Kong, 2016b). In atopic dermatitis, investigators have explored the impact of topical treatment with various microbes, but to my knowledge, the only applications of viable bacteria that have been tested are *V. filiformis*, *S. thermophilus*, *S. hominis*, and *S. epidermidis* (Gueniche et al., 2006, Di Marzio et al., 2003, Nakatsuji et al., 2017). However, the numbers treated so far have been small and indirect putative beneficial effects of the intervention have been measured. Furthermore, investigators have not examined the role of such interventions on the residual skin microbiome.

To better understand the impact of microbial perturbation of the skin microbiome we set out to examine the effects of a two-week application of *N. eutropha* D23 (AO+ Mist™, MotherDirt™, AOBiome LLC) to the skin of the arm and hand of healthy volunteers.

Based on results detailed in Chapter 3 we used Tape sampling and 16S rRNA analysis using the V1-3 region. Additionally, we evaluated the impact of *N. eutropha* application on skin NO emanations and correlated these with changes in the microbiome.

6.1.1 Aims

Overall, we set out to explore the effects of application of *N. eutropha* D23 on the skin of healthy adult volunteers. The specific aims and objectives are as follows:

Aim 1

A. To observe the changes on the skin microbiome composition after application of *N. eutropha* D23 for 14 days, in specific body areas, namely the hand and arm (inner elbow), compared with baseline assessments.

B. To measure changes in NO emanation after application of *N. eutropha* D23 for 14 days as above.

Aim 2

To correlate the abundance of *Nitrosomonas* with NO emanation of healthy adult human skin.

6.2 Results

6.2.1 Sequence quality and taxa frequency per sample pre and post AO mist application

Skin samples were collected from 16 healthy volunteers using tape pre (day 0) and post application (day 14) of *Nitrosomonas eutropha*. Samples were collected from the arm (inner-elbow) in triplicate.

After filtering for quality and amplicon length, samples total frequency was calculated = 3,391,618. Per samples statistics are shown in Table 6.1.

Table 6.1 Frequency per sample table. List showing minimum frequency, 1st quartile, median frequency, 3rd quartile, maximum frequency and mean frequency.

Frequency per sample	
Minimum frequency	10,222.0
1st quartile	43,480.0
Median frequency	52,497.0
3rd quartile	61,431.0
Maximum frequency	77,890.0
Mean frequency	52,178.7

6.2.2 Taxonomic *Nitrosomonas eutropha* species identification for arm and hand.

Nitrosomonas eutropha D23 (AO+ Mist™, MotherDirt™, AOBiome LLC) were applied on the arm and hand of 16 healthy volunteers (Table 5.1). *N. eutropha* D23 presence were expected to be seen in the post samples (day 14) of AO+ Mist application. As illustrated in Fig 6.1, Nitrosomonas was detected pre visit (day 0) in five samples (two arm and three hand), which was deemed to be due to contamination. Because we know that the *N. Eutropha* in AO mist is from a single strain, it was possible to confirm whether or not these samples were contaminated through sequence similarity analysis. Here, a Basic Local Alignment Search Tool (BLAST) analysis, using NCBI blast and default parameters, was done using the *N. eutropha* sequences thought to be contaminants. The program compares the sequenced nucleotides to sequence databases and calculates the statistical chance of the sequences being from the same organism. A 99.9% similarity was found between the sequences on the pre visit samples (day 0) and the *N. eutropha* sequence from the AOBiome AO+Mist bottle which the participants were instructed to apply for 14 days on arm and hand. This result suggested that these 5 pre-application samples were contaminated with *N. eutropha*. As a result, these samples were excluded from further analysis (Fig 6.2).

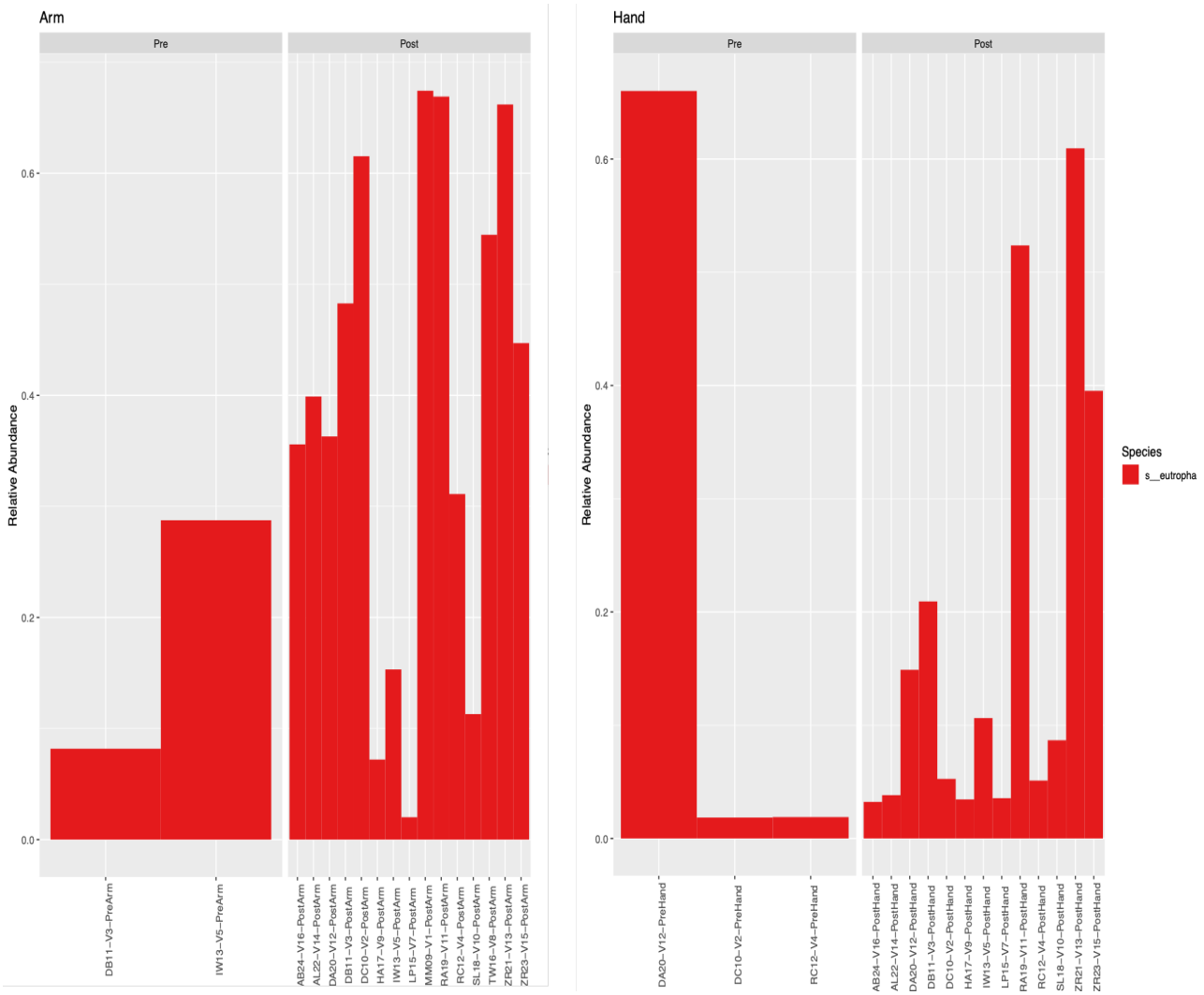


Figure 6.1 Relative abundance of N. eutropha pre and post application of AOB mist. Graph showing *Nitrosomonas eutropha* abundance for pre and post visits for both arm (n= 15, Left panel) and hand (n=13, Right panel). Red colour shows Nitrosomonas eutropha bacteria present at days 0 (baseline) and 14 (post-AOB application) for healthy participants.

To confirm the contaminated samples exclusion, *N. eutropha* D23 presence was again detected the pre (day 0) and post samples (day 14) of AO+ Mist application. As illustrated in Fig 6.2, Contaminated pre samples were successfully excluded.

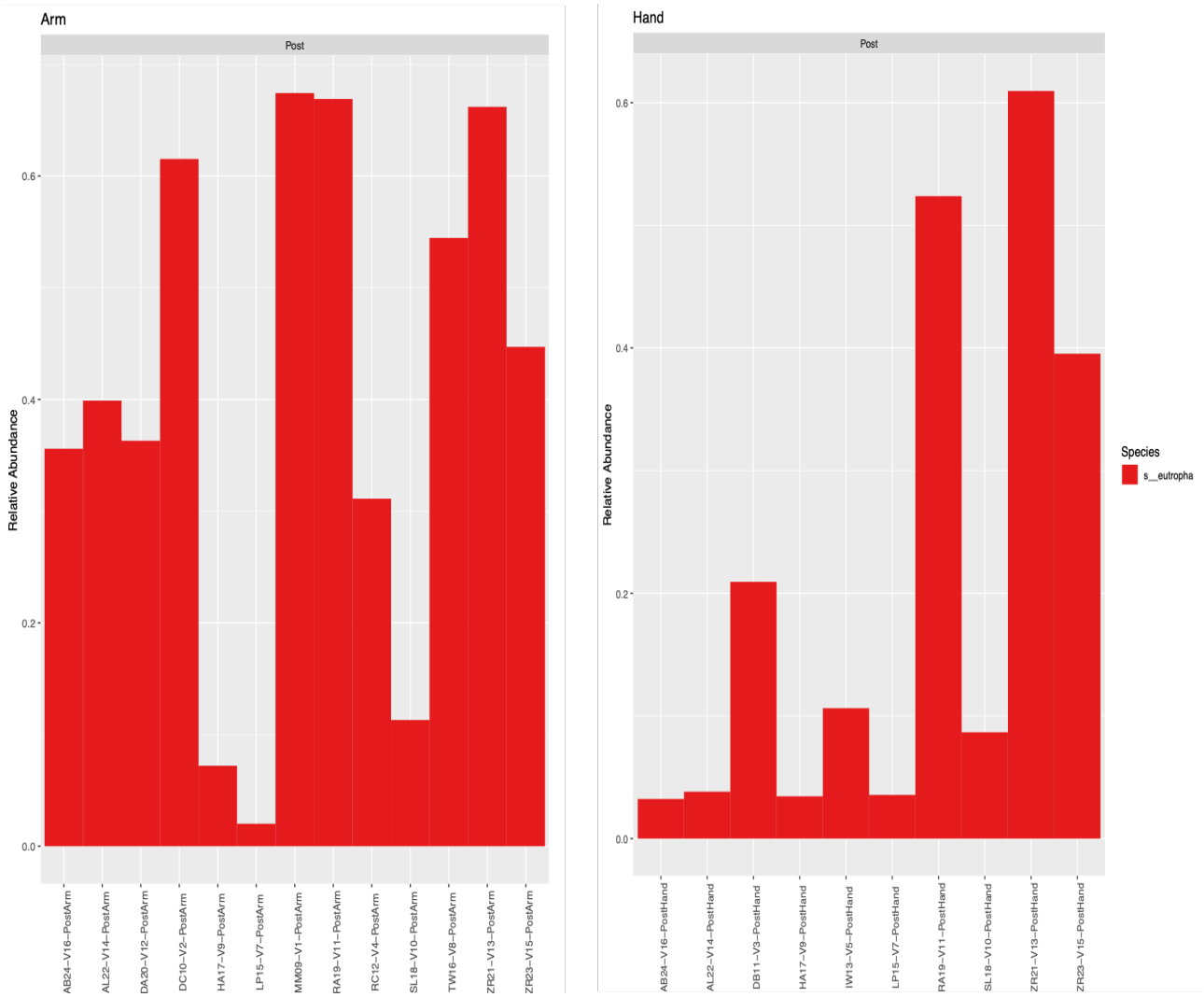


Figure 6.2 Relative abundance of N. eutropha pre and post application of AOB mist. Graph showing that the contaminated (day 0) pre samples were excluded from both arm (Left panel) and hand (Right panel). Red colour shows Nitrosomonas eutropha bacteria present only 14 (post-AOB application) for 16 participants.

6.2.3 Alpha diversity pre and post AO+ Mist application

The alpha diversity of the skin microbial community was measured using four different estimators to compare the microbial communities before and after 14 days of daily *N. eutropha* application. To calculate richness, Observed OTU (total number of species, amplicon sequence variants (ASVs) in this case and Chao1 (a richness estimator for low abundance OTUs) were used. In addition, Shannon (a quantitative measure of community evenness) and the Simpson index (a measure of number and diversity of species) were used to explore the structure of the microbial community.

Comparisons of alpha diversity for the arm revealed no statistically significant differences between the samples of pre (day 0) and post (day 14*) N. eutropha* application in species richness as determined by

Observed OTU and Chao1 (p =0.97 for both). However, Shannon and Simpson showed a statistically significant difference as assessed by paired t-test (p = 0.0023 and p=0.0099, respectively) (Fig 6.3).

In contrast, the alpha diversity of the hand microbiota revealed no statistically significant differences between pre (day 0) and post (day 14) *N. eutropha* application (Fig 6.4).

Aside from outliers in the arm sampling the community richness and structure appeared similar between both body sites.

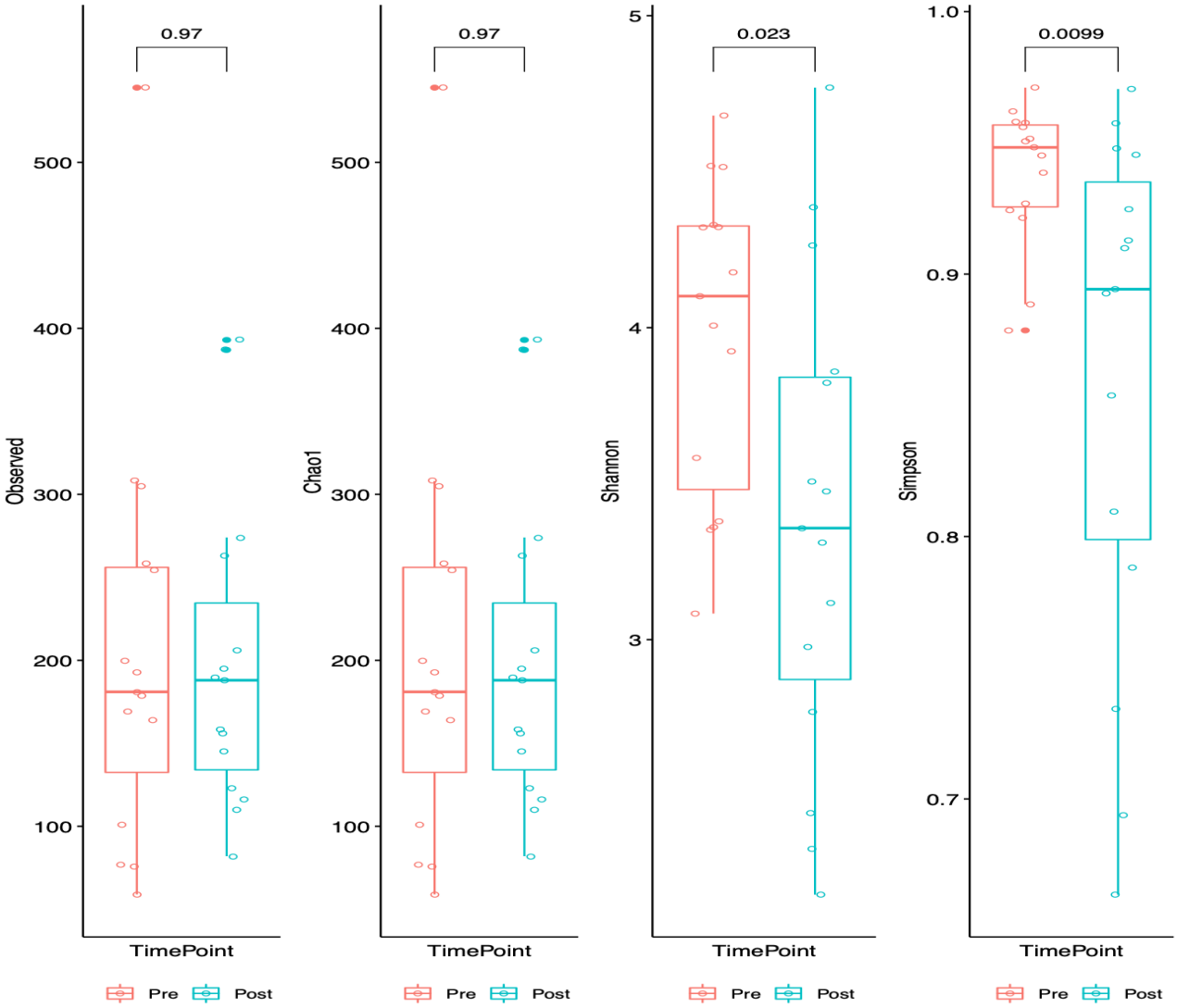


Figure 6.3 Alpha diversity of microbiota from the arm (antecubital fossa). Box and whisker plots showing alpha diversity (as determined by Observed OTUs, Shannon Evenness, Simpson Index and Chao1) of the skin microbiota of 14 healthy volunteers pre and post application of *N. eutropha* for a period of 14 days. Statistical significance was assessed using paired t-test. Significant decreases were seen in evenness and diversity of the microbial communities using Shannon and Simpson (p = 0.0023 and p=0.0099 respectively).

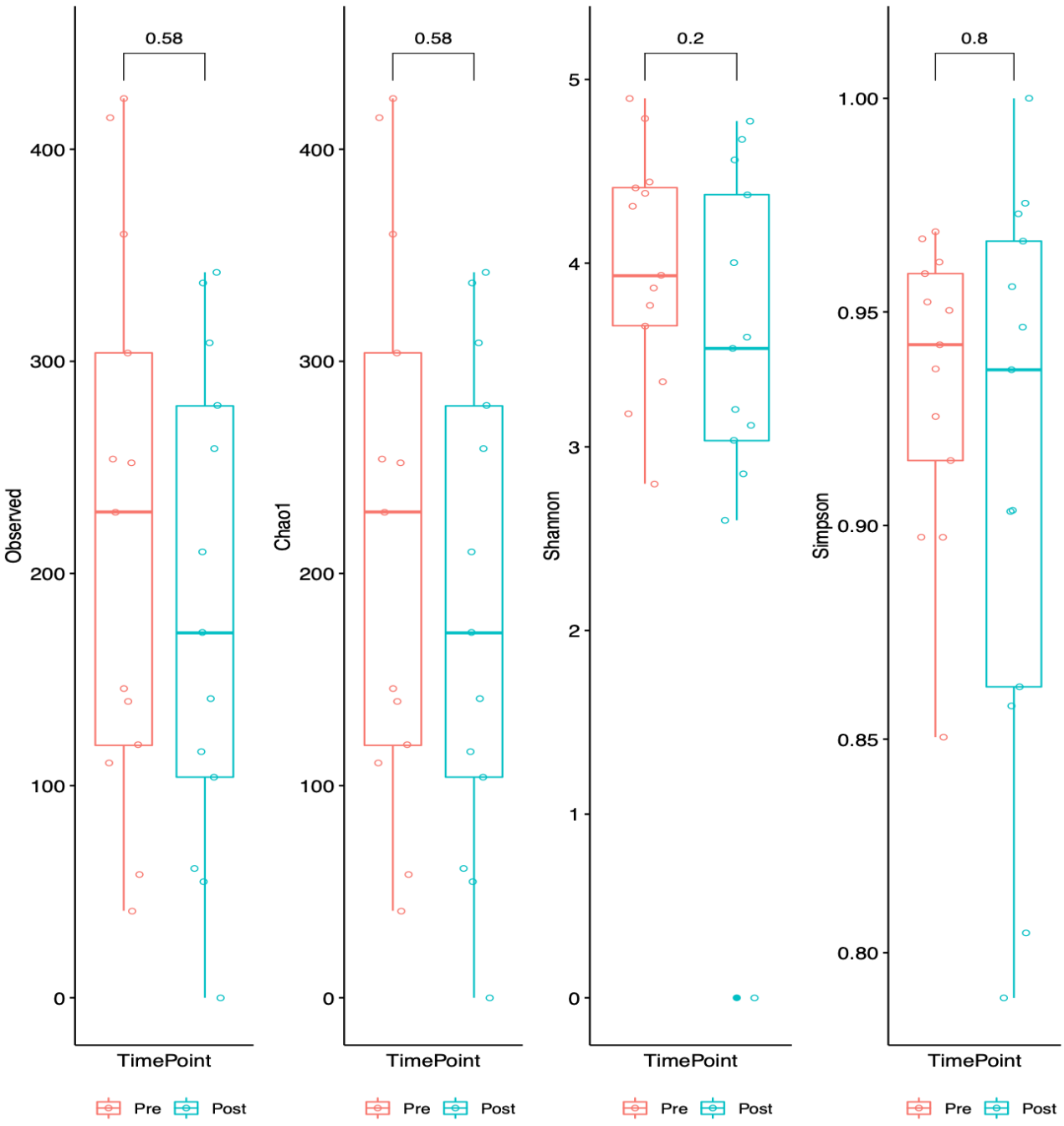


Figure 6.4 Alpha diversity of microbiota from the hand. Box and whisker plots showing alpha diversity (as determined by Observed OTUs, Shannon Evenness, Simpson Index and Chao1) of the skin microbiota of 13 healthy volunteers pre and post application of N. eutropha for a period of 14 days. Statistical significance was assessed using paired t-test. Non-significant decreases were seen in evenness and diversity of the microbial communities using Shannon and Simpson (p = 0.23 and p=0.8 respectively).

6.2.4 Beta diversity pre and post AO+ Mist application

Distance matrix analysis using both weighted and unweighted UniFrac analyses were used to compare the microbiota composition between samples pre and post *N. eutroph*a application. Despite the introduction of a new species with a detectable level of colonisation (Fig 6.1), no statistically significantly differences between pre (day 0) and post (day 14) application was observed for either the arm (weighted p= 0.99 and unweighted p=0.85) (Figure 6.5) or hand (weighted p= 0.48 and unweighted p=0.62) (Fig 6.6).

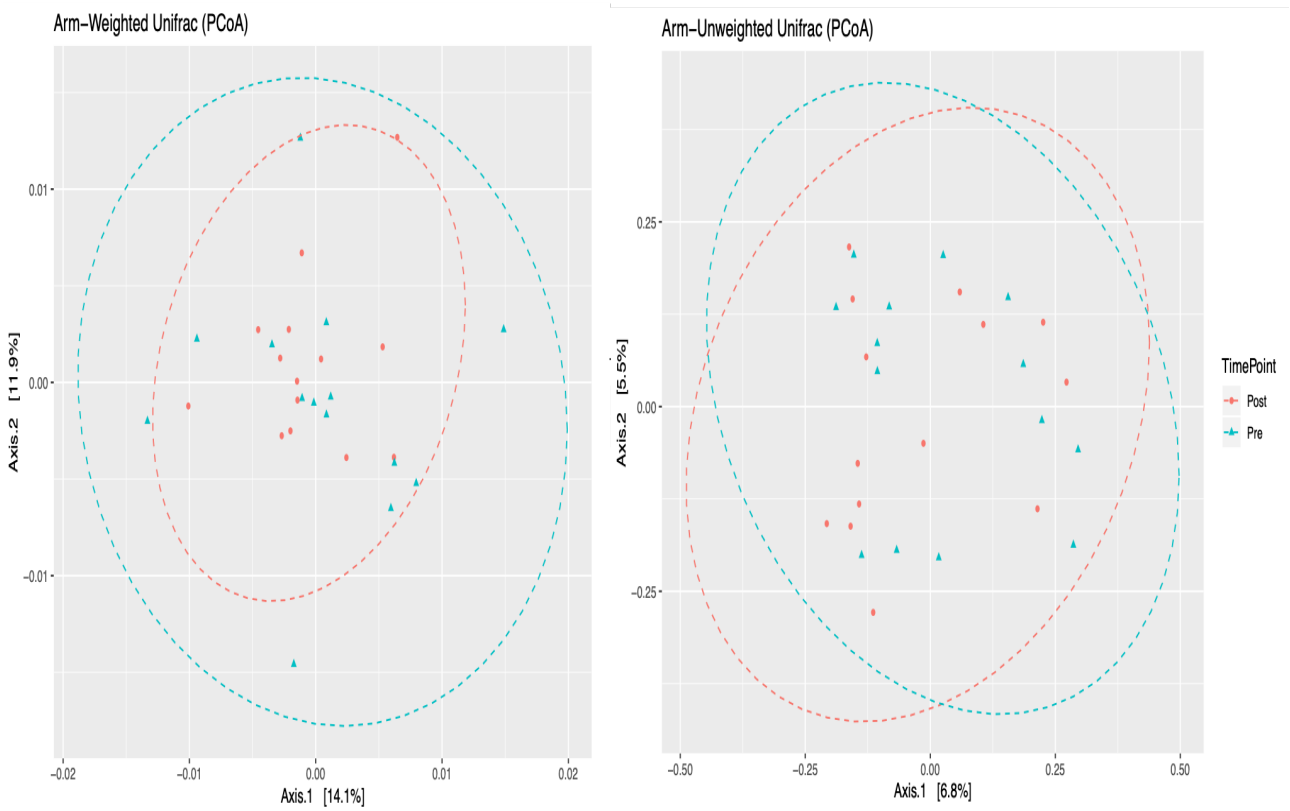


Figure 6.5 Beta Diversity of Arm (antecubital fossa) Microbiomes following *N. eutropha* application (n= 14). Principle Co-ordinates Analysis (PCoA) plots of weighted (left) and unweighted (right) UniFrac. There were no Significant differences were seen for the pre (red dots) and post (blue triangles) application. Ellipses show 95% CI.

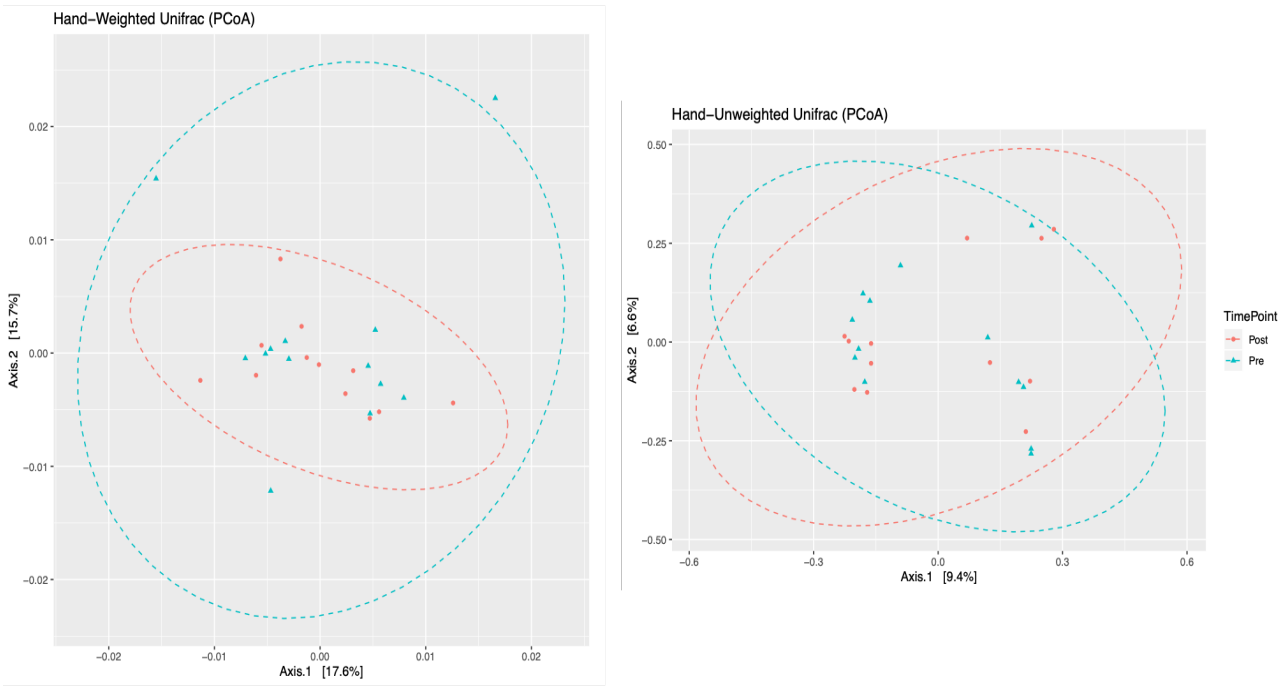


Figure 6.6 Beta Diversity of Hand Microbiomes following *N. eutropha* application (n= 13). Principle Co-ordinates Analysis (PCoA) plots of weighted (leff) and unweighted (right) UniFrac. There were no Significant differences were seen for the pre (red dots) and post (blue triangles) application. Ellipses show 95% CI.

To examine the microbiome changes at an individual level, we compared weighted UniFrac analysis from participants arm and hand microbiota composition changes between pre (day 0) and post (day 14) *N. eutroph*a application as illustrated in (Figure 6.7) and (Figure 6.8). As indicated, most paired samples showed greater similarity post treatment.

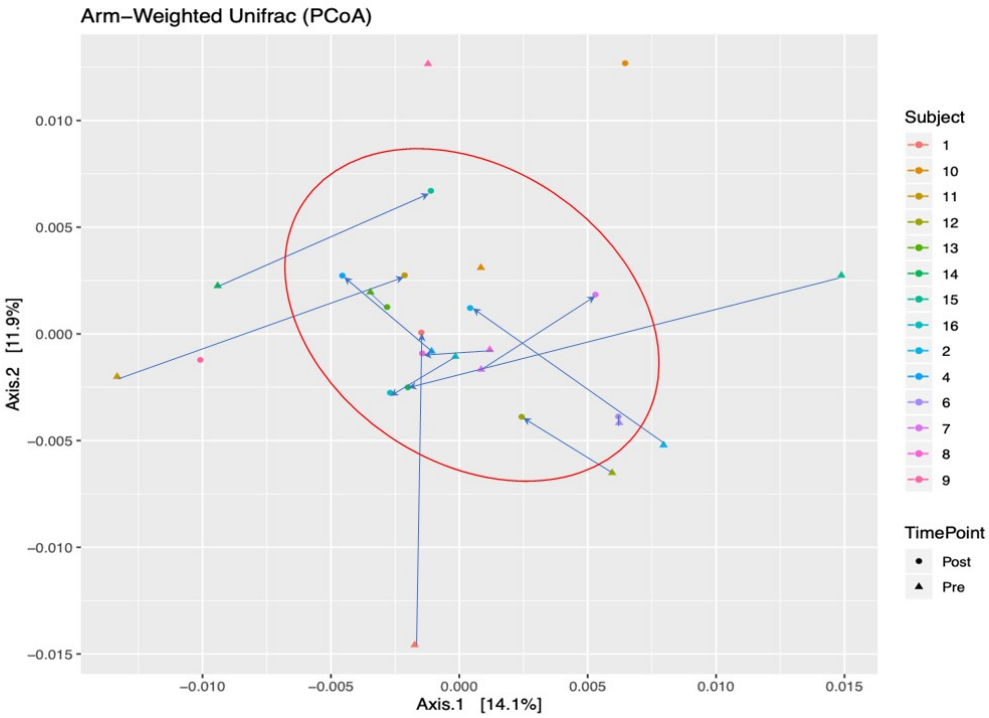


Figure 6.7 Change in microbiota beta diversity pre- and post-application in arm antecubital fossa) samples. PCoA plot showing paired samples with arrows indicating pre- to post application sampling timepoints. Each point is based on weighted UniFrac for the 14 healthy volunteers (coloured individually) with pre (triangles) and post (circles) application of *N. eutropha* shown. Red circle represents clustering of post samples. Ellipses show 95% CI.

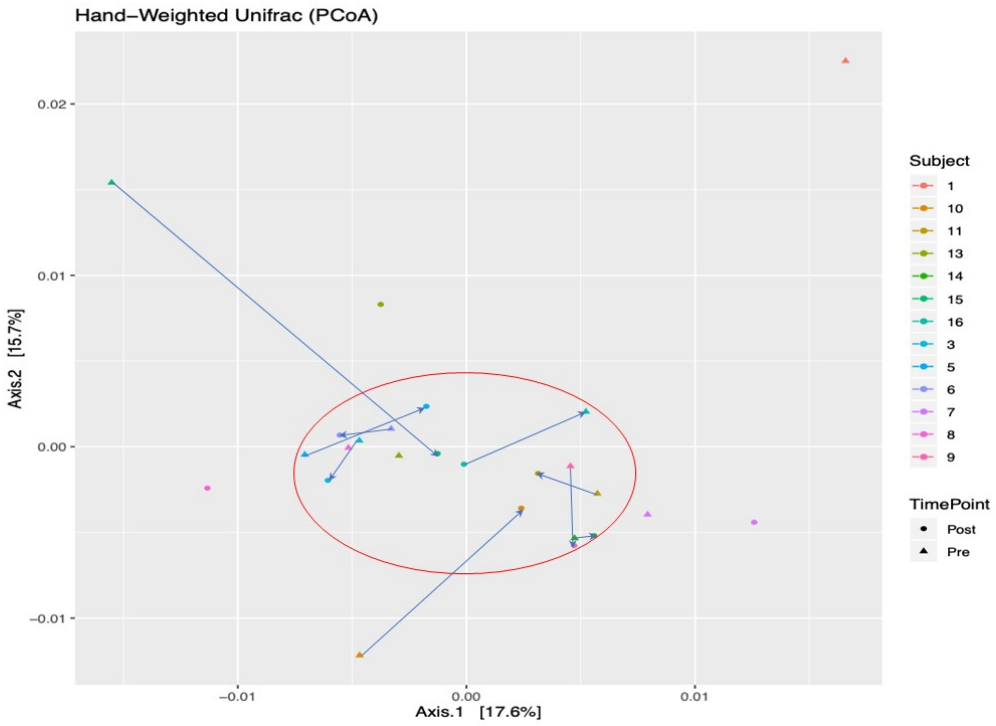


Figure 6.8 Change in microbiota beta diversity pre- and post-application in hand samples. PCoA plot showing paired samples with arrows indicating pre- to post application sampling timepoints. Each point is based on weighted UniFrac for the 13 healthy volunteers (coloured individually) with pre (triangles) and post (circles) application of N. eutropha shown. Arrows represents paired samples (arrow direction points to post sample. Red circle represents clustering of post samples. Ellipses show 95% CI.

6.2.5 Taxonomy pre and post AO+ Mist application for arm (antecubital fossa) and hand- Phylum level.

Differences in the relative abundance of bacterial phyla pre- and post-application of *N. eutropha* are shown in Figure 6.9 and Table 6.2. Dominant phyla were *Actinobacteria, Proteobacteria, Firmicutes, Bacteroidetes* and *Fusobacteria* in both arm and hand. Two tailed t- test showed no significant change in the mean relative abundance of skin microbiota between pre (day 0) and post (day 14) *N. eutropha* application on either arm (p= 0.92) or hand (p= 0.93) of healthy adult human skin was observed. Even though there were no significant differences, the mean of *Actinobacteria* and *Firmicutes* phylum relative abundance were slightly decreased post (day 14) *N. eutropha* application on both arm (45.3% to 32.6% and 20.9% to 16.6) and hand (48.9% to 43.1% and 20% to 18.3), Respectively. In contrast, *Proteobacteria* phylum showed a slight increase on arm (43.6% to 53.7%) and hand (46.6% to 51.7%) this increase were expected as *N. eutropha* were applied in large quantity on the arm and hand skin over 14 days. *Bacteroidetes* and *Fusobacteria* abundance did not change on arm (2.3% to 2.5% and 1.7% to 1.5%) and also on the hand (3.6 to 2.4% and 1.1% to 1.6%). Additionally, 3.8% of Thermi bacteria were detected at the day 0 visit on the hand of one volunteer as shown in Fig 6.9.

Nitrosomonas eutropha mean relative abundance were calculated for post (day 14) N. eutropha application samples on both arm = 40.4% and hand = 20.7% (Table 6.2). The different observed between the *N. eutropha* abundance on the arm and hand suggest that the hand washing frequency may affected the *N. eutropha* abundance on the hand.

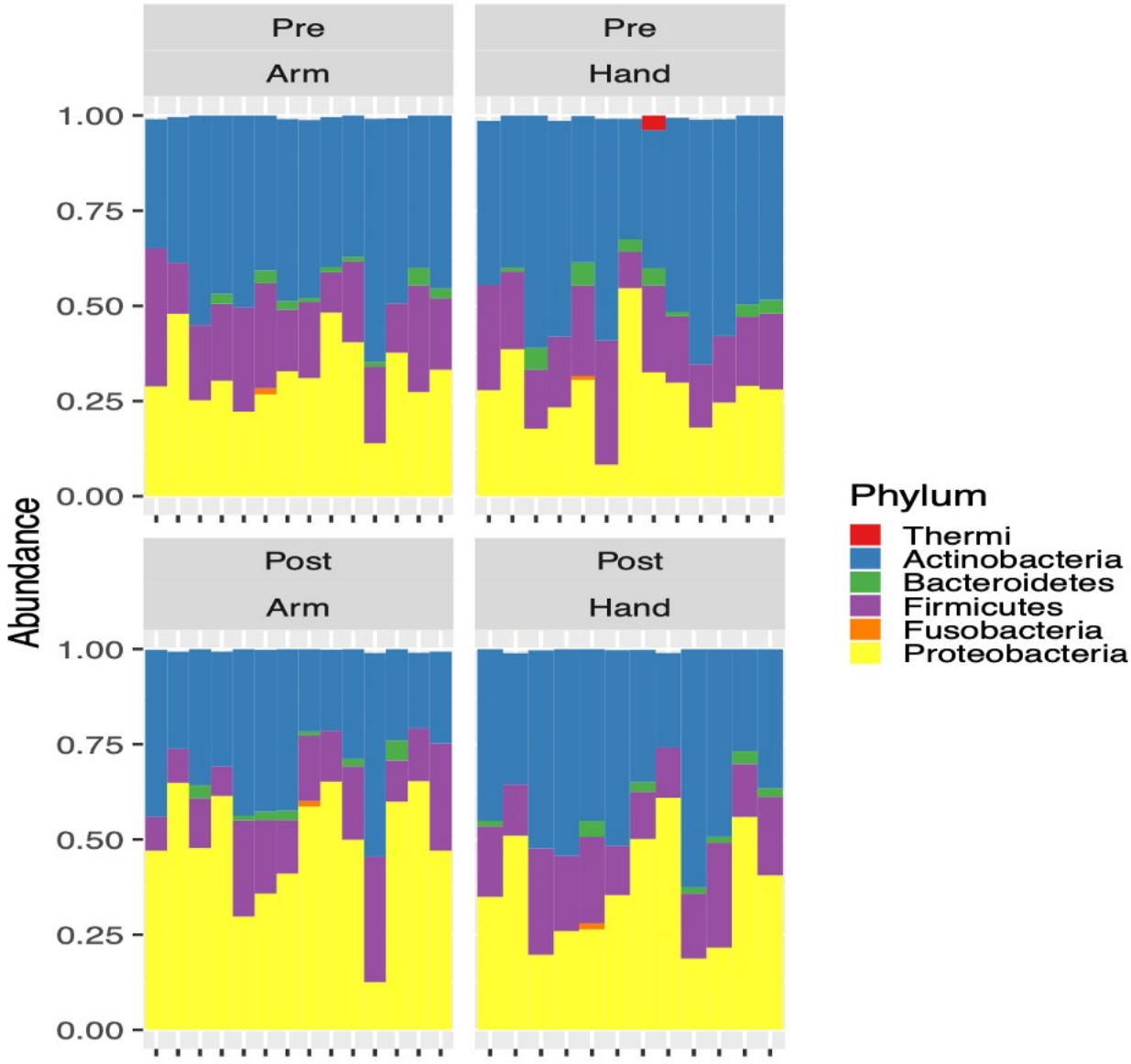


Figure 6.9 Phylum-level Taxonomy of pre and post AO+ Mist application for arm (antecubital fossa) and hand samples. Bar plot showing the relative abundance of dominant bacterial phyla for pre and post-application visit for arm (n= 14, Left panel) and hand (n= 13, Right panel). There were no significant differences in the phylum for the pre (day 0) and post (day 14) *N. eutropha* application on both arm (p= 0.92) or hand (p= 0.93).

Table 6.2 Phylum relative abundance of arm (antecubital fossa) and hand pre and post *N. eutropha* application.

		Mean of Relative Abundance (Range)					
	Actinobacteria	**Proteobacteria**	**Firmicutes**	**Bacteroidetes**	**Fusobacteria**	**Thermi**	***N. eutropha***
Pre-arm phylum	45.3% (33.9% 63.8%)	43.6% (37.7% 48.2%)	20.9% (10.6% 36.3%)	2.3% (1.1% 4.6%)	1.7%	0%	0%
Post-arm phylum	32.6% (19.8% 53.5%)	53.7% (35.8% 65.3%)	16.6% (7.8% 33.0%)	2.5% (1.1% 5.2%)	1.5%	0%	40.4% (2.0% 67.4%)
Pre-hand phylum	48.9% (31.7% 64.3%)	46.6% (38.6% 54.6%)	20.0% (9.5% 32.6%)	3.6% (1.1% 6.1%)	1.1%	3.8%	0%
Post-hand phylum	43.1% (24.8% 62.6%)	51.7% (40.6% 61.0%)	18.3% (12.4% 28.0%)	2.4% (4.2% 1.5%)	1.6%	0%	20.7% (3.2% 60.9%)

6.2.1 Taxonomy pre and post AO+ Mist application for arm (antecubital fossa) and hand-species level.

Bacterial phylogenetic taxonomy is a rank-based classification of bacteria that was developed by Carl Linnaeus. Species are assigned to a genus that is a lower level of a hierarchy of ranks including: family, suborder, order, subclass, class, division/phyla, kingdom and domain.

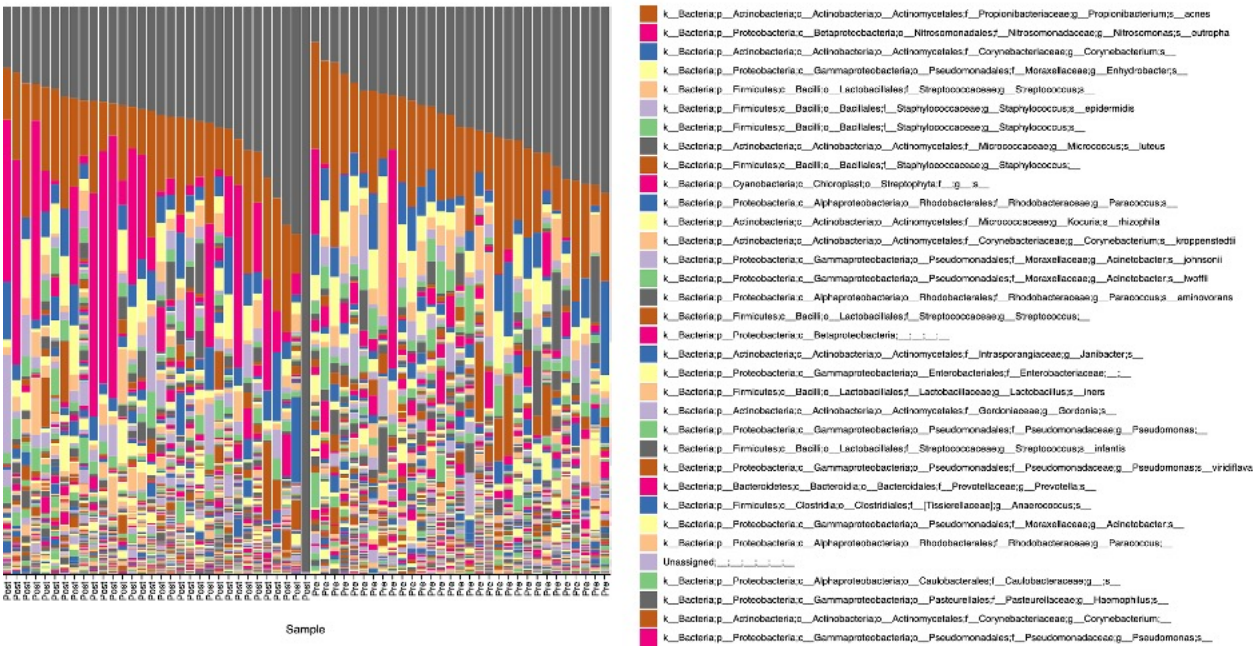


Figure 6.10 Taxonomy identification for pre and post AO+ Mist application for arm and hand-species level.

6.2.2 Impact of AO+ Mist Application on *Nitrosomonas* and *Staphylococcus* genus.

In order to further characterise the effect of *Nitrosomonas* on the microbiome at a genus level, the *Staphylococcus* genus was examined (Figure 6.11). The relative abundance of Nitrosomonas ranged from 0% to 57.6%%, with a mean of 30.2%f or Arm and from 0% to 43%, with a mean of 14.3%f or the Hand. Genus relative abundance percentages were calculated for pre (day0) and post (day14) of arm (Table 6.3) and hand (Table 6.4). Although a slight increase in the mean relative abundance of *Staphylococcus* genus after 14 days of Nitrosomonas application was observed on the arm (9.0% to 10.0%) and hand (7.8% to 10.2%), neither was statistically significant (arm= 0.97 and hand p= 0.78).

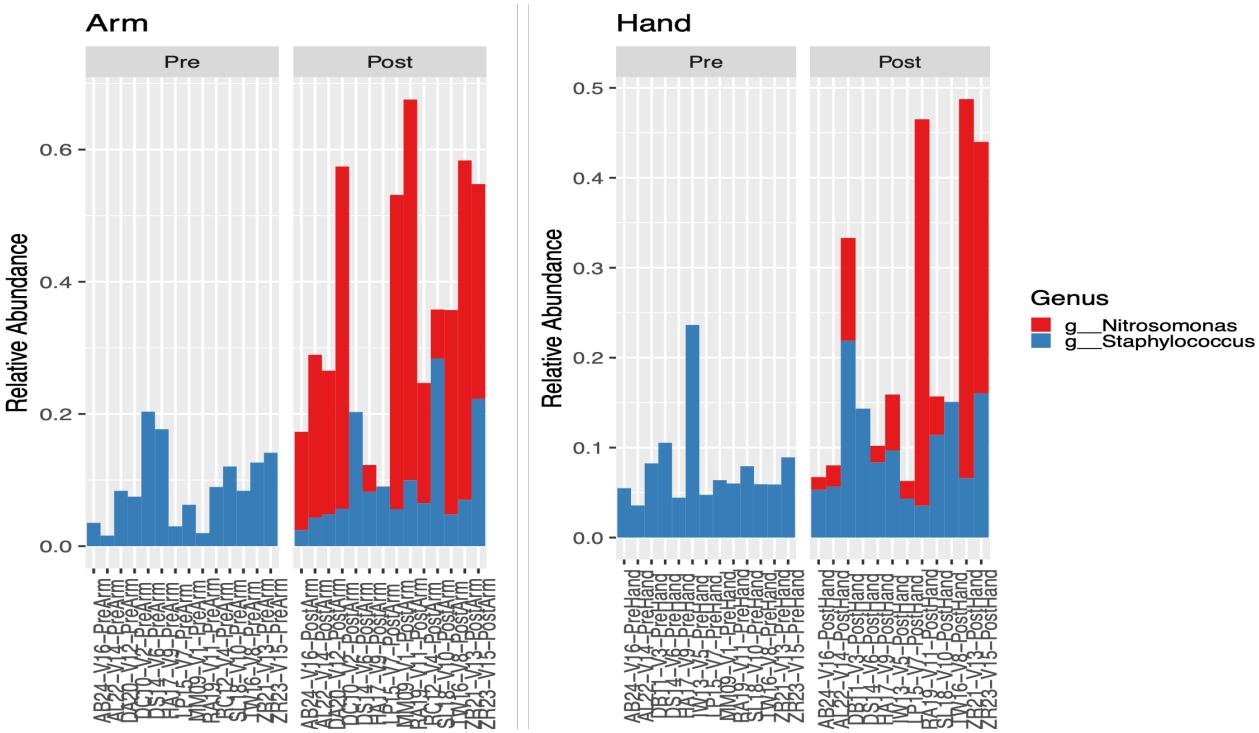


Figure 6.11 Relative Abundance of Nitrosomonas and Staphylococcus genus. Graph showing the relative abundance against the pre and post visit for both arm (n= 14, Left panel) and hand (n=13, Right panel). Red denotes *Nitrosomonas* where blue represents the *Staphylococcus* abundance.

Table 6.3 Relative abundance of *Staphylococcus* and *Nitrosomonas* in arm (antecubital fossa) samples.

	Genus	
	Staphylococcus	**Nitrosomonas**
Pre-application	9.0% (1.6%- 20.3%)	0%

Post-application	10.0% (2.4%- 28.4%)	30.2% (4.0%- 57.6%)

Table 6.4 Relative abundance of *Staphylococcus* and *Nitrosomonas* in hand samples.

	Genus	
	Staphylococcus	**Nitrosomonas**
Pre-application	7.8% (3.6%- 23.6%)	0%
Post-application	10.2% (3.6%- 21.9%)	14.3% (1.4%- 43.0%)

6.2.1 Impact of AO+ Mist Application on *Staphylococcus* species.

In order to further characterise the effect of *Nitrosomonas* on the microbiome at a species level, the *Staphylococcus* species was examined (Figure 6.12). The relative abundance of *Staphylococcus epidermi*s ranged pre-*N. eutropha* application from 1.8% to 19.8% with a mean of 6.9% and post application from 1.9% to 27.1%, with a mean of 8% for Arm. As similar as the arm, *Staphylococcus epidermi*s relative abundance on the hand was calculated, pre-application from 1.9% to 26.7%, with a mean of 6.8% and from 1.5% to 16.4%, with a mean of 8.8% for post-application. Genus relative abundance percentages were calculated for pre (day0) and post (day14) of arm and hand (Table 6.5). Although a slight increase in the relative abundance of *S. epidermis* species after 14 days of Nitrosomonas application was observed on the arm (6.9% to 8.0%) and hand (6.8% to 8.8%), neither was statistically significant (arm= 0.87 and hand p= 0.92).

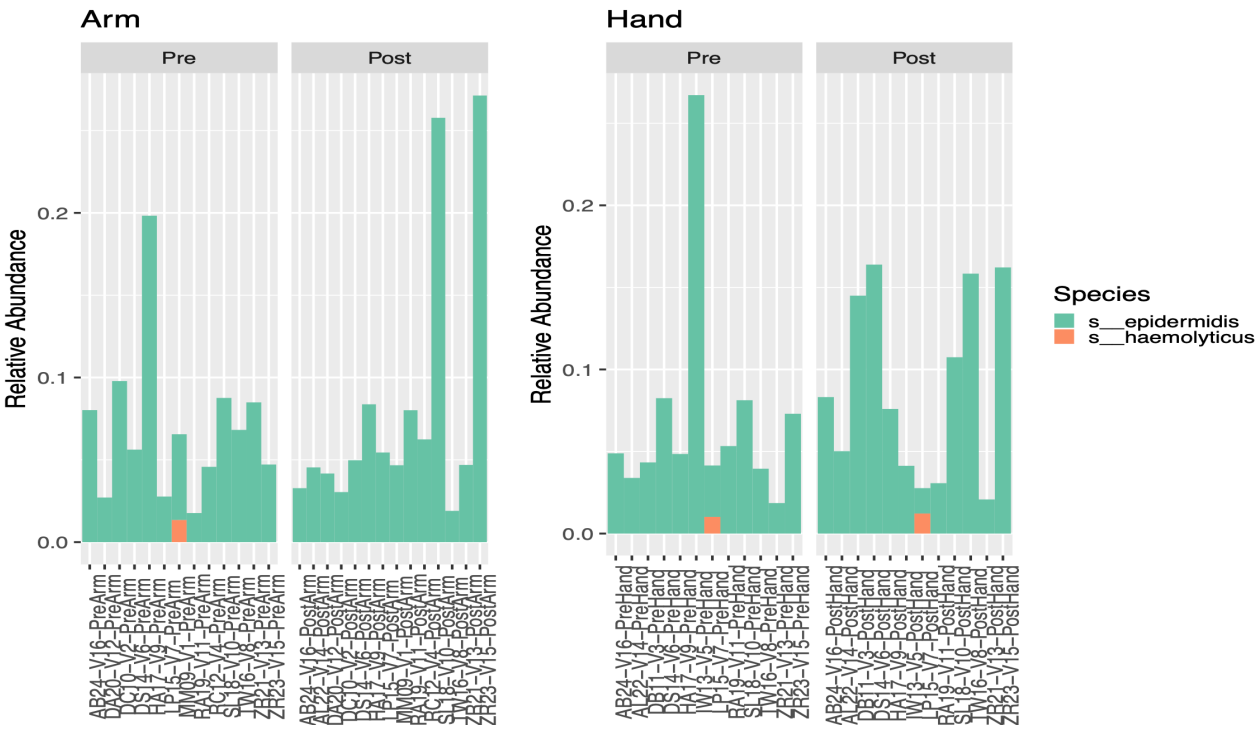


Figure 6.12 Relative Abundance of *Staphylococcus* species. Graph showing the relative abundance against the pre and post visit for both arm (n= 14, Left panel) and hand (n=12, Right panel). Green denotes *S. epidermis* where orange represents the *S. haemolyticu* abundance.

Table 6.5 Relative abundance of *Staphylococcus* species in arm (antecubital fossa) and hand samples.

	Staphylococcus Species	
Arm	**"Staphylococcus epidermidis"**	**"Staphylococcus haemolyticus"**
Pre-application	6.9% (1.8%- 19.8%)	1.3%
Post-application	8.0% (1.9%-27.1%)	0%
Hand	**"Staphylococcus epidermidis "**	**"Staphylococcus haemolyticus"**
Pre-application	6.8% (1.9%- 26.7%)	1.0%
Post-application	8.8% (1.5%- 16.4%)	1.2%

6.2.2 Fold change of genus upon *Nitrosomonas* application

To better visualise the bacterial genus composition changes upon application of *N. eutropha* D23 the top 20 most significant ($p \leq 0.05$) fold changes (FC) compared to baseline are presented as a summary in Figure 6.13 below. Aside from the expected increase in Nitrosomonas, both Acinetobacter and Novosphingobium increased markedly. Conversely, Staphylococcus and Dermabacter both exhibited >20-fold reduction in abundance post-application.

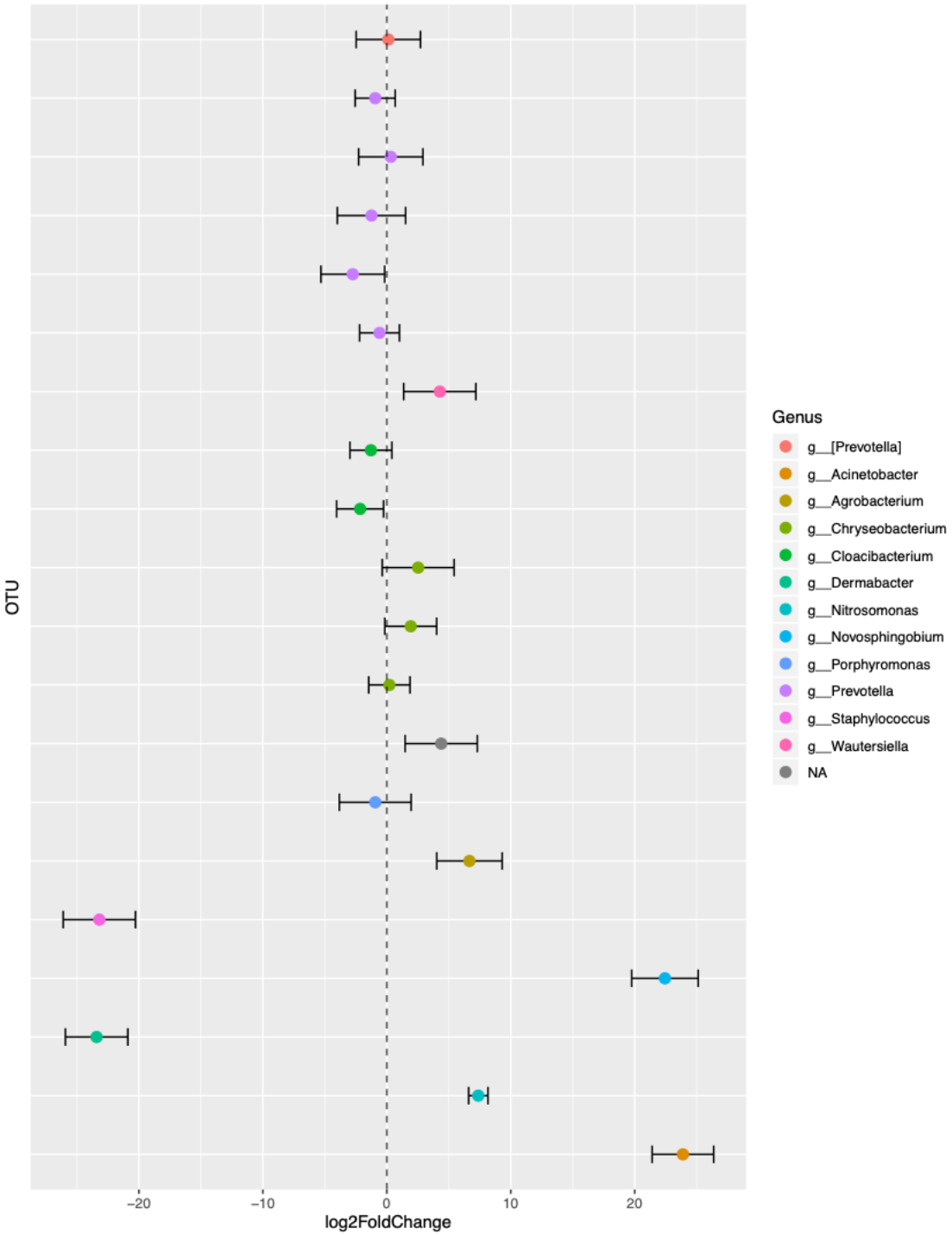


Figure 6.13 Fold change (compared to baseline levels) of genus composition in arm (antecubital fossa) and hand.

6.2.3 Correlation between NO emanation and *Nitrosomonas* abundance

To assess the possible relationship between *Nitrosomonas* abundance and NO emanations, the *Nitrosomonas* abundance values were plotted against their respective NO emanations for each of the body sites examined. Linear regression was carried out to detect any statistically significant differences. As illustrated in Figure 6.14, these data indicate no correlation between semi-quantitative *Nitrosomonas* measurements and NO emanation in the arm (R^2= 7.267e-008, p = 0.99) or hand (R^2= 0.08, p = 0.25).

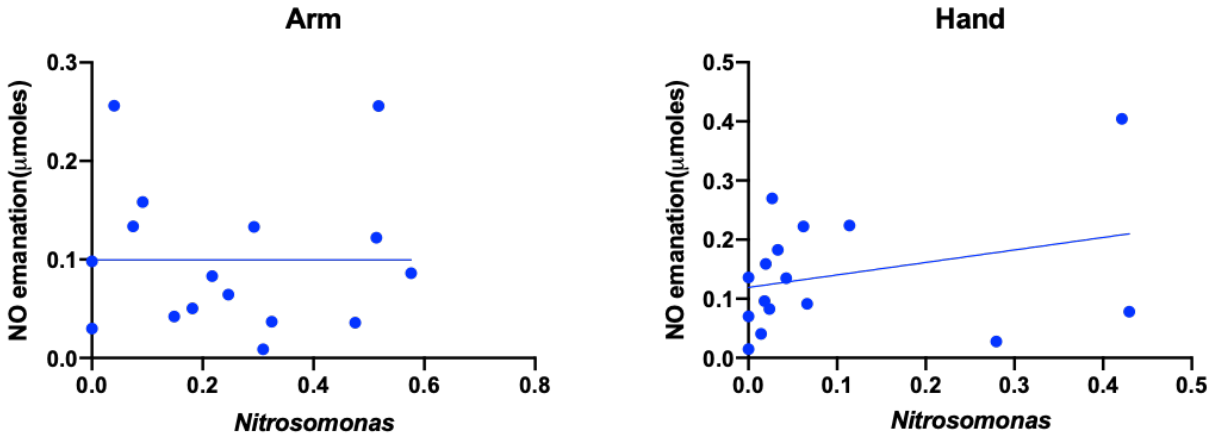


Figure 6.14 Correlation between *Nitrosomonas* present on NO emanations. Linear regression plots showing the effect of 14 days *Nitrosomonas* application on NO emanations in the arm (Left panel) and hand (Right panel). There was no statistically significant correlation was detected in either the arm or hand.

6.3 Discussion

The impacts of pro- and/or pre-biotic formulations on skin microbiomes is an area with a number of unknowns. Principal among these is the impact of a live bacterium on the pre-existing resident microflora of the skin. Using an optimised sampling technique (Chapter 3), analysis of the V1-3 hypervariable regions of 16S rRNA was used to capture the changes in the bacterial composition on the arm and hand of 16 healthy volunteers before and after 14 days of daily *Nitrosomonas eutropha* D23 (AO+ Mist™, MotherDirt™, AOBiome LLC) application.

The bacterial taxonomic identification revealed that the data generated from the arm and hand skin of 16 healthy volunteers after 14 days of daily AO+ Mist application was similar to the Grice 2009 findings which showed at the phylum level Actinobacteria (51.8%), Firmicutes (24.4%), Proteobacteria (16.5%), and Bacteroidetes (6.3%) and chapter 3 results at the phylum level (a similar abundance of bacteria phyla on both visit and body region was identified). The four main bacterial phyla in the skin identified pre and post were unchanged in relative abundance including: *Actinobacteria, Firmicutes, Bacteroidetes* and *Proteobacteria.* This composition remained largely unchanged by perturbation with *N. eutropha*, but most samples showed a loss of evenness and diversity (alpha diversity) after application – a consequence of the abundance of a single new species being introduced.

Interestingly, while the microbiome remained grossly stable, at the Genus level the data suggested that *Nitrosomonas* application led to slight decrease (not statistically significant) in the *Staphylococcus* abundance for both arm (Table 6.3) and hand (Table 6.4) after 14 days of daily AO+ Mist application (Figure 6.11). In contrast, looking at *S. epidermidis* in isolation showed a slight increase (not statistically significant) in abundance. However, if examining hand and arm data together, there was a significant overall decrease in Staphylococcus abundance (Figure 6,13). This suggests a complex interaction which requires greater resolution at the species level. Also observed were decreases in Dermabacter, a common coloniser of the skin. In contrast, increases in Acinetobacter, another coloniser of the skin (Berlau et al., 1999) and Novosphingobium were also observed. The beneficial impact of the former is intriguing given that it has been previously associated with anti-inflammatory responses to allergens (Fyhrquist et al., 2014). Whilst the functional relevance of these alterations is not clear, it does appear that application of *N. eutropha* D23 may potentially alter the microbiome.

Finally, the relationship between *Nitrosomonas* abundance and NO emanations indicated that there is no evidence of correlation between the semi-quantitative measurement of *Nitrosomonas* abundance and NO emanation in the arm. Whereas, on the hand weak correlation was identified. This may be a consequence of key regulatory factors that link the function of AOB with NO emanation. A primary suspicion would lie with the increased sweating capacity of the hand. Sweat is likely to provide an abundant source of ammonia which would enhance AOB NO generation.

There are limitations that need to be addressed in future studies. Firstly, whilst there is clear evidence of sustained presence of *N. eutropha* during periods of application, longitudinal data of persistence would determine if longer-term colonisation had occurred. This was not possible in the timeframe of this project, although samples to examine this have been taken. In addition, several volunteers had to be excluded from the analysis owing to *N. eutropha* contamination in some samples. This was determined by sequence identity matching using blast. It is likely this was a result of cross-contamination when sampling individuals who were at different timepoints. Laboratory cross-contamination is less likely given the samples were processed in batch. Stricter controls over the timing and location of visits could be implemented in future to avoid this issue.

To our knowledge this is the first example of the sustained application of a Nitrosomonas sp. onto human skin with an in-depth examination of the impacts on the skin microbiome.

Chapter 7 Conclusions and future plan

Understanding the skin barrier is of central importance to human physiology. The skin is the largest organ and demonstrates a wide spectrum of physiological functions which facilitate its various biological characteristics including host defence, water regulation, thermoregulation and micronutrient synthesis. However, in settings of cutaneous inflammation, these processes are significantly disrupted and can result in disordered systemic inflammation and biochemistry. Over the last 20 years it has become increasingly apparent that epithelial surfaces harbour a diverse population of microbes which form an ecosystem known as the microbiome. Using culture independent techniques such as ribosomal RNA 16s analysis, the true diverse structure of human microbiome systems has been uncovered. However, as yet, no consensus exists on the precise technological approach to optimal skin sampling. Unlike invasive pathogens, the healthy skin microbiome offers specific advantages to the host including fine regulation of the epithelial barrier itself and defence against infection. Yet, microbes can be exquisitely sensitive to environmental factors and for that reason, significant interindividual differences have been demonstrated in the microbiome populations of different body sites, individuals, populations, and climates. However, human social development has imprinted significant differences in the environmental exposures of skin at a pace which has outstripped that of evolution. In pre-industrial development, human skin contact with soil microbes was significantly greater through direct contact but also social habits such as washing with soap were very different and likely to facilitate cutaneous survival of bacterial species that may not survive modern exposomes.

The common soil bacteria, Nitrosomonas sp. have been well characterised and are known to facilitate the oxidation of ammonia to form nitrite, which is a key component of the pathway to NO formation by further reduction in the epithelial surface. This process has labelled them within the group of ammonia oxidising bacteria (AOB). Recent published analyses of cutaneous microbiome samples have not identified AOBs on human skin. The potential to modify cutaneous NO, by variations in the cutaneous microbiome is of significant physiological interest through its known regulation of epithelial barrier function, inflammatory pathways and biofilm formation. Recently a commercial preparation licensed for application to the skin of a of Nitrosomonas eutropha D23 in a cosmetic formulation called AO+ Mist™, MotherDirt™, AOBiome LLC has been manufactured for general use.

In this thesis, the hypothesis that AOB would be able to participate in the skin microbiome, and that they may regulate skin NO metabolism was tested. I first set out to determine the optimal sampling methodology for cutaneous microbiome analysis using culture independent techniques. To approach this in Chapter 3, I compared previously reported sampling techniques (swab, scrape, post scrape swab, and tape) as well as analysis of the hypervariable 16s RNA regions: V1-3 versus V3-4. V1-3 showed significantly greater amplicon frequency, throughout all sampling methodologies suggesting a greater sensitivity for detection of low frequency species. This was reflected in analyses of alpha diversity which showed that V1-3 analyses

demonstrated a greater richness and diversity. Taxonomic identification showed that V1-3 provided a stronger representation of the fusobacterial class, whereas V3-4 favoured Actinobacteria. Bacteria of specific interest including firmicutes (including Staphylococci) were evenly represented in both systems but at the species level, V1-3 was superior at distinguishing staphylococci. Between the sampling methods employed it was apparent that skin scrape assays were inferior, but of the others, minimal difference was identified and therefore for the rest of the study, skin swab sampling was employed.

In chapter 4, I utilised a novel approach to measurement of NO emanation from the skin and characterised the NO emanation from arm, hand, head and foot of volunteers. Whilst non-significant, the NO emanation on the foot showed the highest levels. In addition, although pH was constant across the various body sites, the foot also showed the highest trans-epidermal water-loss measurements (TEWL). This was likely due to basal sweating rather than poor epidermal function as the skin barrier on the foot is known to be more effective than the other sites studied. It was postulated that the foot NO emanation may be facilitated by increased sweat from the high density of sweat glands on the foot. In support of this hypothesis, after a sweat challenge test (post exercise), NO emanations were seen to rise significantly, and it is likely that this process is via chemical reduction of nitrite in sweat. AO+ mist, a commercial application of viable *N. eutropha* (AOBs) sprayed on the skin also caused an upregulation of NO emanation. Importantly, it was concluded that this effect was distinct from water application to the skin.

To study the possibility that AOB application to the skin may modify NO emanation and cutaneous function, data from a challenge study following 2-week application of AOBs to volunteers' skin is reported in Chapter 5. Firstly, as hypothesised, NO emanations were higher after AOB application, although this didn't reach statistical significance and was not seen on the foot. Interestingly, 2-week application of AO+ mist provided a profound shift increasing skin pH at all sites. This is important, as it is known that increased alkalinity of skin is detrimental to the skin barrier. In line with this TEWL measurements increased in the arm and hand following AO+ application, but this difference did not achieve statistical significance. Because the body areas measured were of different surface areas, the hand was used to correlate skin surface area with NO emanation. Interestingly hand surface area did not correlate with NO emanation thereby suggesting that individual cutaneous factors were more important in regulating NO emanation. It has previously been suggested that cutaneous acidification regulated NO emanation, however, data presented here did not show a correlation between skin pH and NO. Additionally, there was little evidence of correlation between skin TEWL and NO emanation after AOB application suggesting that in the context of AOBs, increased sweating provided no additional resource for NO generation on the skin. The cutaneous effects of AOB application were generally small, therefore it is likely that any down-stream systemic inflammatory effects would be smaller. Indeed, systemic circulatory levels of nitrite, nitrate, cGMP and RXNO were unaffected by AO Mist application as measured in saliva, sweat, RBC and plasma. Although no significant changes were found in

oxidative pathways, as indicated by the concentrations of specific biomarkers (e.g. 4-HNE, TBARS) related to oxidative stress and the anti-oxidative marker (FRAP), a statistically significant elevation of plasma total free thiols (TFTs) was identified, which is a new finding. This is of note, because TFT elevation has been associated with reduced cardiovascular morbidity and mortality (Frenay et al., 2016, Koning et al., 2016), which may suggest that investigation of AOB application to the skin should be further explored.

In Chapter 6, I confirmed that *N. eutropha* can survive on human skin, which is a new finding. Although introduction of Nitrosomonas to the skin microbiome modified the Shannon and Simpson alpha diversity measurements of the microbiome from the arm, the differences were small and not demonstrated in the hand. Interestingly, the measurements of community beta diversity, reflecting microbiome chanes between sampling points showed an increased similarity after AOB application. This may suggest that AOB can contribute to skin microbiome stability. In addition, taxonomic evaluation showed no evidence for disruption of the skin microbiome at the phylum or genus level as a consequence of Nitrosomonas application on the skin. These are important findings as they suggest that were application of AOB to the skin be employed to regulate skin physiology including NO pathways, there would be minimal disruption to the skin microbiome expected. Furthermore, this offers a proof of concept that perturbation of the skin microbiome with one species does not always lead to dramatic changes in relative abundance of others. This is significant as it has been shown that during flares of atopic dermatitis, relative abundance of *S. aureus* increases at the expense of loss of diversity of the rest of the community.

The findings presented here reflect an important advancement in our understanding of the role of the microbiome in regulation of skin inflammation and NO physiology. The potential for exogenously applied bacteria to survive in the skin microbiome is demonstrated for the first time. Additionally, that microbiome modification can regulate key skin physiology including Skin pH was demonstrated. The impact on systemic inflammation of skin AOBs is also indicated by the induction of a TFT response which has been shown to offer cardioprotection. In addition, studies of the microbiome confirm that whilst significant differences in diversity exist between different body sites, these are stable in the presence of challenge with *Nitrosomonas eutropha.*

There were important limitations to this work. As discussed in Chapter 3, the use of a small number of volunteers (n=4) and only one body site for the comparisons of sampling methods and 16S rRNA target makes extrapolation difficult. Additional quantitative data on bacterial biomass and/or the detection of specific commensals using targeted qPCR assays would have given greater detail about the efficacy of the methods chosen. To overcome these limitations, a larger cohort to analyse using 16S rRNA and NO/TEWL/pH testing including post-exercise, would be beneficial. Additionally, the lack of a control group and the existence of only one time-point for outcome assessment post AOB application would be important aspects to address in

future work. Additionally, 16S rRNA analysis does not distinguish viable from non-viable species and this would be an important point of clarification for *N. eutropha* on the skin.

Future work is warranted to underscore and advance the findings presented here. Principally, this work will take three approaches:

1. Investigation of survival of Nitrosomonas on the skin
    a. Whilst this thesis provides convincing evidence for N. eutropha survival following daily application. It is important to understand how long the bacteria can survive without replenishment and whether long-term incorporation into the microbiome is achievable. A long-term study of volunteers post AOB application is underway.
    b. It is likely that certain body sites are better suited to N. eutropha adaption, and comparison of survival in various regions will be explored e.g. wet versus dry versus sebaceous
    c. Different washing practices are likely to modify N. eutropha survival, and these will be explored in a clinical trial
2. Optimisation of NO regulation by modification of the skin
    a. The increase in NO emanation by AOBs was convincing but not statistically significant. The manufacturer has since released a higher density AOB spray, and early work suggests a much higher biological effect of this approach. Therefore, it will be important to compare the two spray applications in terms of ability to regulate NO emanation.
    b. In this thesis, the co-regulation of NO emanation between sweating was proposed. Whilst circumstantial data, including body site and exercise supported this possibility, it is important to address the question in detail. Therefore, injection of anticholinergic blocking agents (Botox) will be employed to completely abrogate sweating in small areas of skin. NO emanation will then be compared pre and post exercise, and application of AOBs. Additionally, components of sweat will be applied to non-sweating areas in a dose response to measure the effect on NO emanation.
3. Validation of skin microbiome robustness to external pathogen introduction
    a. While introduction of Nitrosomonas to the skin microbiome has been convincingly demonstrated here, the level of analysis was not possible below the genus level. Therefore, a comprehensive species level analysis will be undertaken post Nitrosomonas application.
    b. Nitrosomonas is likely to show species specific effects and may not reflect the outcome of introduction of pathogenic bacteria to the skin. Using in vitro skin models and artificial microbiome compositions with species of interest including *S. aureus*, *S. epidermidis*, *Cutibaterium* (formerly *Propionibacterium*) *acnes*, and Nitrosomonas, we will study the microbial composition following addition of Pseudomonas sp. and *Staphylococcus capitis*.

Appendix A

A.1 Ethical Approval

HSC Business Services Organisation

Office for Research Ethics Committees Northern Ireland (ORECNI)

Customer Care & Performance Directorate
Lissue Industrial Estate West
Rathdown Walk
Moira Road
Lisburn
BT28 2RF
Tel: 028 95361400
www.orecni.hscni.net
HSC REC B

HSC REC B
14 November 2016

Professor Martin Feelisch
Faculty of Medicine, University of Southampton
Mailpoint 801, South Academic Block, Tremona Road
Southampton
SO16 6YD

Dear Professor Feelisch

Study title:	**Investigations into the emanation of nitric oxide from adult human skin: rates of release, regional heterogeneity and impact of the presence of ammonia oxidizing bacteria.**
REC reference:	**15/NI/0180**
Amendment number:	**Substantial Amendment #1**
Amendment date:	**04 November 2016**
IRAS project ID:	**185688**

The above amendment was reviewed by the Sub-Committee in correspondence.

Ethical opinion

The members of the Committee taking part in the review gave **a favourable ethical opinion** of the amendment on the basis described in the notice of amendment form and supporting documentation.

Approved documents

The documents reviewed and approved at the meeting were:

Document	*Version*	*Date*
Covering letter on headed paper [_23883_16-10-28_100758_elplconfirmationofcoverletter201617]		14 July 2016
Notice of Substantial Amendment (non-CTIMP) [Notice of substantial amendment #1]		04 November 2016
Other [_23883_16-10-04_054249_poster21-2]	V2.1	27 September 2016
Other [_23883_16-10-04_054332_pigmentationphenotypeassessmenttoolmodfh21]	V2.1	27 September 2016
Other [_23883_16-10-	V2.1	

Appendix B

B.1 R&D Approval

University Hospital Southampton NHS Foundation Trust NHS

Please reply to: Research and Development
SGH - Level E, Laboratory & Pathology Block, SCBR - MP 138
Southampton University Hospitals NHS

Telephone: 023 8120 5078
Fax: 023 8120 8678
E-mail: sharon.davies-dear@uhs.nhs.uk

Professor Martin Feelisch
University of Southampton
Faculty of Medicine
Mailpoint 801, South Academic Block
Tremona Road
Southampton
SO16 6YD

03 March 2017

Dear Professor Feelisch

ID: RHM CRI0320 **Investigations into the emanation of nitric oxide from adult human skin: rates of release, regional heterogeneity and impact of the presence of ammonia oxidizing bacteria.**

Thank you for sending us a copy of amendment 1 dated 04 November 2016, which has been assessed by R&D. We are pleased to inform you that this amendment does not affect local management approval of your research. We have therefore updated our database and your project file.

Please forward all future amendment applications and approvals to us.

Yours sincerely

Sharon Davies-Dear
Research Governance Officer

Appendix C

C.1 Consent Form

UNIVERSITY OF Southampton

CONSENT FORM (*Version2.3*)

Study title: Investigations into the emanation of nitric oxide from adult human skin: rates of release, regional heterogeneity and impact of the presence of ammonia oxidizing bacteria.

Researcher name: Principle Investigator: Professor Martin Feelisch
Ethics reference: IRAS 185688 ERGO 14838

Please initial the box(es) if you agree with the statement(s):

I have read and understood the information sheet (19 October 2018 Version 2.3) and have had the opportunity to ask questions about the study.	☐
I agree to take part in this research project and agree for my data to be used for the purpose of this study	☐
I understand my participation is voluntary and I may withdraw at any time without my legal rights being affected	☐
I am happy to be contacted regarding other unspecified research projects. I therefore consent to the University retaining my personal details on a database, kept separately from the research data detailed above. The 'validity' of my consent is conditional upon the University complying with the Data Protection Act and I understand that I can request my details be removed from this database at any time.	☐

I understand that information collected about me during my participation in this study will be stored on a password protected computer and that this information will be used for the purpose of this study and will be retained for contact about future research studies.. All files containing any personal data will be made anonymous.

Name of participant (print name).....

Signature of participant.....

Date.....

[19/10/2018] [2.3]

Appendix D

D.1 Poster

Version2.2

Healthy skin & pro-biotics

What is this study?

- *We're investigating what happens when healthy volunteers use a special cosmetic on their skin that contains bacteria, compared to a high-street moisturiser.*
- *We would like to test how healthy skin is functioning before and after two weeks of cosmetic application. In particular, we're looking at nitric oxide on the skin, which is a normal component of the healthy circulatory system including skin microbiota.*
- *In order to get the full picture of the nitric oxide in the body we want to take different samples including blood, saliva, urine, sweat, skin swab and skin scrape.*
- *People of all ages can be part of this*

Who is Eligible?

- *≥ 18 years*
- *Fit to perform exercise*
- *Not known to be pregnant*

What is involved?

- *3 visits to the Wellcome Trust Physiology Laboratory at Southampton General Hospital*

How to get involved?

Researcher Contact

Rfeef Alyami 07426718095

Rya1r14@soton.ac.uk

Faculty of Medicine Ethics Committee reference: *14838*
27/09/2016

UNIVERSITY OF Southampton

University Hospital Southampton NHS
NHS Foundation Trust

This student project is sponsored and funded by the University of Southampton

Rfeef Rya1r14@soton.ac.uk 07476985637	*Rfeef Rya1r14@soton.ac.uk 07476985637*	*Rfeef Rya1r14@soton.ac.uk 07476985637*	*Rfeef Rya1r14@soton.ac.uk 07476985637*	*Rfeef Rya1r14@soton.ac.uk 07476985637*	*Rfeef Rya1r14@soton.ac.uk 07476985637*	*Rfeef Rya1r14@soton.ac.uk 07476985637*	*Rfeef Rya1r14@soton.ac.uk 07476985637*	*Rfeef Rya1r14@soton.ac.uk 07476985637*	*Rfeef Rya1r14@soton.ac.uk 07476985637*	*Rfeef Rya1r14@soton.ac.uk 07476985637*	*Rfeef Rya1r14@soton.ac.uk 07476985637*	*Rfeef Rya1r14@soton.ac.uk 07476985637*

Appendix E

E.1 Participant information sheet

UNIVERSITY OF Southampton

Version 2.3

Participant Information Sheet

Study Title: Investigations into the emanation of nitric oxide from adult human skin: rates of release, regional heterogeneity and impact of the presence of ammonia oxidizing bacteria.

Researcher: **IRAS Number: 185688** **ERGO number: 14838**

Please read this information carefully before deciding to take part in this research. If you are happy to participate you will be asked to sign a consent form.

What is the research about?

This is a Ph.D. student project; this research is being conducted by the department of clinical & experimental sciences based at Southampton General Hospital. This research is trying to help us understand more about the normal functioning of the molecule nitric oxide (NO) and related signalling molecules in the skin of healthy volunteers. We want to test to see if regularly applying an existing cosmetic product to the skin can change the NO levels in the skin of a healthy person. The role that the skin and the bacteria that live on it play in normal NO functioning has not been explored thoroughly in any previous study. If it is found that NO levels vary between people and are affected by the application of cosmetics, it could pave the way for future research in people who suffer from skin problems or other medical conditions.

Why have I been chosen?

As a healthy adult who fits our inclusion criteria, you have volunteered to be part of this study. At the moment we don't know of any direct benefit to you from taking part in this study.

What will happen to me if I take part?

Taking part in this study will involve 2 visits to the physiology laboratory at Southampton General Hospital after you have given your express written consent. The first visit will be a maximum of 3 hours and the second visit will be a maximum of 2 hours i.e. 5 hours in total.

At visit one, a trained researcher will record your height, weight, blood pressure, waist circumference, Hand and Foot size skin acidity (skin PH) and transepidermal water loss. We will ask you to fill in a short questionnaire about your skin. We also would want to collect a spit sample, a 10ml blood test, a skin swab and a skin scrape sample. After this we will ask you to do some moderate intensity exercise (<70% of your maximum heart rate) on a treadmill or on a stationary bicycle for up to 15 minutes. The exercise is not a test. We would like to collect samples of sweat. If that sort of exercise doesn't make you sweat, we might ask you to put on a nylon sweat suit which means that sweat collects more rapidly than normal. After we have

collected sweat the exercise part will finish. We will then take another another skin acidity (skin PH) measurements.

The third part of visit one involves testing how much nitric oxide is coming from different parts of your skin. This is entirely painless and just involves you resting that area of skin, e.g. the hand, in a plastic container for no more than 20 minutes. You can choose to listen to music, read, chat or any other relaxing activity during this time. We will then do a test application of the cosmetic to check you don't react to it.

You will then be free to leave and return in two weeks, during which time you will be asked to apply either the cosmetic product to your skin around your normal routine. During visit 2 you'll come back to the lab and we will repeat the same set of the tests as visit 1 to see if there have been any changes.

That is the end of the main study.

Optional Measurements

Analysis of foot and forehead NO emanations as well as pH,TEWL and sweat samples collection..

In order monitor the skin mircrbiome changes after administration of AOB, we would like a subset of the participants to return for additional skin sampling. (using tape adhesion sampling) will consist of three additional skin microbiome samples over a span of 14 days.

Are there any benefits in my taking part?
There are no known benefits to you taking part in this study. The findings from this study will contribute to a better understanding of skin physiology and the composition of the bacterial flora on the skin.

Are there any risks involved?
The main burden on you as a participant is the time taken out of your own time in order to attend the testing sessions in the laboratory 4 hours in total)

Other possible risks to participants

1. Risk of reaction to the cosmetic AO^+ Mist™ (AOBiome). As far as the Feelisch laboratory group is aware there has thus far been no adverse event associated with application of AO^+ Mist™ (AOBiome) in any previous study. Nevertheless, there remains a theoretical a risk of skin or systemic reaction to AO^+ Mist™ (AOBiome) application.
2. Risk from venous sampling: pain, skin trauma, bruising, fainting, nausea. *Rarely*: extravasation, phlebitis. Total volume of blood throughout study = 20ml.
3. Risk of fatigue from moderate exercise
4. Risk of discomfort from: sitting still for cutaneous NO emanations or wearing sauna suit: (including sweating, feeling hot)
5. The skin scrapes will be the same as a gentle scratch from a finger nail. Only the surface is a braided and no discomfort is felt. The tape strips are equivalent to removing an adhesive plaster.

Will my participation be confidential?
Yes. All data from the participating centre will be entered in an anonymised database on NHS computers. No identifiable participant data will be released to the public. The study coordinator will receive anonymised physiological data and will be blinded to outcome and questionnaires. Caldecott principles will be adhered to.

Data will be entered onto a spreadsheet with all direct participant identifiers removed; participants will be identified by codes. All physiological data is held in an encrypted format, ensuring linked anonymity. Hospital laptops may be used by the research team. These will be password protected and have the same security features as the hospital computers. All manual files will be kept in a locked filing cabinet in a locked office. Data will be stored on password protected computers contained within a secure University server, with hard copies stored separately in dedicated laboratory notebooks that remain the property of the University during and after experiments have ceased.

What happens if I change my mind?
You have a right to withdraw at any time without your legal rights being affected.

What happens if something goes wrong?
Throughout the duration of your involvement of the study we encourage you to ask questions to the researchers involved and the research team will provide you with a contact number. Furthermore, in the case of concern or complaint please contact Rfeef Alyami (rya1r14@soton.ac.uk) – or the Research Governance Manager (02380 595058, rgoinfo@soton.ac.uk)

Where can I get more information?
For information contact:

Rfeef Alyami (rya1r14@soton.ac.uk)

Prof. Martin Feelisch (Principal Investigator – 02381206891)

Appendix F

F.1 Samples Collection Sheet

Record of Participant Information/Plan of Data collection

Inclusion Criteria Verified:

Time/Date of Consent Obtained: Participant ID:

Temperature in Room: Degrees

Venous sample obtained? : Yes/No Collection time: Storage time:

Age:

Height (cm):

Weight (kg):

Waist Circumference (cm):

Resting Blood pressure (mmHg): 1.

2.

3.

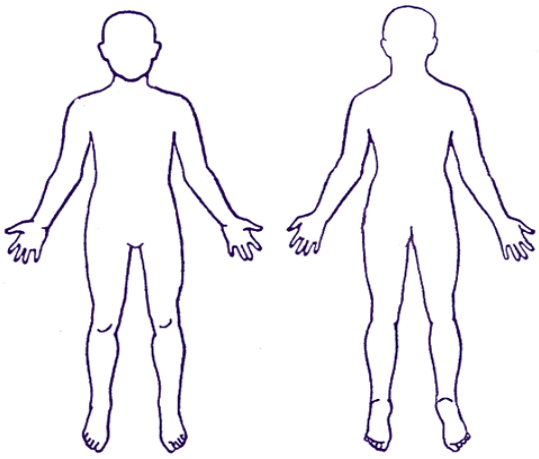
Skin Phenotype Questionnaire completed? Yes/No

Breakfast? Yes/No Note:

NO emanation : Arm Hand

Saliva sample obtained? : Yes/No Collection time: Storage time:

Location of TEWL, pH and skin microbiome samples [Mark 'x' on diagram]



Appendix G

G.1 Phenotype Assessment Tool

Version 2.1 27th Sep 2016

PIGMENTATION PHENOTYPE ASSESSMENT TOOL

(Please circle the most appropriate answer or write the answer on the line provided)

Name of Individual completing form: _____

Role of this individual in the study: _____

1. Subject identification:

Study Participant
Name: ……. Date of birth: …….. Unique Identification number: …….

Where was the participant recruited from? _____

2. Participant Contact details (Telephone number): _____

3. Gender: Male Female

4. Age: _____

5. How would you describe your ethnicity? (Circle the most appropriate answer under one of the subheadings or give further details in the space provided)

a. White
- White British
- White Irish
- White Other - _____

b. Mixed
- White and Black Caribbean
- White and Black African
- White and Asian
- Other _____

c. Asian or Asian British
- Indian
- Pakistani
- Bangladeshi
- Other _____

d. Black or Black British
- Caribbean
- African
- Other _____

e. Other ethnic group
- Chinese
- Other _____

6. Were you born in the UK (England, Scotland, Wales, Northern Ireland)?

Yes (Go to 8.)	No (Go to 7.)

7. What is your country of birth? _____

1

8. a. Current weight (in Kg) _____

b. Height (in metres) _____

9. a. Current Occupation _____

10. Please identify your natural hair colour from the attached sheet *(pick from A-L on the accompanying supplement)*

a . Scalp hair - Child (at 0-5 years) _____

- Adult (at 20 years) _____

b. Beard (at 20 years) _____

c. Armpit hair (at 20 years) _____

d. Pubic hair (at 20 years) _____

11. Researcher to document the participant's eye colour. (See supplement)

Background/peripheral	Blue	Grey	Green	Yellow	Tan	Brown
Peri-pupillary	Blue	Grey	Green	Yellow	Tan	Brown
Flecks	Blue	Grey	Green	Yellow	Tan	Brown

13. Do you get freckles after sun exposure?

Yes	No

14. Where do you get the freckles and approximately how many (please see supplement)?

Face	None	Few	Moderate	Many
Shoulders	None	Few	Moderate	Many
Arms	None	Few	Moderate	Many
Legs	None	Few	Moderate	Many

15. Which of the following is most similar to your natural skin colour on a non-sun exposed site e.g. buttocks? *(**See supplement**)*

I	II	III	IV	V	VI

UV exposure

16. How many times after exposing your skin to sunshine have you developed painful or blistering sunburn of your skin that lasted 2 or more days in your lifetime?

Never	1-5	6-10	>10

17. Have you ever lived outside of the UK for longer than 2 months in a 12 month period (this can be made up of individual weeks, for example if related to work, but would exclude travel to meetings, conferences etc where minimal time was spent outdoors)?

Yes	No

18. During your adult working-life, how many hours per day on average do you spend outside between

10am and 4pm in the Summer (June, July, August)?

	< 1hour	**1 hour**	**2 hours**	**3 hours**	**4 hours**	**5 hours**	**6 hours**
Weekdays							
Weekend							

19. During your adult working-life, how many hours per day on average do/did you spend outside between

10am and 4pm in the Non-Summer months (September to May)?

	< 1hour	**1 hour**	**2 hours**	**3 hours**	**4 hours**	**5 hours**	**6 hours**
Weekdays							
Weekend							

20. How many weeks of annual leave/holiday did you/do you get a year?

1-2	3-4	5-6	7-8	9-10	10+

21. Over the past 5 years on a **hot sunny day**:

a. How often do you wear sunscreen?

Never (0%)	Rarely (1-25% of times)	Sometimes (26-50% of times)	Often (51-75% of times)	Always or nearly always (76%-100% of times)

b. What Sun Protection Factor (SPF) is your sunscreen?

Less than 15 SPF	15-30 SPF	More than 30 SPF	Don't know	I generally don't use sunscreen

c. How often do you/did you wear a hat or have hair that shades your face, ears and neck?

Never (0%)	Rarely (1-25% of times)	Sometimes (26-50% of times)	Often ((51-75% of times)	Always or nearly always (76%-100% of times)

d. How often do you/did you stay in the shade or under an umbrella?

Never (0%)	Rarely (1-25% of times)	Sometimes (26-50% of times)	Often ((51-75% of times)	Always or nearly always (from 76%-100% of times)

22. How many times have you used an artificial sunbed to date (Number of individual exposures)?

Never	1-10	11-50	51-100	101-250	250+

<u>Past medical history</u>

23. Have you ever been diagnosed with a skin disease?

	Yes	No	
Psoriasis			
Eczema			
Acne			
Xeroderma Pigmentosum			
Epidermodysplasia verruciformis			
Viral Warts			Site (e.g. hands, face, lip, other):
Chronic ulcers			Site:
Other /Allergy?			Detail:

24. Have you had a skin cancer?

Yes	No

Type of skin cancer	**Age at diagnosis**	**Method of treatment (surgery, radiotherapy, other – please specify)**	**Strength of evidence (patient history, casenotes or histology report)**

25. Have you ever had any of the following treatments for other skin lesions?

	Yes	No
Cryotherapy		
Aldara (Imiquimod)		
Efudix (5-Fluorouracil)		
Photodynamic therapy		
Radiotherapy		
Surgery		

26. Have you ever had any of the following topical treatments for a skin rash?

	Yes	No
Topical steroid		
Calcineurin inhibitor (e.g. protopic (tacrolimus) or elidel (pimecrolimus))		
Other (please specify) _____ _____ _____		

27. Do you have a history of cancer?

Yes	No

Type of cancer	**Age at diagnosis**	**Method of treatment (surgery, radiotherapy, chemotherapy, other – please specify)**	**Strength of evidence (patient history, casenotes or histology report)**

28. Have you had an organ transplant?

Yes	No

Organ: _____

Age at Transplant: _____

29. Do you have any other significant medical history?

Yes	No

If yes please provide more details below:

System	Details
Cardiovascular	
Respiratory	
Gastrointestinal/liver/pancreas/biliary tract	
Endocrine and Diabetes	
Rheumatological / Musculoskeletal	
Renal	
Haematological	

Neurological	
Psychological	
Ear, Nose and Throat	
Opthalmological	
Urological/Reproductive	
Other	

30. Do you smoke?

Yes – smoker Number/day: _____ Number of years:_____	No – ex-smoker Stopped (age):_____ Number/day: _____ Number of years: ____	No – never smoked	Significant passive smoker Details: _____

31. How many units of alcohol do you drink per week? (see supplement)

_____

32. Have you ever been prescribed immunosuppressive medication, including oral steroids?

Yes Details (Type/Duration): _____ _____ _____ _____ _____	No

33. Please list your current medications:

_____ _____

_____ _____

_____ _____

_____ _____

Thank you for answering these questions.

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