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EFFECTS OF DIFFERENT FACE WASHES ON THE DIVERSITY AND COMPOSITION OF THE SKIN MICROBIOME

MASTER'S THESIS

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AFFIDAVIT

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„Phantasie ist wichtiger als Wissen, denn Wissen ist begrenzt. “

Albert Einstein

Acknowledgements

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Abstract

Cosmetic products are part of our daily routines and are causing interactions with our skin. That's why it is important, to know how this product influence our skin in function and microbiome. Because of that, we observed the influence of three different face washing products on human skin.

32 women, aged between 20 and 45 years, applied one of three products twice a day on their volar forearm for a total duration of four weeks. We than measured the changes of following skin parameters of their skin: Transepidermal water loss (TEWL), skin moisture and pH as well as the changes in the microbiome. Measurements and samples for the analysis of the microbiome were taken prior to the first use of the products, after two weeks and after four weeks of continuous use of the product. The devices used for measuring the skin parameters were, a Tewameter® for measuring the TEWL, a Corneometer®, for measuring the moisture of the skin and a Skin-pH-Meter. For taking samples of the skin microbiome the tested area was swabbed with a sterile, pre-moisturised swab, subsequently extracting the DNA with the Fast Spin DNA Extraction Kit, followed by multiplying the area of the 16S r RNA Gene in a PCR. This product was used for library construction and Next Generation sequencing (NGS). These data were processed in QIIME, using the Greengenes Database, and afterwards analysed by using CALYPSO. The statistical evaluation of the obtained data from skin measurements was carried through, using a mixed ANOVA model, run on SPSS.

Over the period of the study, the skin physiology, as revealed by the processed measurement-data, changed significantly for all groups, irrespective of the used product.

The epidermal water loss decreased over the duration of study and the skin of the participants became drier by and by, while there was no statistical context to be found relating to the values of pH.

As to the microbiome, there were no significant changes in α-diversity to be detected, when comparing the different product groups. However, for all groups of products a significant increase in the α-diversity appeared over time. Other changes, concerning the skin microbiome, could only be found in low-abundance skin bacteria species.

Kurzzusammenfassung

Kosmetische Produkte sind Teil unserer täglichen Routine und stehen durch direkte Anwendung vor allem in Kontakt mit unserer Haut, weshalb es besonders wichtig ist herauszufinden, inwiefern solche Produkte unsere Hautfunktionen und unser Hautmikrobiom beeinflussen. Um diese Effekte genauer betrachten zu können, haben wir den Einfluss von drei verschiedenen Gesichtswaschprodukten auf die menschliche Haut getestet.

32 Frauen, im Alter von 20 bis 45 Jahren, verwendeten im Zuge unserer Studie eines der drei Produkte zweimal täglich zum Reinigen ihres inneren Unterarms, über einen Zeitraum von insgesamt vier Wochen. Hierbei wurden die Veränderung der Haut mittels Messungen des transepidermalen Wasserverlusts, der Hautfeuchtigkeit und des Haut-pH-Werts festgestellt und zusätzlich wurden Untersuchungen über allfällige Veränderungen des Hautmikrobioms angestellt. Die Messungen der besagten Hautparameter und die Probennahme vom Mikrobiom der Haut fanden jeweils vor der ersten Produktanwendung, nach zwei Wochen und nach vier Wochen einer kontinuierlichen Produktbehandlung statt. Für die Hautmessungen wurden ein Tewameter, ein Corneometer und ein Haut-pH-Meter verwendet. Zur Testung des Hautmikrobioms wurde die jeweilige Hautstelle mit einem sterilen, angefeuchteten Tupfer abgestrichen und anschließend die DNA mittels Fast Spin DNA Extraktions Kit gewonnen. Der Bereich der 16s rRNA wurde dann mittels PCR vervielfältigt und zur Bibliothekskonstruktion und Next-Generation Sequencing verwendet. Die gewonnen Daten wurden dann mittels QIIME unter Verwendung der Greengenes Datenbank aufbereitet und abschließend mit CALYPSO analysiert. Zusätzlich wurden die gewonnen Hautphysiologieparameter statistisch, mittels eines mixed ANOVA Models, in SPSS ausgewertet.

Während der Studie veränderten sich die Hautphysiologieparameter signifikant für alle Gruppen, unabhängig davon welches Produkt verwendet wurde. Es konnten eine Verminderung des transepidermalen Wasserverlusts und eine Austrocknung der Haut der Probanden festgestellt werden, wo hingegen keine statistisch signifikanten Veränderungen des pH-Werts beobachtet wurden. Bezüglich des Hautmikrobioms konnten weder Produkt-, noch-gruppenspezifisch signifikante Veränderungen in der α-Diversität festgestellt werden. Die α-Diversität zeigte jedoch für alle Gruppen gemeinsam einen signifikanten Anstieg nach den vier Wochen kontinuierlicher Produktanwendung. Es konnten auch noch weitere Veränderungen des Hautmikrobioms beobachtet werden, wobei diese aber ausschließlich Bakterien Spezies mit geringer Häufigkeit betrafen.

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1 Introduction

1.1 The Origin of Microbiology

In 1664 Robert Hooke, a physicist, natural philosopher and mathematician, first described microbial life. By using a hand-crafted microscope, he examined different objects, finding and describing a fungus on a book cover, representing the first described microorganism so far. His findings, including a depiction and drawing of the fungus, were published 1665 in his book "Micrographia" (Hooke, 1665; Madigan et al., 2013). A little later, in 1676, Antonie van Leeuwenhoek analysed natural substances with regard to their microbial contents, with a simple, self-made microscope and found the first Bacteria, which he described as "Wee Animalcules" at that time (Madigan et al., 2013).

With the knowledge from Hooke's and van Leeuwenhoek's discoveries, as well as through technological advancements in the field of microscopy, microbial research developed, led by famous scientists as Ferdinand Cohn, Robert Koch and Louis Pasteur (Madigan et al., 2013).

Especially Robert Koch played an important role in developing the research on microorganisms and their cultivation. At that time, it had already been discovered, that bacteria cause putrefaction and that diseases can be caused by living transferable entities. Furthermore, the first steps in the development of antiseptic surgical methods were already taken by Joseph Lister. Koch, on the contrary, concentrated on the anthrax disease, that posed a threat to humans and animals during Koch's active period. He observed a rod-shaped structure in the blood of affected animals and found, that it can be transmitted by transferring blood from a sick individual to a healthy one. He also observed, that the contaminated blood lost its ability to cause disease after a few days, what was contrary to his former finding, that the disease could remain dangerous for long periods of time. To find out more about this loss of pathogenicity, Koch began to develop cultivation techniques. Thus, he was able to find dividing *Bacillus anthracis* cells and "refractile spheres"- today known as *Bacillus* spores. He also witnessed vegetative cells emerging from the spores and therefore hypothesised, that these spheres were responsible for the long lasting infectious potential of contaminated soil, despite the absence of living bacteria (Blevins & Bronze, 2010).

This was a remarkable discovery, as Robert Koch was the first person ever to link a specific bacterium to a specific disease. Although he also made investigations on cholera and the cure of tuberculosis, his discovery of the Anthrax causing bacteria, as well as his microbiological

methods and techniques, remained his most important achievements. Koch's methods, like the utilization of solid culture media are, even though a little adapted over time, still used by today's biologists (Blevins & Bronze, 2010).

1.2 Development of Molecular Analysis

Cultivation still is an important tool to assess characteristics of microorganisms, but since about 99% of microorganisms from natural habitats are not easily cultivable, most microorganisms were not accessible for research until further development (Amann et al., 1995). With the first molecular techniques a proper tool was found, to replace the phenotypic approach as main tool for characterising microorganisms (Zuckerkandl & Pauling 1965). Based on the sequences of 16S and 18S ribosomal ribonucleic acid (rRNA) the concept of the phylogenetic tree of terrestrial life (Fig. 1), and accordingly a reliable concept for the classification of life, was introduced (Woese & Fox, 1977).

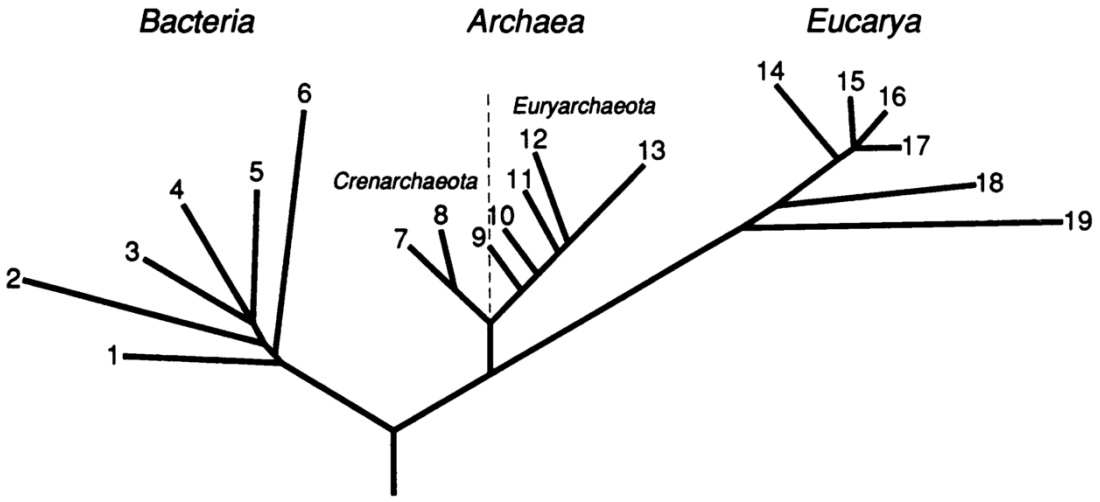


Figure 1: The phylogenetic tree of life as proposed by Carl Woese et al. 1990. It shows the three domains of life, Bacteria, Archaea and Eucarya, divided in three branches with numbers corresponding to different groups of organisms (Woese, Kandler, and Wheelis 1990).

Along with the introduction of this phylogenetic tree of life, many other molecular biological tools like the polymerase chain reaction (PCR) (Mullis, 1990), the sanger sequencing method (Sanger, Nicklen & Coulson, 1977) and the implementation of public databases like the NCBI GenBank (Mizrachi, 2003) helped to improve the research in the field of microbiology and led the way to the art of state of studying microbial sphere, as performed today. These methods and many others were the basis for making educated guess about the function of unknown genes and moreover, together with new tools in the field of bioinformatics, offering first approaches in metagenomic analysis, and thus for accessing the genetic content of entire communities of

organisms (Thomas, Gilbert & Meyer, 2012). First the metagenomic projects were mostly based on ”shotgun sequencing”, which is expensive and very time-consuming because of the excessive work flow and therefore was not accessible for most researches at the time (Hugenholtz & Tyson, 2008; Madigan et al. 2013).

1.3 16S ribosomal Ribonucleic Acid (rRNA) Gene

16S rRNA gene sequencing is commonly used to study bacterial phylogeny and taxonomy, since the 16S rRNA gene is present in all bacteria, its function did not change over time and it is large enough for informatics purpose (Patel, 2001). Genus identification with the full length 16S rRNA gene is possible in about 90% of all cases, while species classification is provided only in 65 to 83% of all cases. Accordingly, the 16s rRNA gene is highly useful to classify bacteria, but still has some shortcomings, especially in the phylogenetic species classification. The main reasons for failing are the recognition of novel taxa, incomplete nucleotide databases and the fact, that some species are sharing similar and/or identical 16S rRNA sequences (Janda & Abbott, 2007).

Based on the 16S rRNA sequencing new phylogenetic trees with a more accurate classification were elaborated, as for example in Isenbarger et al., 2008.

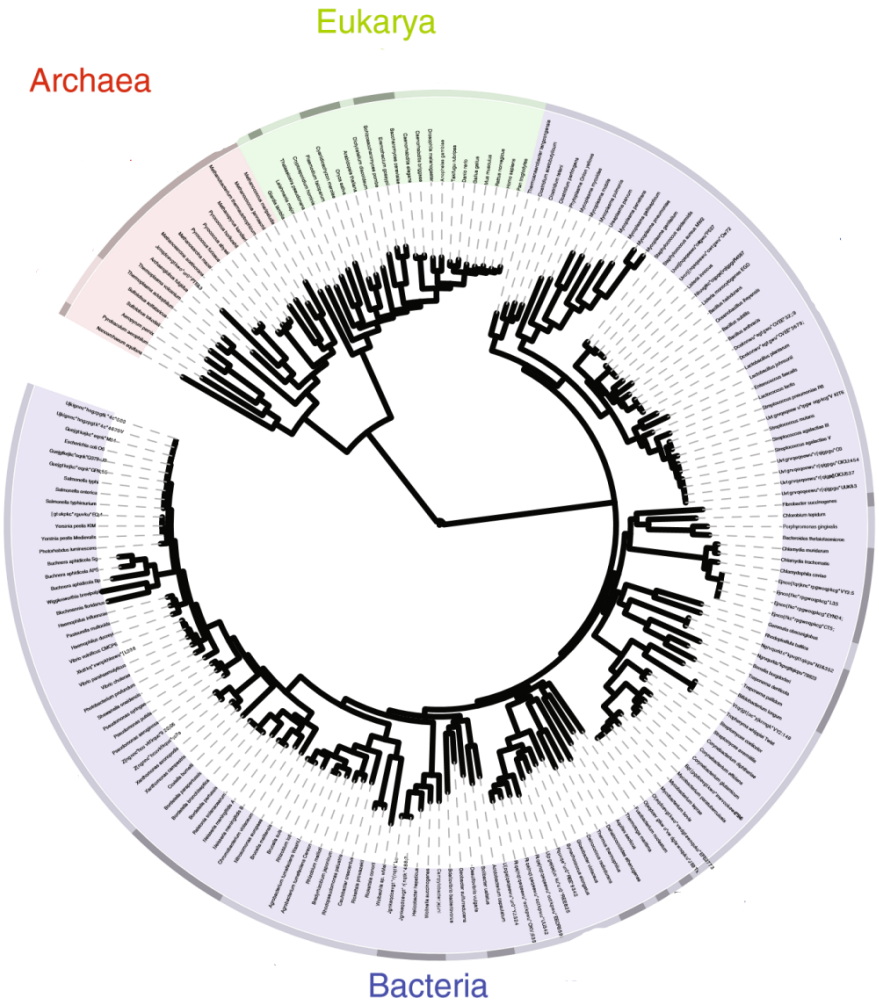


Figure 2: New phylogenetic tree from Isenbarger et al., 2008 (adapted by Diana Farthofer by removing restriction enzymes), representing the Bacteria (blue), Archaea (red), and Eukarya (green) based on an overlay of the whole genome sequences used in this study.

1.4 Sequencing

The so-called "next generation sequencing", including techniques like the ILLUMINA MiSeq®/HiSeq2000®, were developed, making it possible to sequence samples with complex microbial communities with a high throughput at still good sequencing depth (Bokulich et al., 2013). This reduced the operational costs and reduced the time for sequencing significantly, what makes these techniques economically viable for most researches nowadays. Anyhow these techniques are asking for much higher computing capacity to assemble the much larger quantity of shorter reads, than produced from Sanger sequencing (Shendure & Aiden, 2012; Wetterstrand, 2016). That's why Sanger sequencing with its low error rate, provided through the use of fluorescently labelled dideoxynucleotides and chain termination, is still used for sequencing samples that contain only one species (Morey et al., 2013).

As processing of the sequenced data requires quite sophisticated software tools, we decided to use Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUST), which, using the 16S information, recaptures key findings of the Human Microbiome Project and is able to predict the abundance of functional gene families in host-associated and environmental communities accurately (Langille et al., 2013).

1.5 Data Analysis

The 16S rRNA gene sequencing is not only the key tool for studying microbial communities, but also provides information about the community's functional capabilities. According to the big data volume computational data processing is inevitable and requires special software tools. This study was elaborated by using Quantitative Insights Into Microbial Ecology (QIIME) for processing the data analysis and PICRUSt for interpreting the data.

QIIME is an open-source software tool that can be used to analyse and interpret sequencing data from different microbial communities, such as fungi, bacteria or archaea using the PyCogent toolkit6. QIIME is one of few software tools that are able to handle the massive datasets that can be accessed through the new sequencing technologies (Kuczynski et al., 2011; Caporaso et al., 2011).

Using marker gene data and a database of reference genomes PICRUSt predicts the functional composition of a metagenome in a two-step process. In the first step, absolutely independent from microbial communities; a table of predicted gene family abundance for each organism in the 16S-based phylogeny is reconstructed, which is used in the second step to combine the resulting gene content predictions for all microbial taxa with the relative abundance of 16S rRNA genes in one or more microbial community samples, what is called "metagenome inference" (Langille et al., 2013).

1.6 The Skin as Microbial Habitat

The bacterial diversity of human skin is enormous and seems to be even vaster than the diversity found in the stomach environment. Beside the resident skin microbiome, that consists mainly of the three phyla, Actinobacteria, Firmicutes and Proteobacteria, there reside large numbers of rare taxa on our skin, that can be either transient, short term colonisers or more persistent long-term residents (Fierer et al., 2008).

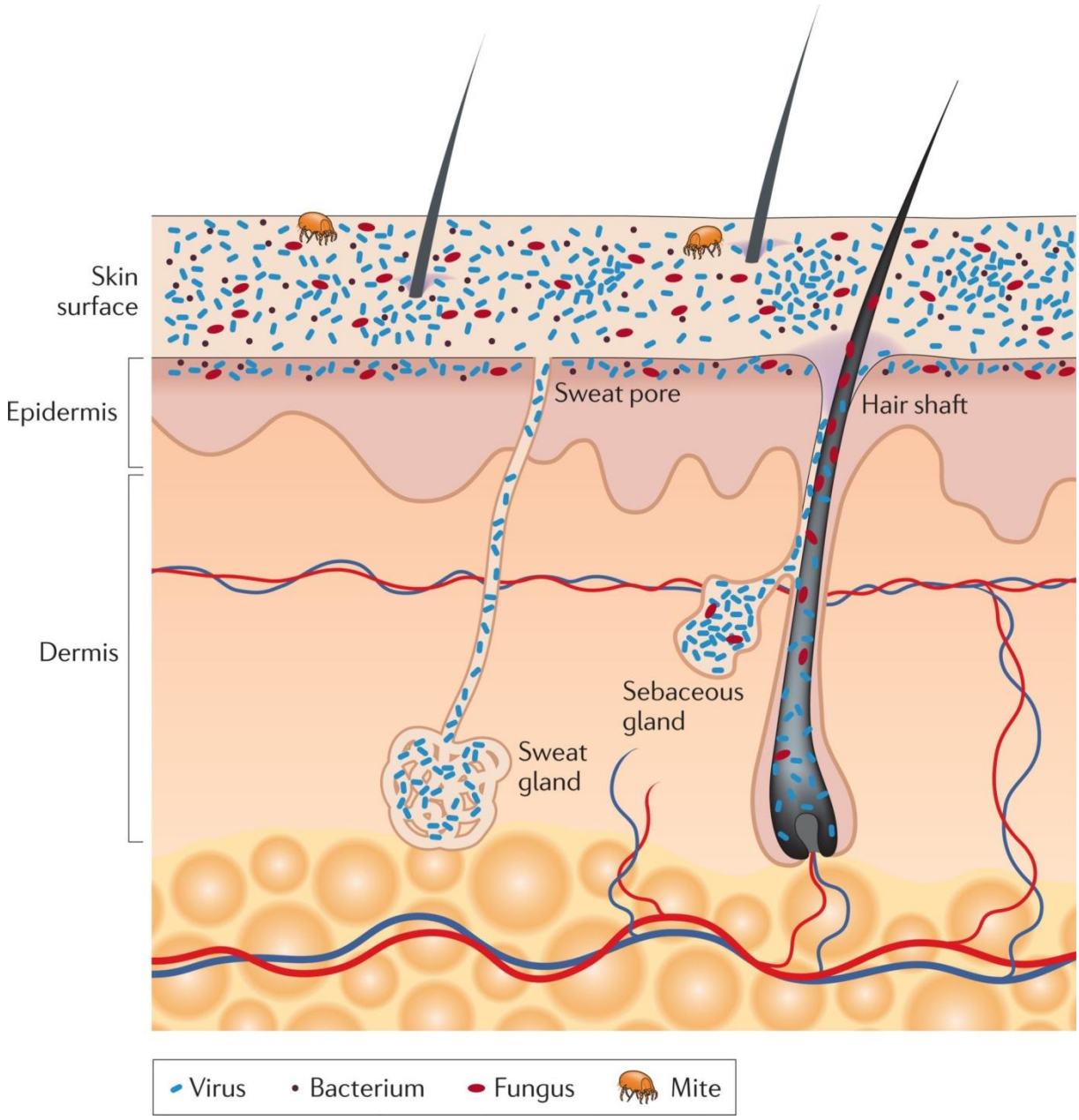


Figure 3: Schematic Figure of the skin histology and it's interaction with microorganism from Grice & Segre, 2013.

A wide range of microorganisms can be found living on these 1.9 m² of human skin. These microorganisms are part of several habitats on the skin of different parts of the body. Many of these organisms are colonisers from the environment as result of its interaction with the skin. Anyhow most of the microorganisms on our skin are harmless and in some cases they even provide vital functions, which have not been evolved by the human genome, like protection against invasion by more pathogenic or harmful organisms(Grice & Segre, 2013).

Human skin is the habitat of aerobic as well as anaerobic bacteria, that vary in number and type on different skin areas. Culture based studies showed, that aerobic bacteria, which often

colonise humid areas such as the axilla, can reach counts up to 107 bacteria per cm^2 and anaerobic bacteria as well show colony counts up to 106 bacteria per cm^2 (Leyden et al., 1987). Furthermore there are unique microorganisms to be found on special skin structures like the stratum corneum (SC), the cellular layer, hair shaft, the follicle and the sebaceous glands (Fredricks, 2001). The density of the local population as well as the constituent species are indicating unique cutaneous environments, since the colonising microorganisms are highly specialised for living in their environment. *Propionibacterium acnes* and the yeast *Pit ovale* for example are predominant in regions of the body with oily skin, while in moist areas like the axillae large numbers of liphophilic diphtheroids and micrococci can be detected. The ability to maintain a reduced environment, the availability of moisture and the presence of sebaceous lipid therefore seem to be the three major determinants that define if skin habitats are oily, dry or wet, what finally is also reflected in the microbial population (Leyden et al., 1987).

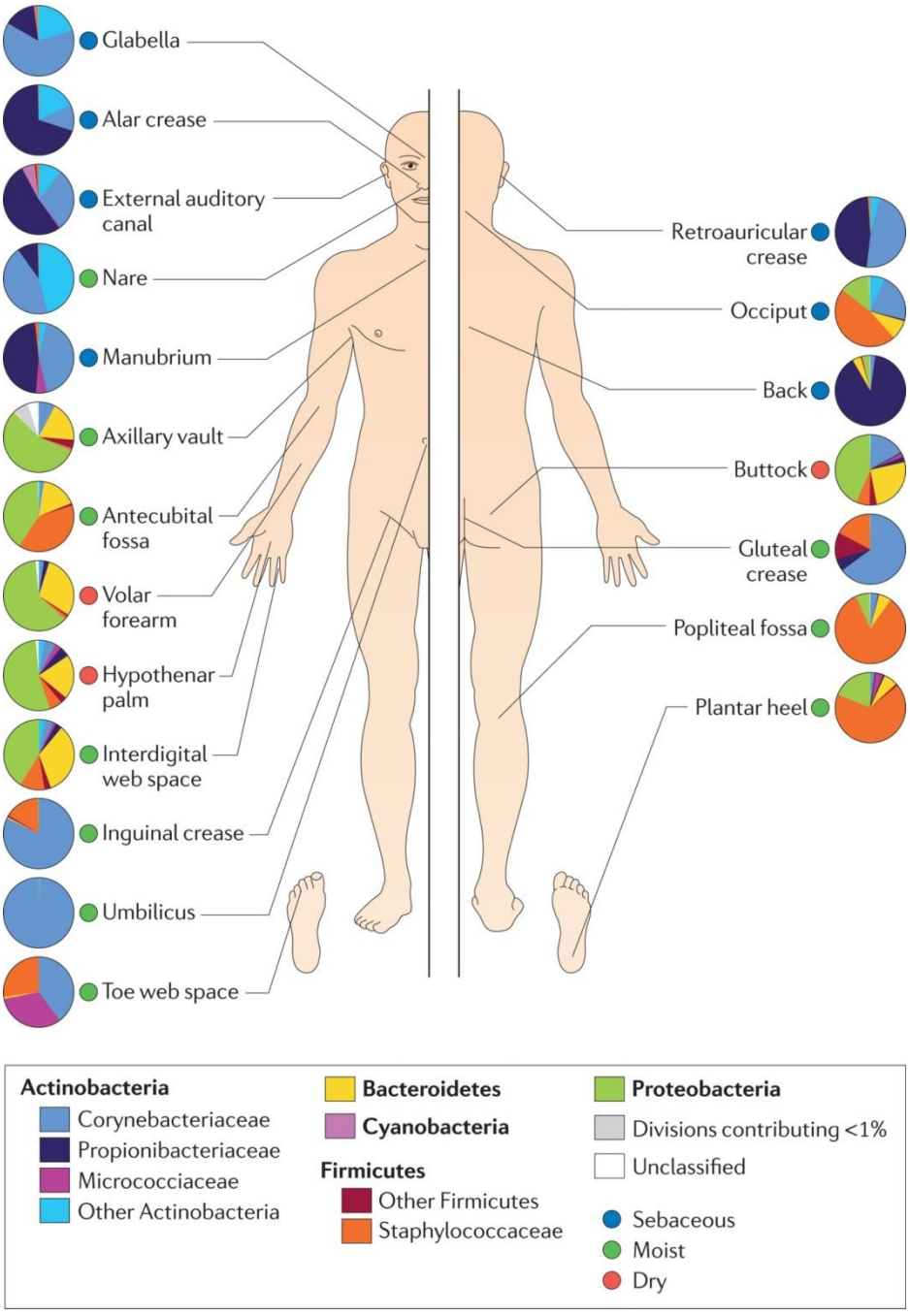


Figure 4: Topographical distribution of bacteria on skin sites from Grice & Segre, 2013.
The picture shows the classification of bacteria colonizing an individual subject, with the phyla in the bold. This also shows that the skin microbiome is highly dependent on the microenvironment of the sampled site. The sites selected were those that show a predilection for skin bacterial infections and are grouped as sebaceous or oily (blue circles), moist (typically skin creases) (green circles) and dry, flat surfaces (red circles).

In addition to the bacterial microbiome human skin is also colonised by different species of Eukaryotes, Fungi and Archaea, that could affect the skin and its bacterial colonisation (Grice & Segre, 2013; Probst et al., 2013).

1.7 The Skin Barrier and Skin Function

There are two different kinds of skin that cover the body, helping to protect the body from the surrounding environment, what are on the one hand the glabrous and on the other hand the hair-bearing skin. While the first skin type has a thick stratum corneum, in order to protect the deeper layers of the skin from strong external forces and is only found on the palmoplantar surface, the second type holds a thinner SC but long or vellus hair. Though the effect of the changes in the SC are a controversial subject, there remarkable regional differences in the SC as well as in the living tissue, according to the function of each anatomical location (Tagami, 2008).

The SC is the interface between the external environment and our internal molecular processes, and therefore is the most exposed human organ. Thus there is a composition of molecules derived from our own cells, our resident microbiota, molecules from the environment and products that directly interact with our skin, like washing detergents and clothing, and the local microbiome (Bouslimani et al., 2015).

SC cells (Corneocytes) protect against environmental factors by maintaining an optimal hydration level, depending on several parameters – first, a lipid gel phase as a protective barrier, which is important for the water passage through the tissue, second the diffusion path length and third the natural moisturising factor (Rawlings & Harding, 2004).

Beside moisture and water loss, also the pH can be changed through different skin conditions. Humid climates for example can lead to a higher pH, while barrier repairs take place at a more acidic pH (Rawlings & Harding, 2004).

Furthermore skin surface lipids are apparently involved in skin processes like thermoregulation, bacterial colonisation and barrier function and maintenance and can affect the skin physiology (Addy, Oliphant, & Harper, 2017).

1.8 Skin Physiology

Non- invasive skin measurement methods have been used to measure, physiological changes in the Stratum corneum, as mentioned above. Using the Tewameter®, as tool for the measurement of the transepidermal water loss, is so far important, as it measures not only the barrier function of the skin, but also the state of hydration and the water retention capacity, which are main

factors, influencing the softness and smoothness of the skin surface. The results of such skin measurements are differing largely, when examining different body sites (Tagami, 2008).

1.9 Influence Factors on the Physiological Skin Parameters and the Skin Microbiome

The skin and its microbiome are influenced by many different host specific factors, like individuality, age, location and sex, as well as by environmental factors, like choice in clothing and use of antibiotics. Weather can also influence the skin through varying temperatures or air humidity. Another important factor is the use of cosmetic and sanitary products that can not only influence the skin barrier, but also the skin microbiota (Grice & Segre, 2013).

1.10 Skin Microbiome and Disease

In addition to its physical barrier function the skin has an immunological barrier function as well. The skin helps to prevent infections and modulates the commensals that colonise the skin. Changes in the skin and its microbiome can therefore negatively affect the health of the human being himself. Especially common skin disorders are often associated with a microbial change. Specific skin diseases can be associated with specific organisms in three different ways: first it could be a direct correlation between skin disease and the microbiota, second a currently undefined microbial component may cause the skin disease and third a skin commensal causes infection in becoming invasive (Grice & Segre, 2013).

The harmless resident microbiome on our skin can be identified by our skin cells and helps to stimulate an immune response to prevent an infection with pathogenic microorganisms when the skin is damaged. The study from Lai et al., 2009 showed that the lipoteichoic acid (LTA) produced by staphylococcal species induce an anti-inflammatory response on keratinocytes when the skin is healthy, while it has a proinflammatory effect on cells that normally exist only in sterile environment like mast cells or macrophages (Lai et al., 2009).

1.11 Motivation

While studying, the microbiome was discussed in many different ways, what made me become interested in this topic was the fact that the microbiome is not only part of us and our surroundings but can also affect our health. That made my decision easy, when being offered the opportunity to take part in doing research on the reaction of the microbiome to our daily use

of skin care products. Being involved in this project, I did not only get the chance to deepen my knowledge and learn a lot about our largest organ – the skin, but also to work with an amazing team of scientists, who helped me in developing personally and professionally.

The influence of cosmetics and other skin care products on our skin microbiome is not only a very interesting field of study, but will also help to understand the genesis of "modern" skin diseases. Continuative research in this field may one day open access to highly effective, personalised treatments of skin problems and thus being disruptive to today's medical practice.

2 Materials and Methods

2.1 Chemicals and Bio-Chemicals

Table 1: List of used chemicals and bio-chemicals.

Chemical/ Bio-Chemical	Supplier	Head Office (Country)
Agarose, SeaKem LE	Lonza	Rockland (US)
Bovine Serum Albumin (BSA)	Roche Diagnostics	Mannheim (GER)
Ethylenediaminetetraacetic acid (EDTA)	Merck	Darmstadt (GER)
Ethanol p.a „Baker analyzed"	J.T. Baker	Center Valley (US)
FastRuler Low Range DNA Ladder, ready-to-use	Life Technologies	Carlsbad (US)
GelRed Nucleic Acid Gel Stain (10000x)	Biotium	Hayward (US)
H_2O (LiChrosolv grade)	Merck	Darmstadt (GER)
H_2O (Milli-Q grade)	Merck	Darmstadt (GER)
HCl 37%	VWR international	Radnor (US)
Loading Dye 6x	Life Technologies	Carlsbad (US)
PCR grade water, nuclease free	Jena Bioscience	Jena (GER)
Tris-base	VWR international	Radnor (US)
Tween 20	VWR international	Radnor (US)

2.2 PCR Chemicals

Table 2: List of used PCR chemicals.

Enzyme/Bio-Chemical	Supplier	Head Office (Country)
10x Taq Buffer	Takara Bio Inc.	Tokyo (JP)
dNTP mix	Takara Bio Inc.	Tokyo (JP)
Ex Taq DNA Polymerase	Takara Bio Inc.	Tokyo (JP)

2.3 Kits

Table 3: List of used kits.

Kit	Supplier	Head Office (Country)
FastDNA Spin Kit	MP Biomedicals	Solon(US)
Qubit dsDNA HS Assay Kit	Life Technologies	Carlsbad (US)

2.4 Consumables

Table 4: List of used consumables.

Consumable	Supplier	Head Office (Country)
BBL Culture Swabs EZ Becton	Dickinson and Company	Maryland (US)
Filter tips (0.1-10 µl; 1-100 µl; 20-200 µl; 100-1000 µl)	Corning	Corning (US)
Gloves, latex	B.Braun Melsungen AG	Melsungen (GER)
Gloves, nitrile (comfort nitrile)	Kimberly-Clark	Irving (US)
Lysing Matrix E tubes	MP Biomedicals	Solon (US)
MColorpHast pH indicator strips	Merck	Darmstadt (GER)
Parafilm "M"	Bemis	Neenah (US)
PCR cup 8-strip (0.2 ml)	Carl Roth	Karlsruhe (GER)
PCR cup single (0.2 ml)	VWR International	Radnor (US)
Reaction tubes (15 ml)	Sarstedt AG & Co.	Nuembrecht (GER)
Reaction tubes (50 ml)	Sarstedt AG & Co.	Nuembrecht (GER)
Rotilabo syringe filter, CME sterile; 0.22µM	Carl Roth	Karlsruhe (GER)
Safe Lock Tubes (1,5 ml)	Eppendorf	Hamburg (GER)
Safe Lock Tubes (2,0 ml)	Eppendorf	Hamburg (GER)

2.5 Oligonucleotides

Oligonucleotide primers were supplied by Eurofins. In the following table the primers used in elaborating this study are listed.

Table 5: List of used oligonucleotide primers.

Primer	Sequence 5' → 3'	Reference
515f	GTGCCAGCMGCCGCGGTAA	Caporaso et al., 2012
806r	GGACTACHVGGGTWTCTAAT	Caporaso et al., 2012

The primers used in this study, were used for Illumina Sequencing, by carrying a specific sequence necessary for Illumina technique. The following Illumina-tags were used for forward- and reverse-primers respectively:

- Fwd. overhang: 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-[primer-sequence]-3'
- Rev. overhang: 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-[primer-sequence]-3'

2.6 Instrumentation

Table 6: List of used instruments.

Autoclave „T-Line"	Fedegari	Albuzzano (IT)
Biofuge fresco (2ml Rotor 7500 3328)	Heraeus	Hanau (GER)
Centrifuge 5418R (Rotor F4-45-18-11)	Eppendorf	Hamburg (GER)
Cutometer dual MPA 580	Courage + Khazaka electronic GmbH	Köln (GER)
Freezer (-20°C) Medline (No. 7083241-01)	Liebherr	Kirchdorf (GER)
Freezer (-80°C)	Heraeus	Hanau (GER)
Fridge (4°C) Profiline	Liebherr	Kirchdorf (GER)
Gel chamber 20ml	Regensburg Werkstätten Biologie	Regensburg (GER)
Hera safe KS9	Thermo Electron LED GmbH	Langenselbold (GER)
MagNA Lyser	Roche Diagnostics	Mannheim (GER)
Multifuge 1_LR (Rotor 75002005)	Hereaus	Hanau (GER)
MyCycler Thermal Cycler System	BioRAD	Hercules (US)
Nanodrop 2000/2000c	Thermo Scientific	Waltham (US)
PowerPac Basic Electrophoresis Power supply	BioRAD	Hercules (US)
Research plus Pipettes	Eppendorf	Hamburg (GER)
Scale EMB 600-2	KERN	B-lingen (GER)
Single Channel Pipettes (0.1-2µl; 0.5-10µl; 2-20µl; 1-100 µl; 20-200 µl; 100-1000 µl)	PeqPette	AL-Hamriyah (OMN)
ThermoMixer Model: HTM 130 R	HLC - Heap Labor Consult	Bonvenden (GER)
Universal Hood II Gel Imager	BioRAD	Milan (IT)

UV Sterilizing PCR workstation	PEQLAB	Erlangen (GER)
Vortex Gene 2 (60 micro tube insert attachment), Modell: SI-T256	Scientific industry	Bohemia (USA)
Vortex 4 basic	IKA-Werke GmbH & CO. KG	Staufen (GER)
Qubit 2.0 DNA Quantification	Inivitrogen	Carlsbad (US)

2.7 Buffers

2.7.1 Sampling Buffer

For sampling, swabs were pre-moistured with a buffer containing:

- 50 mM Tris-HCl pH 7,2
- 1 mM EDTA
- 0,5% Tween
- Ad 1000 ml LiChrosolv grade H_2O

The buffer was sterile filtered with a 0,22 µM Rotilabo syringe filter before use. It was stored at 4°C during the whole study and used for all samplings.

2.7.2 Tris-acetate-EDTA Buffer (TAE)

TAE was used for the agarose gel electrophoresis and was prepared as 50x concentrate. The 50x concentrated TAE contained:

- 242 g Tris
- 100 ml Na_2EDTA (0,5M)
- 57,1 acetic acid (glacial)
- 1000 ml ddH_2O

The buffer was diluted to a 1x TAE with Aqua bidest before use.

2.8 Study Design

For the study 36 female participants in the age between 20 and 45 years were recruited. The participants were first grouped regarding their initial similarities in the skin measurements and then divided into three groups of 12 people, so that the groups were comparable in respect of

the tested individuals. Each group was assigned to one of three different face washing products and the participants were asked to make solely use of the assigned face washing product as their only shower gel for the whole body once a day and to wash separately their volar forearm of their non-dominant arm for a second time each day. All participants were asked to strictly avoid using any other washing or skin caring products during the entire time of the 4 weeks study's period.

Furthermore, an additional Follow-up measurement was done 6 weeks after the end of the study., while all participants had changed to their usual daily washing and skin caring routines, using the products of their choice again. Together with taking the samples from the participants' skin information about the used washing products, the daily washing routines and frequency as well as the use of moistening skin care products were gathered.

2.8.1 Sampling Procedure

Skin sampling for microbiome analysis was conducted 3 times during the study period. Date one prior to the first use of the product as baseline (=T1), second after 2 weeks of continuous product use (=T2) and finally after ending the 4 weeks of using the assigned products (=T3). For the Follow-up study, another sampling was carried out after 6 more weeks (totally 10 weeks from the very start) (=T4). The upper part of the volar forearm of the non-dominant arm was swabbed with BBL culture swabs, pre-moistened with sampling buffer, in three different directions over the whole area. The participants were told, not to wash their volar forearm in the morning of day of fixed sampling.

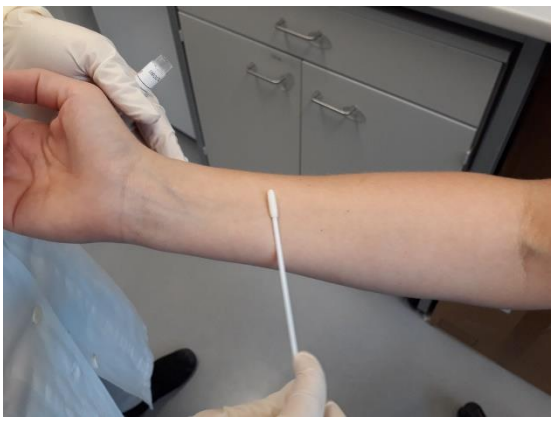


Figure 5: Microbiome sampling procedure with pre-moistened BBL culture swab.

After sampling, the swabs were broken into deoxyribonucleic acid (DNA) free Eppendorf tubes and frozen at -80°C until DNA extraction.
At every single sampling procedure, one swab was only moistened with sampling buffer and used as negative sampling control. In addition, all three products themselves were tested for contaminations before use, by putting a little bit of the product on a pre-moistened swab on broaching.

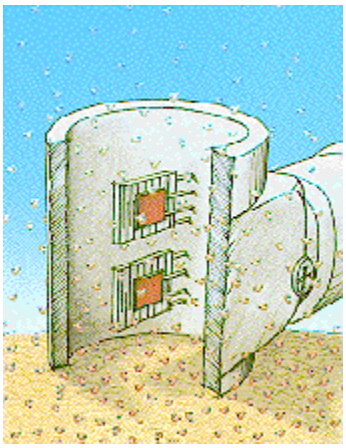
2.8.2 Skin Measurements

The skin measurements were carried out after each of the microbiome samplings with the Cutometer MPA580 from "Courage and Khazaka", starting with the Tewameter (TM300 Courage + Khazaka) measurement, for epidermal water loss, followed by the Corneometer (CM825 Courage + Khazaka) for moisture content, and the Skin-pH-Meter (PH905 Courage + Khazaka) for measuring the pH. All measurements were repeated 3 times.
For statistical analysis and the grouping of the participants the arithmetical mean of the 3 measurements were used.

Figure 6: Cutometer MPA580 from Courage + Khazaka used for the skin measurements.

2.8.2.1 Tewameter

The Tewameter is used to measure the evaporation of the skin to determine its barrier function, characterized as transepidermal water loss as key-parameter, which is already increasing at small damages of the skin, not visible to the bare eye.



The Tewameter works with an open chamber, including humidity- and temperature measuring sensors. The measurement is based on the diffusion theory known as Fick's Law. The measured diffusion flow shows, how much mass per cm^2 is transported per time[1].

Figure 7: Open chamber with the measuring sensors, showing the measurment principle of the Tewameter after Courage + Khazaka (Courage + Khazaka, TM-Sonde Deutsch 2016/12 DK).

Such the Tewameter measures the TWL of the skin and helps to evaluate the barrier function of the skin. Measurements will differ from skin area to skin area and over time. For this reason, the measurements for this study were performed 3 times for 30 seconds on slightly different, but close-by skin areas of the same type, in order to compensate possible inaccuracies. Outer influences like temperature, and body condition were kept to a minimum, since all the participants were asked to keep to a strict routine prior to the sampling and measurement procedure.

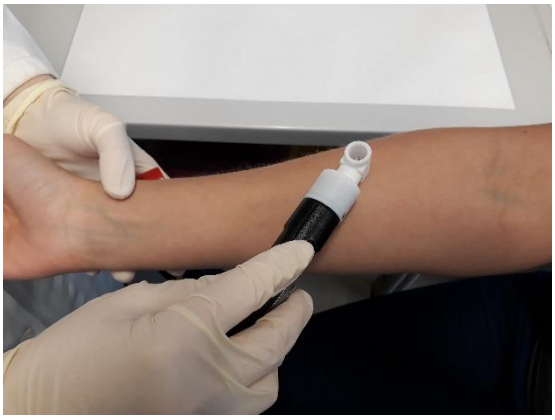


Figure 8: Tewameter measurement procedure with the TM300 Courage + Khazaka for determination of transepidermal water loss.

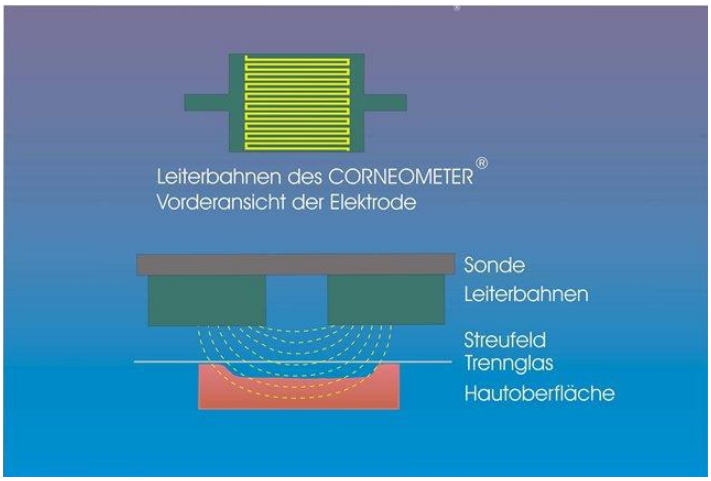
Based on the instructions of Courage + Khazaka the results of the Tewameter were interpreted as followed: 0-10 g/hm^2: very healthy skin; 10-15 g/hm^2: healthy skin; 15-25 g/hm^2: normal skin; 25-30 g/hm^2: stressed skin; >30 g/hm^2: critical skin.

[1] (Courage + Khazaka, TM-Sonde Deutsch 2016/12 DK).

2.8.2.2 Corneometer

The Corneometer is used for measuring the skin moisture and is based on capacitance measuring of a di-electric medium, known as the Corneometer-method.

The stratum corneum serves thereby as di-electric medium, what means that when the SC gets moist, a change in its di-electric properties appears.



The measuring principle is based on electric fields, that builds between the metal conducting paths. During the measurement an electrical stray field penetrates the upper most skin layer and measures the change in the di-electric constant, what is directly correlated to the skin surface hydration.[2]

Figure 9: Construction of the Corneometer measuring electrode, showing the measuring principle of the skin moisture measurement with the Corneometer (Courage + Khazaka, CM-Sonde Deutsch 2016/12 DK).

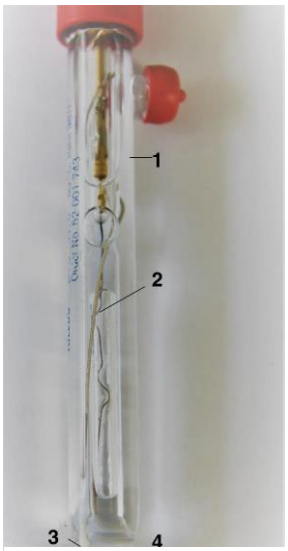
The Corneometer is the chosen method of measuring the skin moisture and was also performed 3 times on 3 slightly different, but correlating skin areas.

Figure 10: Cornemeter measurement procedure with the CM825 Courage + Khazaka for determination of skin moisture.

Based on the instructions of Courage + Khazaka the results of the Corneometer were interpreted as followed: < 30 Corneometer unit (CU): very dry; 30-40 CU: dry; > 40 CU: adequately moist.

[2] (Courage + Khazaka, CM-Sonde Deutsch 2016/12 DK).

2.8.2.3 Skin-pH-Meter



Due to its excretions and moisture content, skin allows reliable and significant measuring of pH directly on the skin surface. The pH as a result of hydrogen ion concentration helps to rate the acid-base status of the skin.

The measurement of the skin-pH is conducted with a special combined electrode. The measuring electrode is in direct contact with the skin, while the reference electrode and the skin are separated by a diaphragm, what leads to measuring differences of the electric potential.[3]

Figure 11: PH- combined electrode, showing the principles of the skin pH measurement. 1 refers to the reference electrode with electrolytes, 2 to the measuring electrode with liquid buffer inside, 3 to the measuring area (the membrane) and 4 to the diaphragm (Courage + Khazaka, pH-Sonde Deutsch 2017/06 DK).

The pH of the skin was measured accordingly 3 times on 3 different but neighbouring skin areas of the same type with the pH-Meter.

Figure 12: pH-Meter measurement procedure with the PH905 Courage + Khazaka for measuring the current skin pH.

Following the instructions of Courage + Khazaka measurements of pH were classified into three categories. 3.5-4.49: acid range, 4.5-5.5: normal range, 5,51-7: alkaline range.

2.9 Negative Controls

In addition to skin swab samples, negative controls were carried out, including PCR and sequencing controls, DNA extraction controls, as well as swab controls at each and every point

[3] (Courage + Khazaka, pH-Sonde Deutsch 2017/06 DK)

in time of sampling. In addition, control examinations of each product were done. Thus, good for excluding effects of potential contamination by laboratory reagents.

2.10 Statistics

The results of the skin measurements were statistically analysed, using a paired T-Test in Excel and a two-way mixed ANOVA model run on IBM SPSS Statistics Version 23.

2.10.1 Paired T-Test in Excel

A paired t-test was used to compare the averages of the values of the measurements at the different points of time of all 3 skin parameters, in order to identify significant differences during the period of product-use in synopsis with the Two-Way mixed ANOVA. All test-results with a p value < 0,05 were classified as significant.

2.10.2 Two-Way Mixed ANOVA

A two-way mixed ANOVA was used for additional statistical analysis of the measurement data. For quality control, the data were also analysed by standard deviation, variance and covariance with the Levene test and the Ljung-Box-Test. In addition, a Mauchly's sphericity test was run with all study data. Outliers, which were defined by a studentized residual larger than +/-3, were not excluded of the analysis. Values $p > 0.05$ for the standard deviation according to Levene and Mauchly test, and $p > 0.001$ for the Box-Test were necessary to fulfil the requirement of equality. Furthermore, Bonferroni correction was used. Also for the ANOVA all results with a p value < 0,05 were classified as significant.

2.11 Molecular Biology Methods

2.11.1 DNA Extraction

DNA extraction was done by Fast Spin DNA Extraction Kit from MP Biomedicals with Lysing Matrix E tubes according to the manufacturer's instructions under following modifications: 10 minutes shaking with a vortex device for the samples of the first two sampling time points, whereas for the samples of the other two sampling time points a Magna Lyser from Roche with 2*30 sec with a cooling and spin down step in-between was used, since the vortex device broke

meanwhile. The first centrifugation step according to the manual was performed for 10 minutes (MP Biomedicals[4]).

The swabs for the analysis were transferred from the DNA free Eppendorf tubes into the Matrix Lysis E tubes using flame sterilised forceps, placing the heads of the swabs upside down, for running the bead beating process.

To be able to exclude possible contamination from the reagents, an extraction control was included, by performing FastDNASpin DNA extraction using the same Kit but without adding any sample.

Afterwards the concentration of DNA was measured with Qubit ds-DNA HS Assay Kit (Life Technologies[5])according to the manufacturer's protocol with 1 µl of DNA per sample.

The extracted DNA was stored at -20°C for further analysis.

2.11.2 Polymerase Chain Reaction (PCR)

For further analysis of the microbiome samples, a 16S rRNA gene amplification was used for amplicon-based Illumina MiSeq sequencing, while DNA amplification was conducted, using Takara ExTag DNA Polymerase. The exact primer sequences can be found in chapter 2.6. The protocol for the 16S rRNA gene amplification is shown in Table 8 below and was performed in a 30 µl reaction mix.

Table 7: PCR reagent mix for 16S rRNA gene amplification with Takara ExTaq Polymerase for Illumina Sequencing.

Reagent	**Concentration**	**Volume/ Reaction**	**Final Concentration**
Ex Taq® Buffer (with $MgCl_2$)	10x	3 µl	1x
llu_515f	10.0 µM	0.9 µl	450 nM
Illu_806r	10.0 µM	0.9 µl	450 nM
BSA	20 mg/ml	1.5 µl	1.0 mg/ml
dNTP mix	2.5 mM each	2.4 µl	0.2 mM each
Ex Taq Polymerase	5 U/µl	0.1 µl	0.025 U/µl
template		2 µl	
LiChrosolv H_2O		19.2 µl	

Since all extracted DNA samples were under the detection limit of the Qubit ds-DNA Assay Kit, all PCR reactions were performed with 2 µl of DNA solution. If samples did not show any results, we repeated the protocol with 1 µl of DNA.

[4] MP Biomedicals: FastDNA® SPIN Kit, Instruction Manual

[5] Life Technologies: Qubit dsDNA HS Assay Kit, Instruction Manual

Extracted DNA from *Escherichia coli* or *Bacillus subtilis* cultures were used as positive controls during each PCR. For this purpose only 0,5 µl of DNA mixed with 1,5 µl LiChrosolv H_2O was added as template. For negative controls, the template was replaced by the same amount of pure LiChrosolv H_2O.

The PCR thermocycler conditions were chosen according to the protocol from Caporaso et al., 2011 and are shown in Table 8.

Table 8: Thermocycler protocol for the 16S rRNA amplification.

Step	**T [°C]**	**t [sec]**	**Cycles**
Initial Denaturation	94	180	1x
Denaturation	94	45	35x
Annealing	50	60	
Elongation	72	90	
Final Elongation	72	600	1x
Storage	4	∞	

The PCR product size was confirmed by agarose gel electrophoresis and the product was stored at -20°C until processing.

2.11.3 Agarose Gel Electrophoresis

Agarose gel electrophoresis was used to verify the PCR amplifications. 1,5% agarose was mixed with 1x TAE buffer in an Erlenmeyer flask and microwaved until all agarose was dissolved and the mixture appeared completely transparent. Since during this boiling process a lot of water evaporates, the mixture was weighed out before microwaving and evaporated water was replaced with buffer to keep the concentration of the gel at 1,5%. 20 ml of the hot agarose-solution were poured into a 50 ml Falcon tube and allowed to cool down to about 60°C before 2,0 µl of GelRed Nucleic Acid Stain (x10000, Biotium) were added. The solution was mixed and the gel was then directly transferred into a custom made 20 ml gel chamber. Afterwards a comb was inserted, the gel chamber covered with the lid and the gel left approximately 10 minutes for drying.

After the gel was solidified the comb was removed and the chamber filled with 1x TAE buffer as a running buffer, till the gel was covered. For the electrophoresis 4 µl of sample were mixed 1:4 with 5x loading dye (Life Technologies) and subsequently pipetted into the gel pockets. 2,5 µl of FastRuler™ Low Range DNA Ladder (Invitrogen Life Technologies) were used as a size-marker and pipetted at the outer right and outer left pocket. The power source was set to 400 mA and 70 V for 35 minutes. Afterwards the gel was imaged with a Universal Hood II Gel Imager (Bio-Rad).

2.12 Sequencing and Data Analysis

2.12.1 Next Generation Sequencing (NGS) Procedure

Illumina MiSeq sequencing was carried out bythe Core Facility Molecular Biology at the center for medical research (ZMF), Graz (Austria). 20 µl of each PCR product were pipetted into 96 well plates and sealed with an adhesive foil. This was performed under a laminar flow. The plate was then handed over to the Core Facility Molecular Biology team, where the library preparation and the actual sequencing were carried out. Below a Workflow[6] description from Core Facility Molecular Biology is shown, describing the sequencing procedure.

Workflow overview:

- Normalization: 15 µl of PCR product were normalized according to the manufacturer's instructions on a SequalPrep™ Normalization Plate from Life Technologies.
- Indexing PCR: 15 µl of the normalized PCR product were used as template for indexing PCR (shown in table 9 and 10). This was performed in a 50 µl single reaction to introduce barcode sequences to each sample (according to Kozich, et al., 2013).
- Purification: 5.0 µl of each indexed sample were pooled. Then 50 µl of the unpurified library were loaded on a 1% agarose gel and purified from the gel with a QIAquick Gel Extraction Kit (Qiagen) according to manufacturer's instructions.
- Quantification & validation: The pool was quantified using the QuantiFluor® ONE dsDNA Dye on the Promega Quantus™ instrument according to manufacturer's instructions. Size of the sequencing library was validated on an Agilent 2100 Bioanalyzer (Agilent, Santa Clara (US)) using a high sensitivity DNA assay according to manufacturer's instructions.
- Sequencing: The pool containing all samples was run at 6 pM final concentration with version 3 600 cycles chemistry (Illumina) according to manufacturer 's protocol and with 20% PhiX control DNA (Illumina).
- Data output: FASTQ.GZ files are provided and are, after unpacking them to FASTQ files, used for data analysis.

[6] Workflow description adapted from Core Facility Molecular Biology, ZMF, Graz, Austria

Table 9: PCR reagent mix for Illumina sequencing by the Core Facility Molecular Biology Graz.

Reagent	Concentration	Volume
Fast Start High Fidelity Buffer	Not specified	5 µl
Forward primer	10 pmol/µl	2 µl
Reverse primer	10 pmol/µl	2 µl
deoxyribonucleoside triphosphates (dNTPs)	Not specified	1 µl
High Fidelity Enzyme	5 U/µl	0.5 µl
PCR-product normalised	Not specified	15 µl
PCR-grade water		24,5 µl

The used Thermocycler protocol is shown below in Table 10.

Table 10: Thermocycler protocol for the indexing PCR performed by the Core Facility Molecular Biology, Graz.

Step	T[°C]	t[sec]	Cycles
Initial Denaturation	95	180	x1
Denaturation	98	45	x8
Annealing	55	45	
Elongation	72	60	
Final Elongation	72	420	x1
Storage	10	∞	

2.12.2 Processing of Sequencing Raw Data

The raw Illumina MiSeq® sequencing data were analysed on an in-house server provided by the ZMF. The easy to use graphical interface "Galaxy" (Afgan et al., 2016), runs on the server and makes it easy to modify, share and execute implemented workflows. The data obtained from QIIME (Caporaso et al., 2010) and PICRUSt (Langille et al., 2013) were further processed using Microsoft Excel and then pictured with Calypso (Zakrzewski et al., 2017).

For the analysis QIIME 1.9.1. (Caporaso et al., 2010) was used. The most important steps of the QIIME-workflow that were used are shown in Table 11.

Table 11: Overview of the QIIME Workflow with parameters and according values.

QIIME workflow step	Parameter	Value
Input Dataset	Data file Parameter file Mapping file	Fastq txt, tab delimited txt, tab delimited
Count sequences		standard
Multiple join paired ends		standard
Multiple split libraries fastq		standard
Count sequences		standard
Cutadapt	Maximum error rate Match times Minimum overlap length Minimum length Maximum length	0,1 1 3 1 1

Count sequences		standard
Cutadapt	Maximum error rate Match times Minimum overlap length Minimum length Maximum length	0,1 1 3 1 1
Count sequences		standard
Identify chimeric sequences	Chimera detection method Reference sequences	usearch61 (Edgar, 2010) GreenGenes_13_8_97_otus
Filter fasta		standard
Count sequences		standard
Pick open-reference OTUs	Reference sequences	GreenGenes_13_8_97_otus
Create summary of BIOM table		Standard
Core diversity analysis	Analysis to run	Parameter file
Convert BIOM		Standard
Convert BIOM to txt	Input BIOM file Including the taxonomy observation metadata	"Pick open-reference OTUs: OTU table with taxonomic assignment without sequencing failing the alignment" Yes
Summarize taxa	Input OTU table	"Pick open-reference OTUs: OTU table with taxonomic assignment without sequencing failing the alignment"
Calculate α diversity	Input OTU table	"Pick open-reference OTUs: OTU table with taxonomic assignment without sequencing failing the alignment"

"standard" refers to values, automatically chosen from QIIME.

The violet highlighted steps were added manually to the standardised workflow.

An analysis with QIIME yields tables containing operational taxonomic units (OTUs) with assigned taxonomies. Absolute counts for each OTU are shown for each sample individually, but since a normalisation step is carried out before NGS, only relative amounts are comparable across all samples.

Different OTUs can represent the same group of organisms. Because of that it is necessary to use the "summarize taxa" step at the end of the workflow to cluster OTUs on a taxonomic level of choice.

Since this approach makes it difficult to extract and exclude the negative controls, this file was used only for the taxa, while a separate OTU table was designed from the original BIOM file after converting it to a txt-file.
The same workflow was rerun for the Follow-up including all samples from T1, T2, T3 and T4 and furthermore it was used for an analysis with the Silva database (silva_128_release_rep_set_all_97_97_otus), for comparative reasons.

2.12.3 PICRUSt

PICRUSt (Langille et al., 2013) is used to predict the genomic functions, based on 16S rRNA sequence diversity and can only be performed based on the GreenGenes database (GreenGenes_13_8_97_otus). The PICRUSt-Workflow is shown in Table 12.

Table 12: Overview of the PICRUSt Workflow steps with parameters and according values.

PICRUSt Workflow Step	Parameter	Value
Input dataset	BIOM table	"Pick open-reference OTUs: OTU table with taxonomic assignment without sequencing failing the alignment"
Filter OTUs from an OTU table	Reference Sequences	GreenGenes_13_8_97_otu
Convert BIOM		standard
Normalize by copy number	GreenGenes Version	13_5
Predict metagenome	GreenGenes Version	13_5
Categorize by function	GreenGenes Version	13_5

"standard" refers to values, automatically chosen from QIIME.

An analysis with PICRUSt results in two large datasets, "predict metagenome" and "categorize by function".
By linking the information contained in both datasets, functions, that are enriched in a specific sample, can easily be picked out.
The PICRUSt file was also adapted in Excel and then used for picturing in Calypso.

2.12.4 Filtering

To remove the data from negative controls from final results, a cut-off of 70% was set for summarized data in terms of genus rank. All genera, of which more than 70% were obtained in the negative controls, were removed. This was only applied on the genus level, while no removal of data at other taxonomic ranks was possible.

For the OUT table the cut-off for removing values of the negative controls was set at 1%. Furthermore, the output was subject to singleton read filtering, removing all OTUs with less than 5 sequences. The output was also filtered off chloroplast, mitochondria, chimeric sequences and unassigned reads.
The PICRUSt file was not filtered at all.

2.12.5 Calypso

The 16S rRNA gene biome table as well as the PICRUSt file were analysed by using Calypso (http://cgenome.net/calypso/) (Zakrzewski et al., 2017)
at following settings:

- square roots normalization (L2 Normalisation (LSE) – working on minimising the sum of the square of the differences) and Total Sum Scaling (TSS) normalisation
- no samples removed
- no taxa removed
- 0,005% cut-off for rare taxa

The taxa files obtained from Galaxy, were already normalized during the "summarize taxa" step in Galaxy and therefore no Calypso normalization was run on these data.
All data were compared in terms of α and β diversity and the abundance of different classification levels were shown. This was done for the different time points for all groups together, the single groups and for the third sampling time point of all groups, as well as for the Follow-up sampling. The data were also compared using Linear discriminant analysis Effect Size (LEfSe) algorithm to identify taxa that were significantly different in abundance between the different treatments (Segata et al., 2011).

3 Results

3.1 Statistical Analysis of the Skin Measurements

3.1.1 Main Study

32 Participants (11 from group 1, 11 from group 2 and 10 from group 3) completed the study. Only results from participants who completed the study are subject to the following.
Skin physiology, as revealed via the measurements of water loss, pH and moisture changed significantly for all groups, during the study. This happened irrespective of the product, that was used by the participants.

Overall only 3 outliers were identified, all to be found in group 1. One occurring at the third measuring time point of the Tewameter (46,38) and one each at the second (44,5) and the third (31,57) measuring time point of the Corneometer. Since the results did not change significantly, when removing the outliers, they were not excluded from the analysis.

The Tewameter results only showed standard distribution when logarithmised, what was used for further statistical analysis. Still the time point T2 of group 3 did not show any standard distribution ($p = 0.032$), but nevertheless the data were included in the study. As well the variance of the T2 group was below 0.05 ($p = 0.005$) and therefore did not fulfil the requirement of equality, usually used for this kind of statistical analysis.
As to the values of the Corneometer measurements, the standard distribution was not fulfilled for T2 of group 1 ($p = 0.018$) and also the sphericity of this point in time showed a value below 0.05 ($p = 0.044$).
As all other criteria of equality were fulfilled completely and as there was no possibility to harmonise the data in excess of the described manner, the statistical analysis was completed with the existing data.

Significance was found for the parameter time in the Tewameter measurements ($p = 0.015$) and the Corneometer measurements ($p = 0.001$), what means that the water loss decreased and the skin became drier over time (Fig. 13 and 18). No significant differences were observed between all groups, for interaction of time and group regarding the pH-Meter values (Fig.23). The time-

significance concerning the epidermal water loss (Tewameter), which decreased over time, and the moisture (Corneometer), which also became less over time, were found between time point 1 and 3 ($p = 0.001$ for the Corneometer and $p = 0.037$ for the Tewameter) as well as time point 2 and 3 ($p = 0.049$ for the Corneometer and the Tewameter), but not between the time points 1 and 2. In the Multivariante Test, group 3 ($p = 0.035$)and in the Test of "Within-Subjects Effects" group 2 ($p = 0,017$) also showed a significant change over time in the Corneometer values, representing the change of the skin moisture, while that could not be found valid for group 1.
These results were confirmed via paired T-Tests, except the significance related to the epidermal water loss and the difference between the time points 2 and 3, which could not be verified.
Testing on "Within-Subjects Effects" showed significant differences for group 3 ($p = 0.044$) over time in the Tewameter measurements, what could not be confirmed by the T-Tests.
This means, that during product use the skin of the participants became drier and their skin barrier function improved. This effect was detected for all groups and could not be related to any specific product.

3.1.1.1 Tewameter

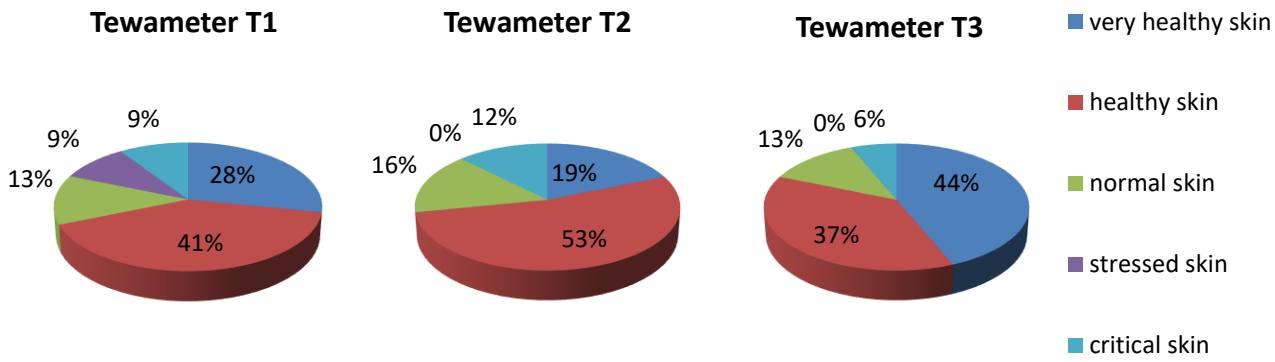


Figure 13: Comparison of the percentage of different skin types throughout all study subjects based on the epidermal water loss measured with a Tewameter before product use (=T1), after 2 weeks of product use (=T2) and after 4 weeks of product use (=T3).

As resumé overall results of all groups epidermal water loss decreased over the sampling time, and more participants showed "very healthy" skin type according to the specified criteria. (Fig.13)
In respect of the average values of specific timepoints, no significant change could be found. Anyway, in Figure 14 a trend of group 1 and group 2, being more similar than group 3, is visible.

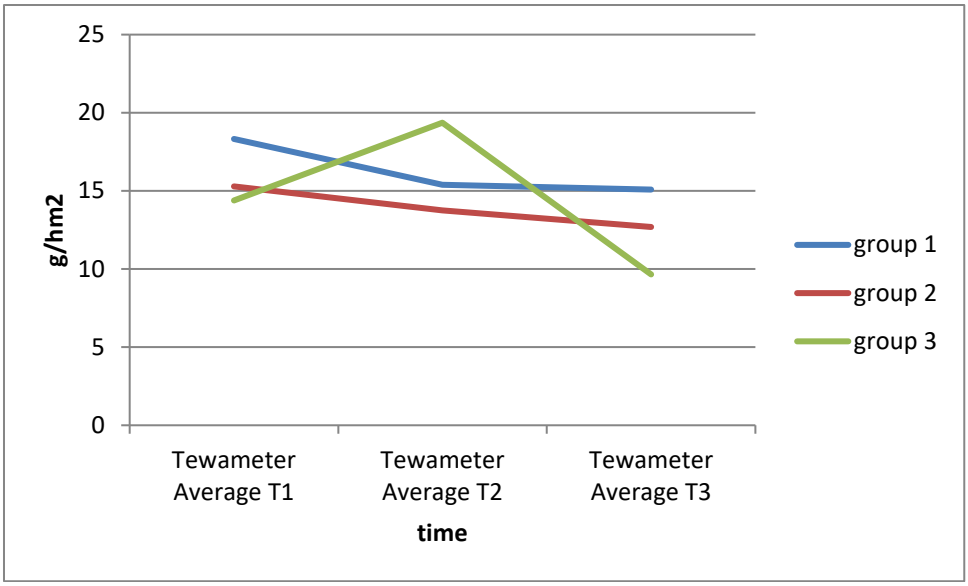


Figure 14: Progression of the water loss (Tewameter averages) from the 3 groups before product use (=T1), after 2 weeks of product use (=T2) and after 4 weeks of product use (=T3).

The values of the skin measurements using the Tewameter are shown for each group individually and prove that the transepidermal water loss decreased over all groups and is therefore not significant for a single one of them (Fig. 15 - 17).

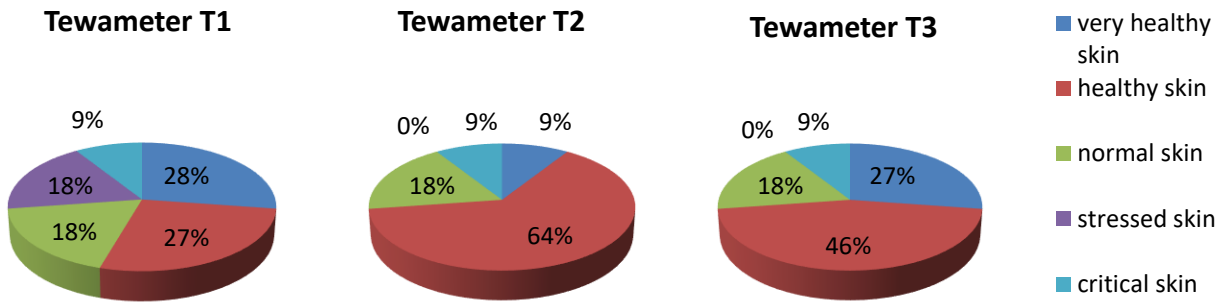


Figure 15: Comparison of the percentage of different skin types of all participants of group 1, based on the epidermal water loss, measured with a Tewameter before product use (=T1), after 2 weeks of product use (=T2) and after 4 weeks of product use (=T3).

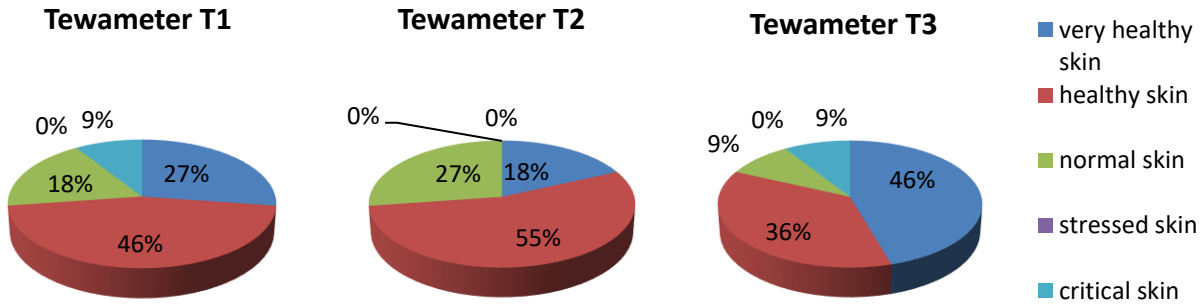


Figure 16: Comparison of the percentage of different skin types of all participants of group 2 participants based on the epidermal water loss measured with a Tewameter before product use (=T1), after 2 weeks of product use (=T2) and after 4 weeks of product use (=T3).

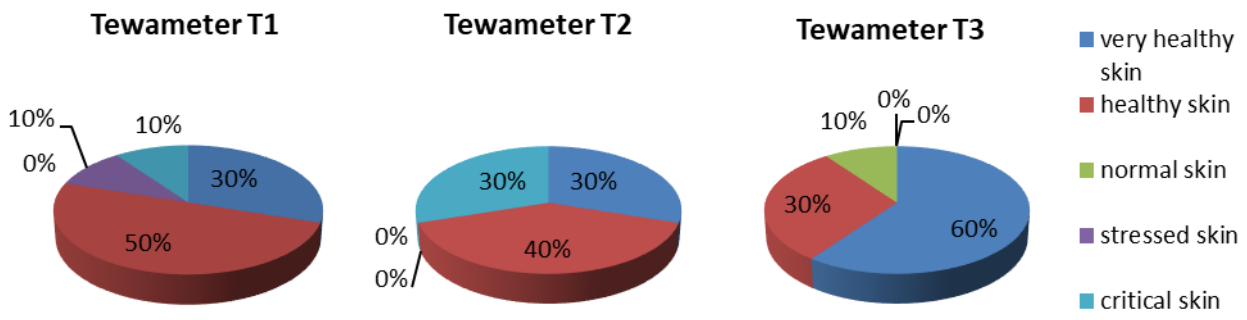


Figure 17: Comparison of the percentage of different skin types of all participants in group 3, based on the epidermal water loss measured with a Tewameter before product use (=T1), after 2 weeks of product use (=T2) and after 4 weeks of product use (=T3).

3.1.1.2 Corneometer

The skin of the participants also became drier over time (Fig. 18). This effect was statistically significant, even if most of the values were already in the range of "very dry" skin from the very beginning of the study.

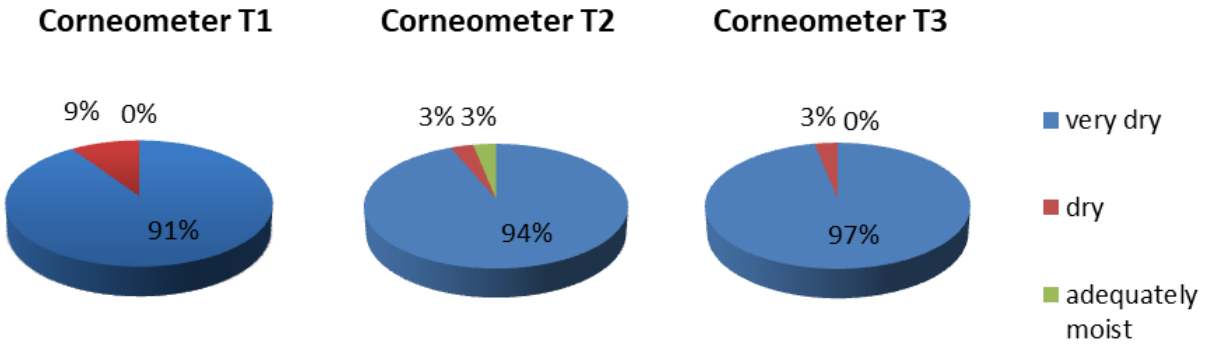


Figure 18: Comparison of the skin moisture of the study subjects, based on the Corneometer measurements before product use (=T1), after 2 weeks of product use (=T2) and after 4 weeks of product use (=T3).

The averages of the group-specific values related to the points in time of measurement showed again a higher congruency between group 1 and group 2, than compared with group 3, even if this difference was not significant (Fig. 19).

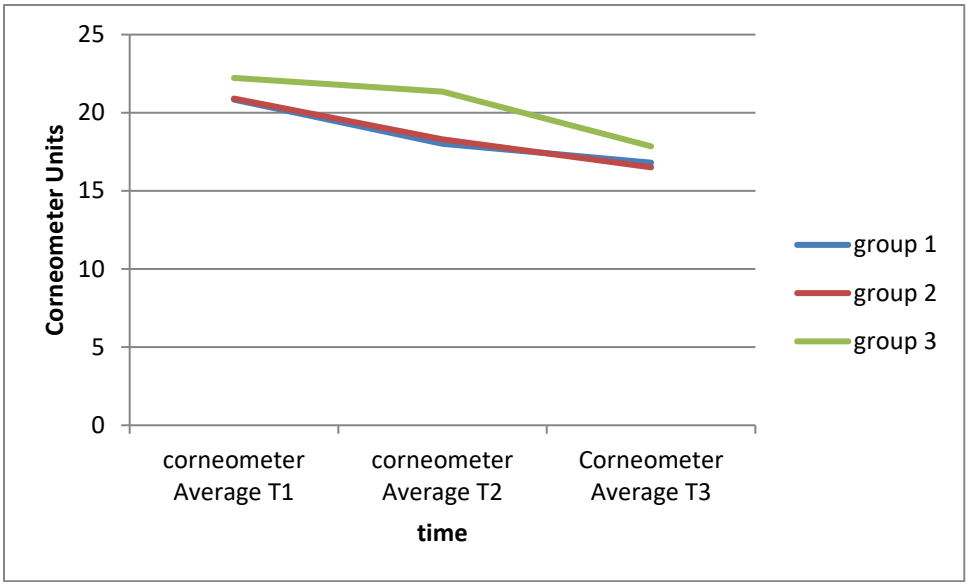


Figure 19: Progression of the skin moisture (average values of Corneometer measurements) for all 3 groups before product use (=T1), after 2 weeks of product use (=T2) and after 4 weeks of product use (=T3).

The distribution of all skin Corneometer measurements in relation to the classification of skin quality, are again shown for each group individually. This analysis shows that the moisture of the skin decreased significantly in group 2 and group 3 but not in group 1(Fig. 20-22). This means that all the participants of group 2 and group 3 ended up in the range of very dry skin. Even if this change was statistically significant, it must be considered with caution, since each group only contains 10 or 11 participants and the statistical value of change is based on the change of the skin parameters of only one single individual per group.

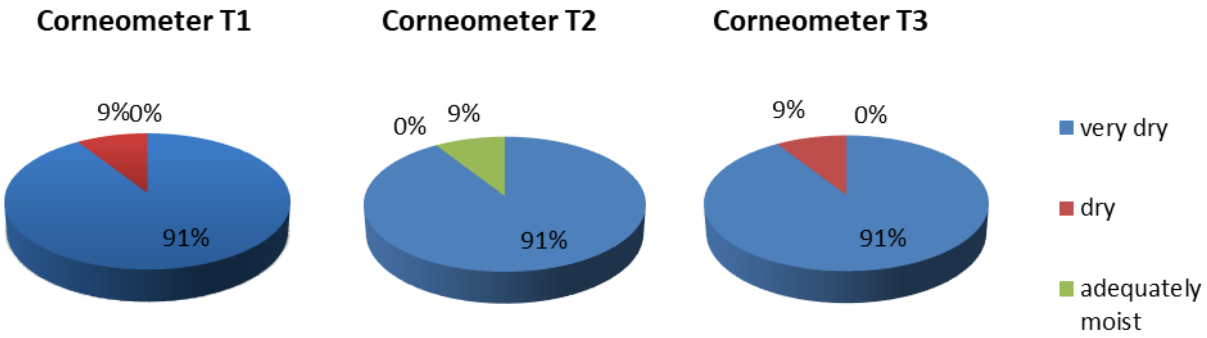


Figure 20: Comparison of the skin moisture of the participants of group 2 based on the Corneometer measurements before product use (=T1), after 2 weeks of product use (=T2) and after 4 weeks of product use (=T3).

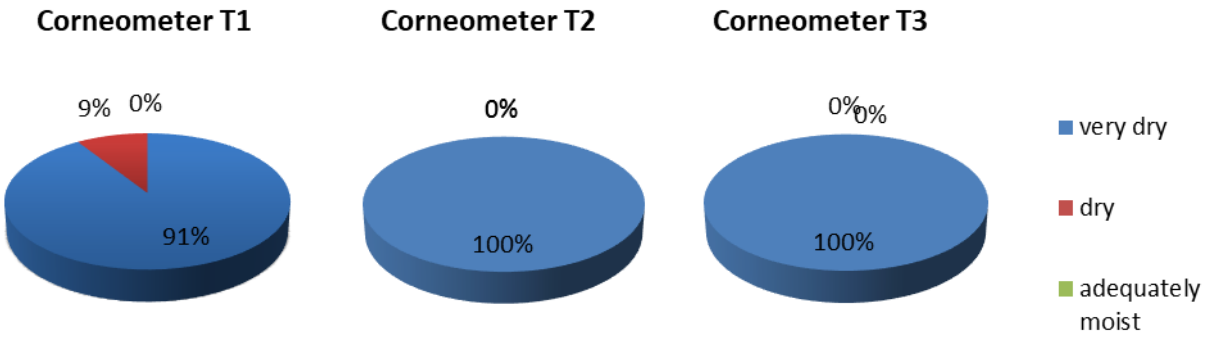


Figure 22: Comparison of the skin moisture of the participants of group 1 based on the Corneometer measurements before product use (=T1), after 2 weeks of product use (=T2) and after 4 weeks of product use (=T3).

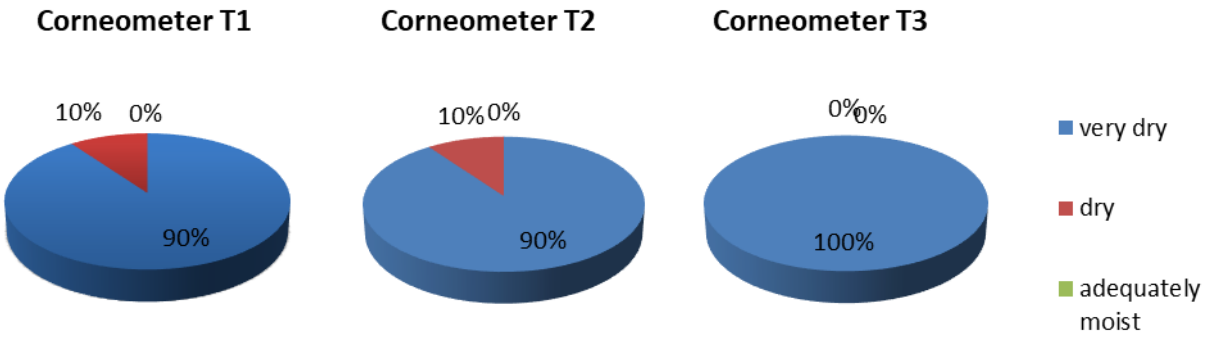


Figure 21: Comparison of the skin moisture of the participants of group 3 based on the Corneometer measurements before product use (=T1), after 2 weeks of product use (=T2) and after 4 weeks of product use (=T3).

3.1.1.3 pH-Meter

The impact on the pH of the skin, related to the use of different products was measured, while the basic average values were 4.5 for group 1 and 6 for group 2 and group 3.

However, no statistically verified effect was found concerning the pH-values of the skin (Fig.23).

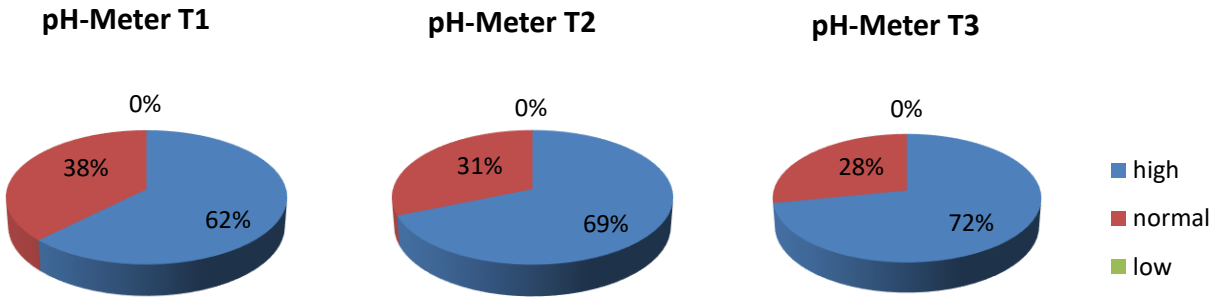


Figure 23: Comparison of the pH of the participants skin based on the pH-meter Measurements before product use (=T1), after 2 weeks of product use (=T2) and after 4 weeks of product use (=T3).

The averages of the pH measurements did not show any trends. Anyhow, the averages from figure 12 show that group 1 started with a much higher pH, which means that a smaller increase in the pH already lead to another ranking, according to the participants' skin physiology. This is also shown in the individual group comparisons, where most high pH ranged participants were found in group 1. However, this was not significant and therefore is not shown.

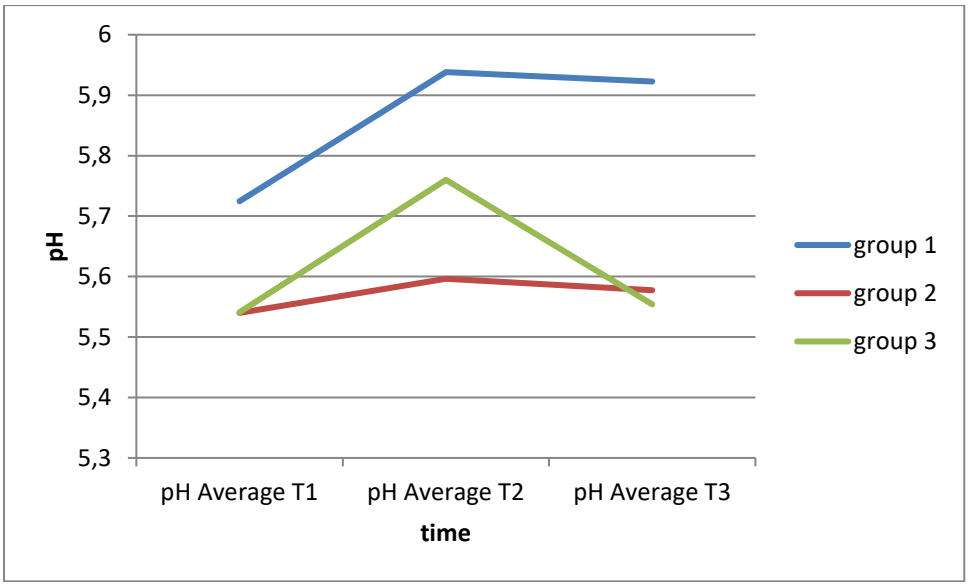


Figure 24: Progression of the average values of pH of the skin for all the 3 groups before product use (=T1), after 2 weeks of product use (=T2) and after 4 weeks of product use (=T3).

3.1.2 Follow-up

As out of the 32 participants, taking part in the main study at the beginning, only 30 (11 participants in group 1, 10 in group 2 and 9 in group 3) completed the study, with showing up at the Follow-up for having final measurements taken. Only values from participants who completed also the Follow-up were taken into account for the results discussed in this chapter.
To provide a reliable basis for comparing the results of all measurements, the data of the two participants, not showing up at the Follow-up, were extracted of all data and a rerun on all statistical analysis over all points in time was performed, according to the procedures and standards applied to in the main study (in the chapters up above).
Even though now less statistically significant changes were found, some important changes still appeared, especially related to skin moisture. Again these effects are visible in particular, when taking into account the data of all participants.

In the values of the measurements done at the Follow-up, there were no other outliers detected, except the three already described in the main study. Again the outliers were not excluded from the analysis.

The Tewameter results again only showed standard distribution when logarithmised, which was used for further statistical analysis. Concerning the Tewameter and the Corneometer the criteria for equality stayed the same as described before. The T2 and the T4 pH-Meter data anyway did not show standard distribution in the Follow-up.

All other equality parameters were again fulfilled and since there were no possibilities to get better results on harmonising the data, even when removing the outliers from the data, the statistical analysis was completed with all existing data.

The significance in the parameter time regarding the measurements of transepidermal water loss and moisture is still obvious, like in the main study (Fig. 25 and 28). Again no significant difference was found between the groups, for interaction of time and group and for the pH-Meter values (Fig.29). The time-significance, related to the epidermal water loss (Tewameter), continued decreasing over time, are for the Follow-up only shown between the timepoint 2 and 3. Regarding the values of moisture, measured with the Corneometer, which also grew less during the product use, but increased during the time of the Follow-up without specific product use, only for the time point 1 and 3 and between 3 and 4 a time dependent significant difference was found in the Follow-up. In the "Multivariate Test", group 3 still showed a significant change over time in the skin dryness values, while such correlation was not found in the data of the two other groups. This effect was especially significant between the time points 3 and 4. In the Follow-up also a significance between group 1 and group 3 concerning the 4^{th} point in time can be found.

These results were again verified in separately run paired T-Tests, except again the significance concerning the epidermal water loss and the difference between the time points 2 and 3, which could not be confirmed.

When testing the "Within-Subjects Effects", this time significance for group 2 and 3 over time was also found in the Corneometer measurements, but this could not be verified by the T-Tests. This means, that during the period between the end of 4 weeks use of the specific products and the Follow-up measurement, the skin of the participants became less dry and their skin barrier function further improved. This effect was found valid for all groups and could again not be related to any of the products.

3.1.2.1 Tewameter Follow-up

When evaluating the data of all participants throughout all groups the epidermal water loss continued to decrease from the last time, using the specified product up to the point in time of the Follow-up sampling, even though the participants did not stick to standardised washing procedures any longer (Fig.25).

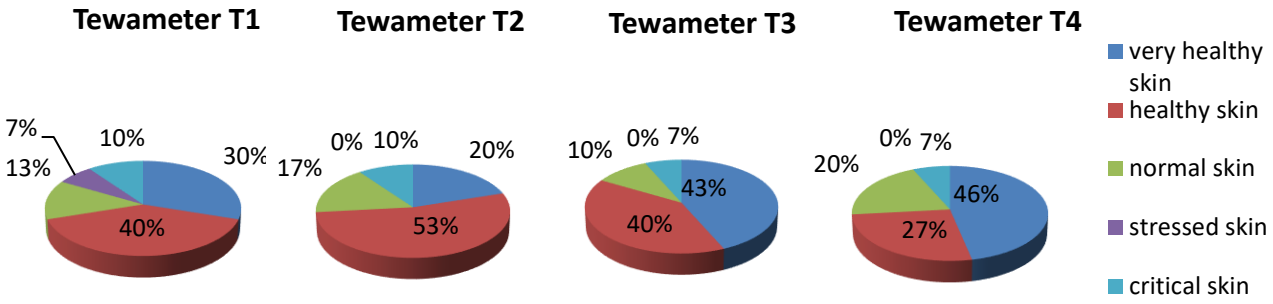


Figure 25: Comparison of the percentage of different skin types throughout all study subjects based on the epidermal water loss measured with a Tewameter before product use (=T1), after 2 weeks of product use (=T2), after 4 weeks of product use (=T3), and after further 6 weeks without the use of specific products (=T4).

The averages of the Tewameter measurements according to the Follow-up revealed an increase of the epidermal water loss for group 1 from T3 to T4 – in fact showing nearly the same values as at the initial measurements at T1, whereas the epidermal water loss still improved in the two other groups. (Fig. 26) This effect however was not statistically significant and can only be seen as a trend. This change is also visible in the individual group analysis, which is shown in the Figures 57 -59 in the Appendix.

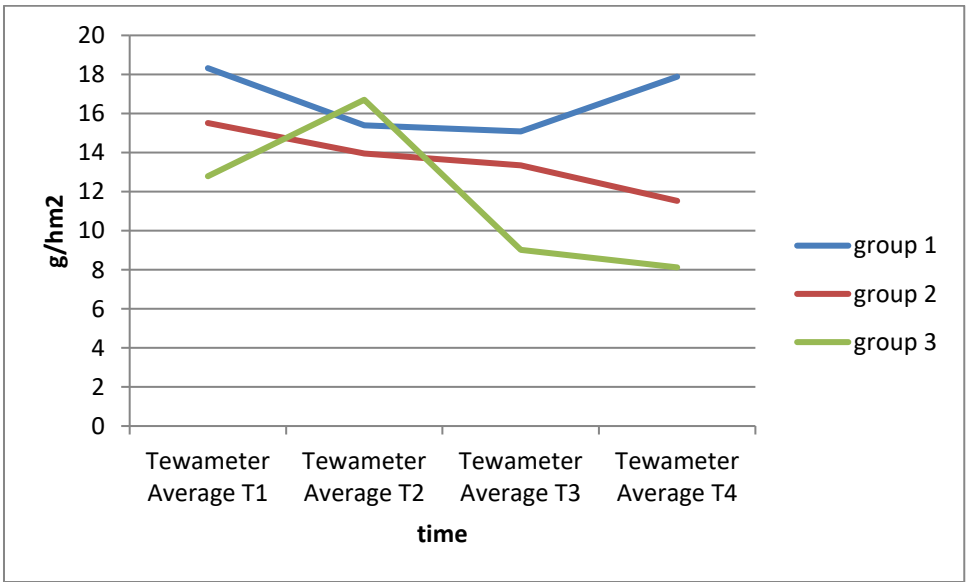


Figure 26: Progression of the average values of the Tewameter measurements for the 3 groups before product use (=T1), after 2 weeks of product use (=T2), after 4 weeks of product use (=T3) and after further 6 weeks without the use of specific products (=T4).

3.1.2.2 Corneometer Follow-up

The skin of the participants became moister from T3 to T4, nearly returning to the basic values of T1 (Fig. 28). This effect is statistically significant, though not appearing in the charts (Fig. 27) obviously, as finally all participants were ranked among "very dry skin".

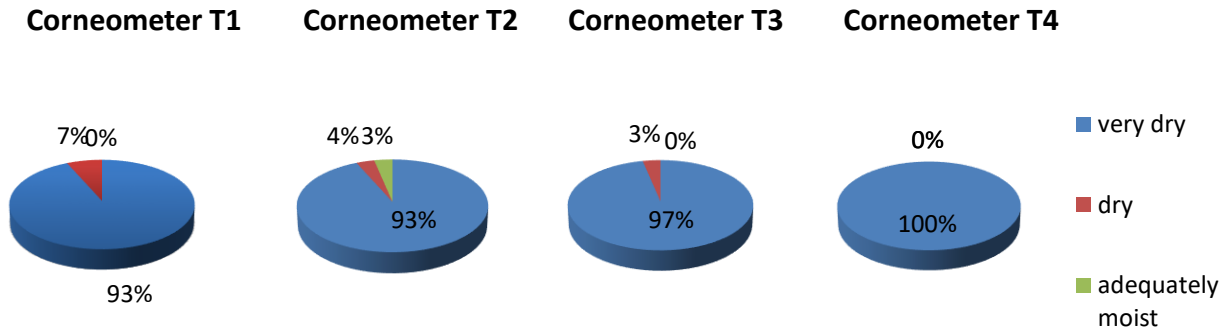


Figure 27: Comparison of the skin moisture of the study subjects based on the Corneometer measurements before product use (=T1), after 2 weeks of product use (=T2), after 4 weeks of product use (=T3) and after further 6 weeks without the use of a specific products (=T4).

The group specific averages of the Corneometer measurements showed an increase of the moisture of the skin of the participants in the 6 weeks of the Follow-up study – T3 to T4, in which they used skin products of their personal choice again (Fig. 28).

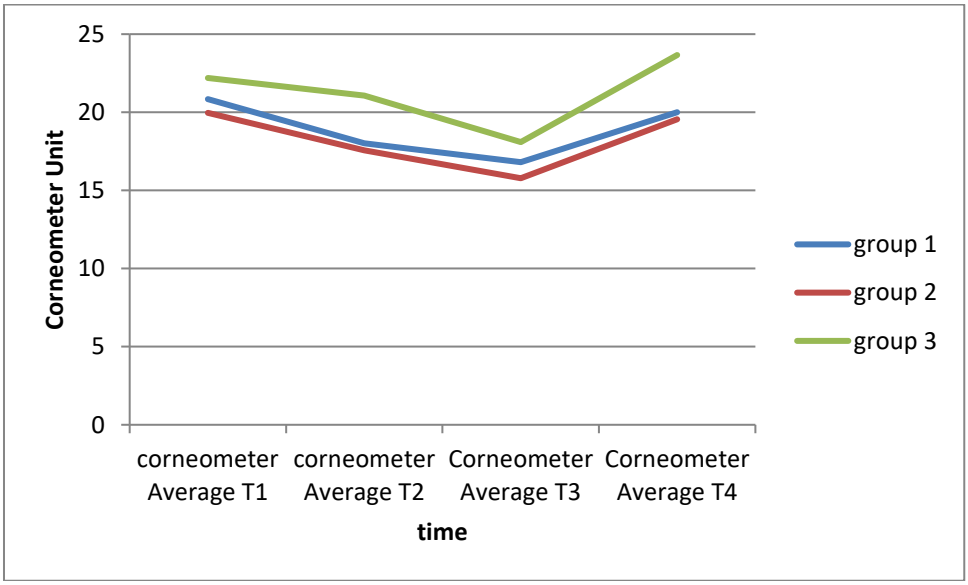


Figure 28: Progression of the average values of the measurements with the Corneometer for the 3 groups before product use (=T1), after 2 weeks of product use (=T2), after 4 weeks of product use (=T3) and after further 6 weeks without the use of specific products (=T4).

3.1.2.3 pH-Meter Follow-up

As in the main study no statistically significant difference was found related to the skin pH values. Anyhow in the pie chart in Figure 29 the higher percentage of participants in the range of normal skin indicates a trend of decreasing skin pH from T3 to T4, somehow returning to the range of values of T1.

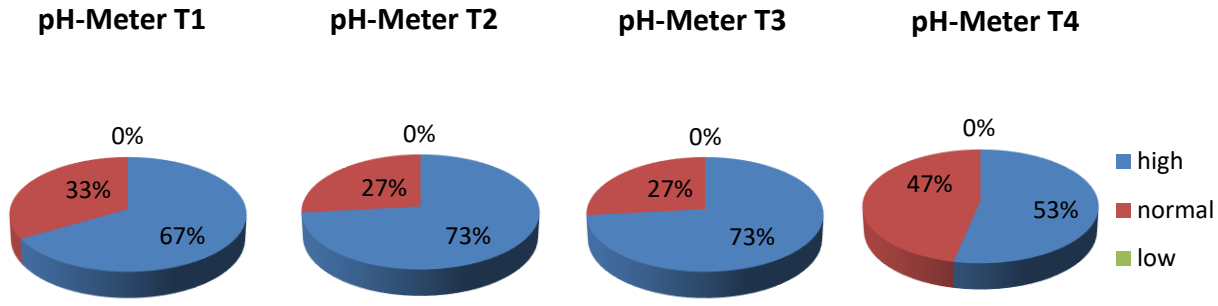


Figure 29: Comparison of the skin pH of the study subjects based on the pH-Meter measurements before product use (=T1), after 2 weeks of product use (=T2), after 4 weeks of product use (=T3) and after further 6 weeks without the use of a specific product (=T4).

The decrease in the pH values from T3 to T4 is also obvious, when looking at the group specific averages of the pH in Figure 30, though this effect only appeared in group 1 and 2, while in group 3 the pH increased (Fig. 30). Since the average values varied only about 0,4 this cannot be told significant, as the range of pH for "healthy skin" lies between 4.5 to 5.5 but is shown to have a broad range from 4.0 to 7.0 in literature (Lambers et al., 2006).

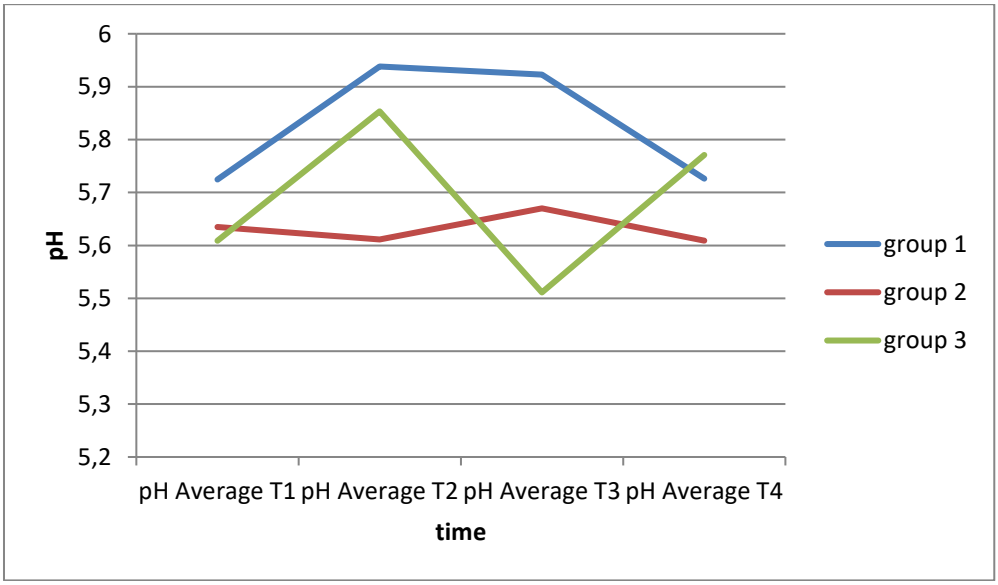


Figure 30: Progression of the pH averages from the 3 groups before product use (=T1), after 2 weeks of product use (=T2), after 4 weeks of product use (=T3) and after further 6 weeks without the use of specific products (=T4).

As shown in Figure 30 the averages of the values of all groups are always higher than 5.5, so within the range of "high skin-pH".

3.2 Microbiome Data of the Skin Samples

3.2.1 Main Study

After DNA extraction, the content of DNA for all samples was under the detection limit of 0.01µg/ml, whereas after PCR, there were visible bands on the electrophoresis gel in all samples.

During the processing, no sample and no negative control was lost because of too less reads.

If reads were found also in negative controls, they were removed from the dataset, according to the predefined percentage stated in the methods part.

The results are shown below split up in α and β diversity, abundance, LEfSe and rarefaction analysis.

3.2.1.1 α and β Diversity

No significant changes were found in α-diversity comparing the different groups.

However, over all groups a significant increase in the α-diversity over time was detected, as shown in the Chao1 and Richness indices (Fig. 31 and 32).

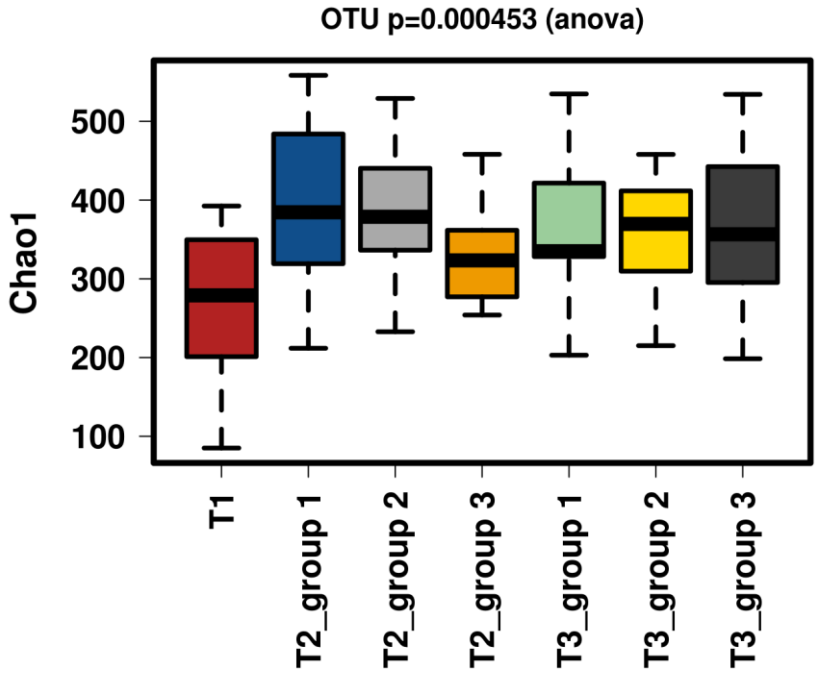


Figure 31: Chao1 indices of the different time points per group and T1 as basic dataset before product use. This figure shows a significant increase of diversity in all groups over time.

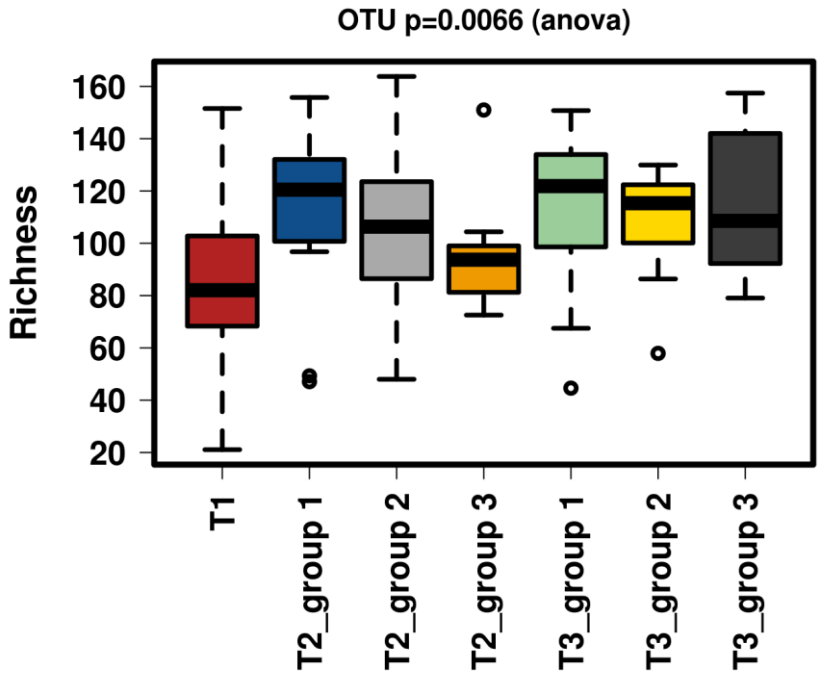


Figure 32: Richness indices of the different time points per group and T1 as basic dataset before product use. This figure shows a significant increase of richness.

In Figure 31 and Figure 32 an increase in diversity for all groups over time is shown for the Chao 1 index, as well as for the Richness index.

In the Appendix the same indices are shown again, with the T1 sample points split for the three different groups. In these figures it is shown, that especially for the Chao 1 index the diversity of the T1 time point between the groups varied strongly (Appendix Fig. 60 and 61).

The effect of increasing diversity in Chao 1 and Richness also appeared regarding the different groups. The increase in diversity for the group 3 and group 2 samples was evident in both α-diversity indices, while for the group 1 this was only to be detected in the Chao1 index (Appendix Fig. 62 - 67). The Chao1 and Richness indices are also shown for the individual groups comparing only T1 with T2 and T1 with T3. These charts show clearly that Chao1 was significant for both compared time points in the group 1, only for T1/T3 in the group 3, and only for T1/T2 in the group 2. The changes in Richness however were not significant for any of the two timepoint comparisons in the group 1, while significant results could be found for both comparisons in the group 2, and T1/T3 in the group 3 (Appendix Fig. 68- 79).

Comparing the β-diversity of the different groups over all time points and for only time point three did not show any significance at all. An example for β-diversity is shown in the Principal Coordinates Analysis (PCoA) Plot for the different time points per each group (Fig. 33).

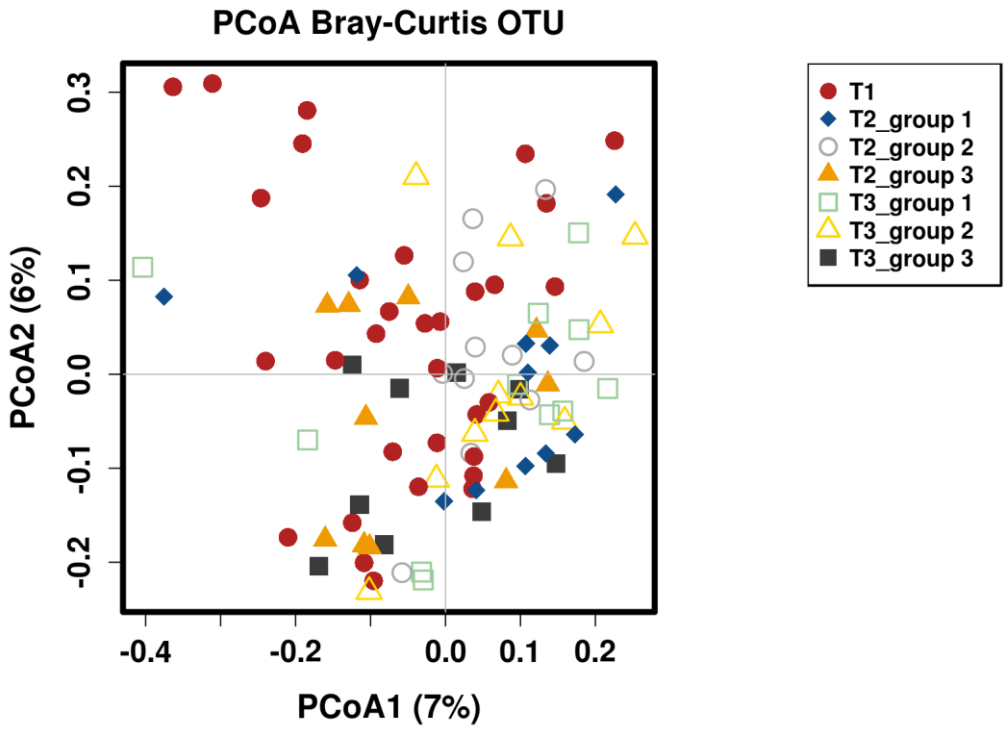


Figure 33: PCoA Plot of the different time points per group. Each point refers to one microbiome measurement. Shapes and colours indicate the different time points and the group (see legend). No grouping could be detected.

This plot is shown again in the Appendix with T1 split between the product groups, where you can see that the outlying T1 sample points moved toward the "crowd" during the product use (Appendix Fig. 80).

Redundancy analysis (RDA -β-diversity) showed that the parameter "time" has a significant effect on the microbiome composition, suggesting that the skin microbial community structure changed during the experiment. However, the percentages of axes in RDA analysis were very low (x-and x%) and therefore explaining only a minor share of the total variance of the data set (Fig. 34).

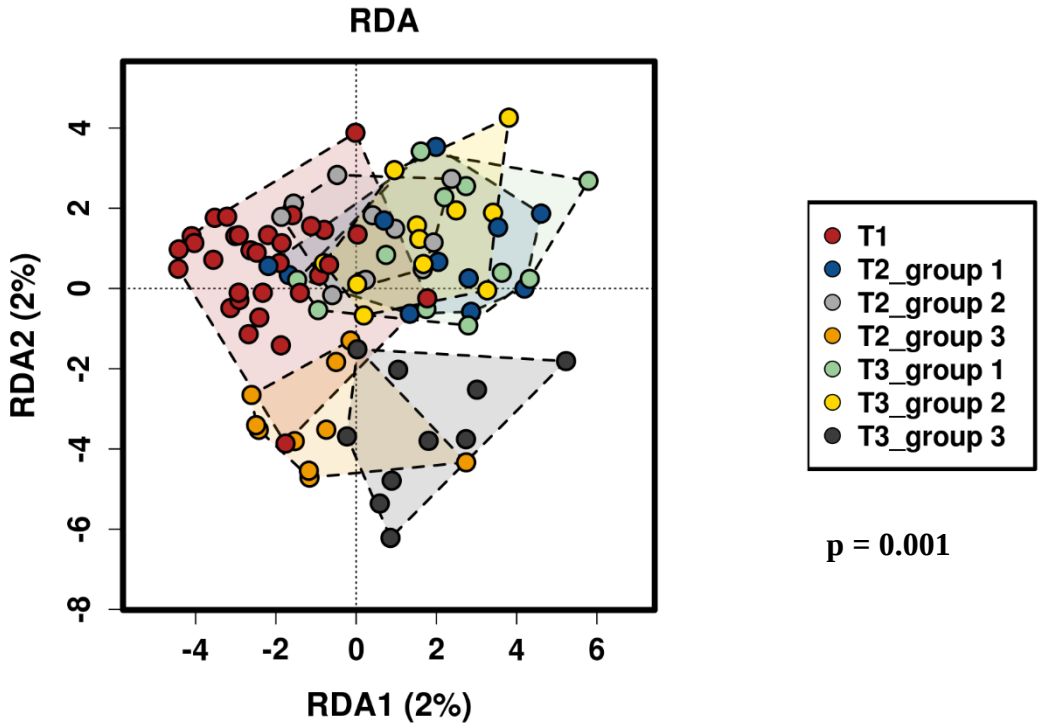


Figure 34: RDA Plot of the different time points per group and T1 as basic dataset before product use.

3.2.1.2 Relative Abundance

The most abundant signatures on phylum level for all samples were Proteobacteria, Firmicutes, Actinobacteria and Bacteroidetes (Fig. 35).

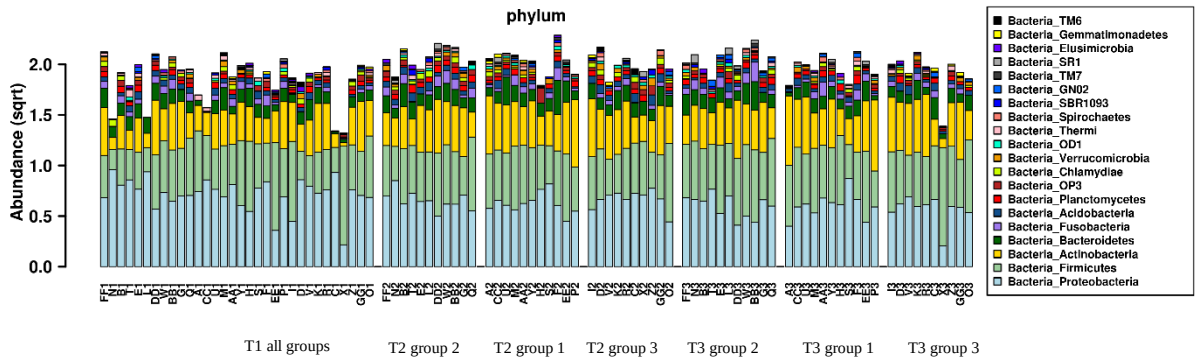


Figure 35: Relative abundance based on phylum level in all samples, grouped per time points and product use.

On genus level signatures of *Staphylococcus*, *Corynebacterium*, *Streptococcus*, *Micrococcus* and *Acinetobacter* were most abundant (Fig. 36).

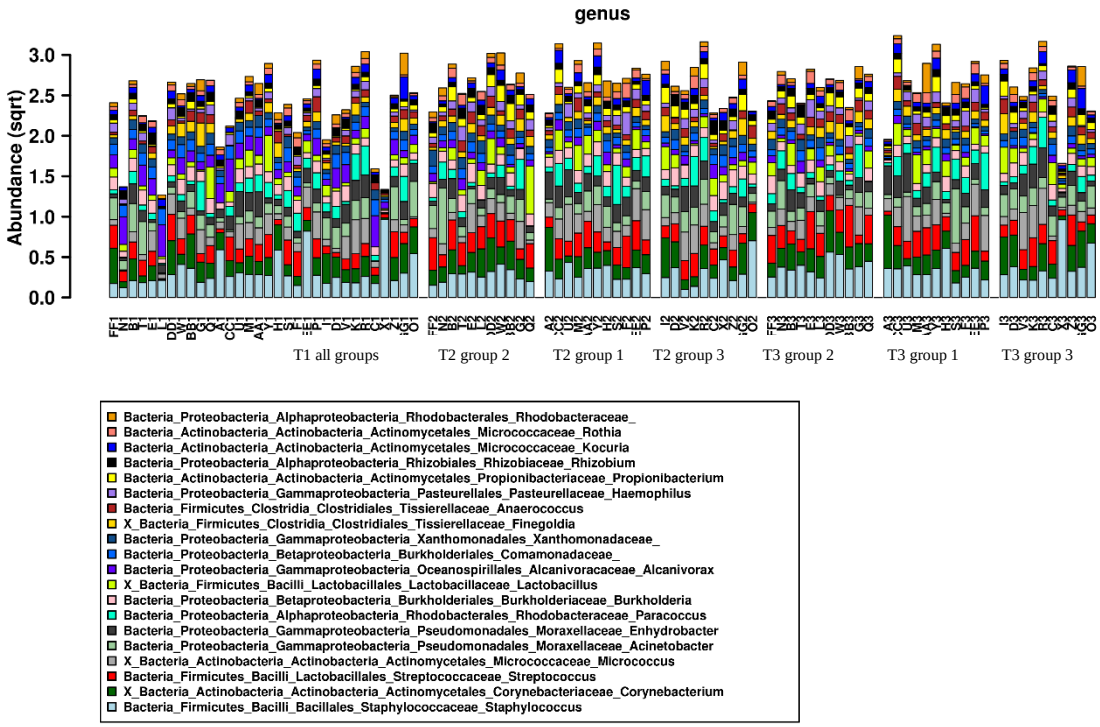


Figure 36: Relative abundance based on genus level in all samples, grouped per time points and product use.

In addition, the relative abundance on OTU level is shown (Appendix Fig. 81).

When interpreting the OTU level, some changes over time could be detected, which are shown in the Appendix in Table 13.

Further research was just performed on species, which are most abundant in Table 13. The chosen OTUs are marked in Table 13. It is shown that signatures of the representatively shown *Micrococcus* OTU, the *Propionibacterium acnes* OTU, the Bacillales OTU and *Staphylococcus* OTU increased in all of the groups over time. The signatures of *Micrococcus* were most abundant in group 1 samples, the Bacillales in the samples of group 3 and group 1, while the *Staphylococcus* OTU was most abundant in group 3 and group 2. Also a *Haemophilus parainfluenza* OTU was significantly increased but only for the second sampling time point of the group 1 and the third sampling time point of the group 2 samples (Fig. 37).

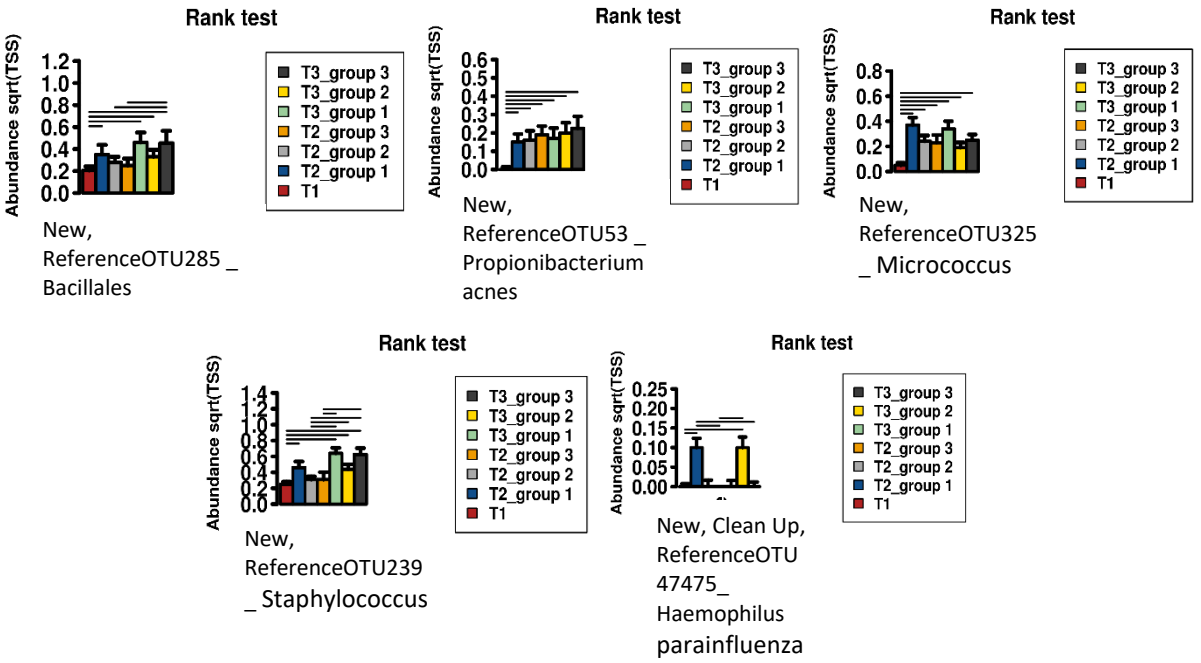


Figure 37: Changes in relative Abundance of selected OTUs, depending on group and timepoint.

Two *Corynebacterium durum* OTU showed significant changes. One was increasing in almost all samples over time, with highest increment for group 2, while the other OTU was only significantly increased in group 2 and group 1 (Fig. 38).

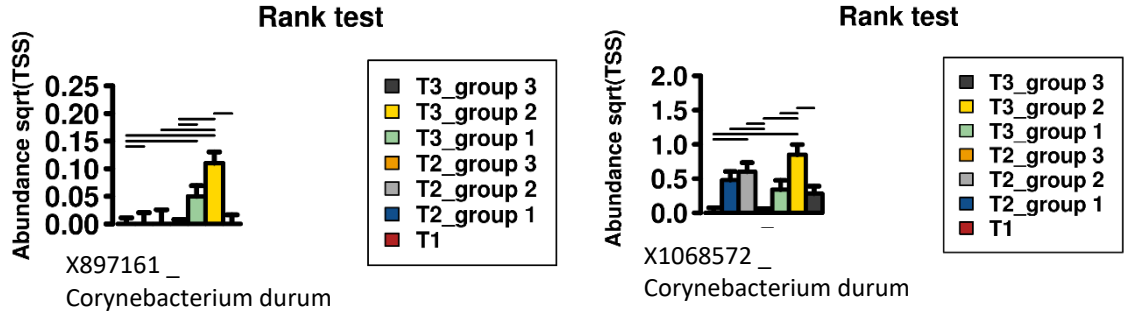


Figure 38: Changes in relative abundance for the different groups at time points T1, T2 and T3 for the two different C. durum OTU.

3.2.1.3 LEfSe

LEfSe analysis reveals group and time point specific OTUs. For T2 of group 1 a *Micrococcus* OTU showed significance, while a *Staphylococcus* and a *Ruminococcacea* OTU are significant for T2 of group 3. More significantly different OTUs were evident for T3 of group 2 and T3 of group 3. For both a *Corynebacterium* and a *Propionibacterium* OTU were Indicators. (Fig. 39).

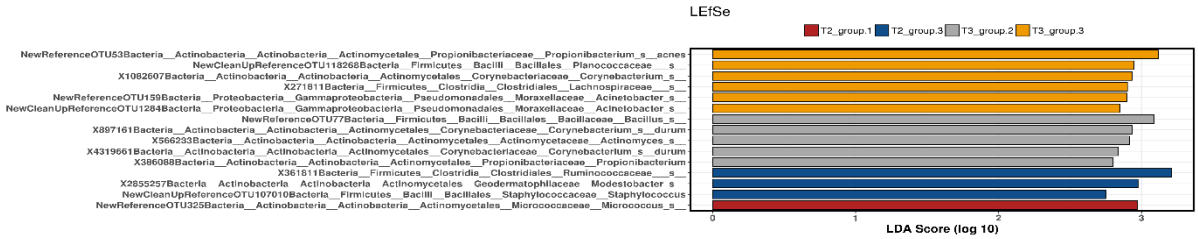


Figure 39: LEfSe analysis, including the top 1000 most abundant taxa, showing specific OTUs for groups and timepoints.

3.2.1.4 Rarefaction

The rarefaction curve in Fig. 40 shows that almost all samples have still a high potential of undetected OTUs, as a plateau was not yet reached. More OTUs to be detected by deeper sequencing.

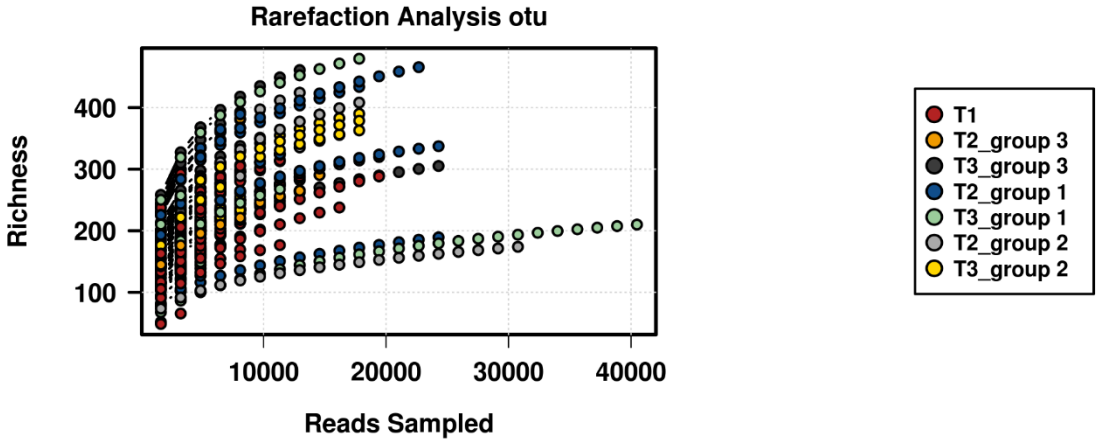


Figure 40: Rarefaction analysis based on OTU level, showing the sequencing potential for the different groups at the specific timepoints.

3.2.2 Follow-up

After DNA extraction, the DNA of all samples was again under the detection limit of 0.01 µg/ml, while there were visible bands in the gel electrophoresis in all samples after PCR.

During sequencing no sample and no negative control was lost because of too less reads.

Reads detected in the negative controls (extraction controls, product controls etc.) were removed from the dataset, up to the predefined percentage, applying the same limits as set in the main study. For the Calypso analysis all data from the main study and the Follow-up were included.

The results from Calypso are shown below split up in α and β diversity, abundance, LEfSe and the rarefaction analysis.

3.2.2.1 α and β Diversity Follow-up

There were again no significant changes in α-diversity comparing the different groups. However, comparing only the values at the different points in time, there was still a significant increase in the α-diversity over time, as shown in the Chao1, the Shannon and the Richness indices. The α diversity of timepoint 4 (T4) anyway increased only minimal in the Chao 1 and the Shannon indices, but decreased in Richness (Fig. 41, 42 und 43).

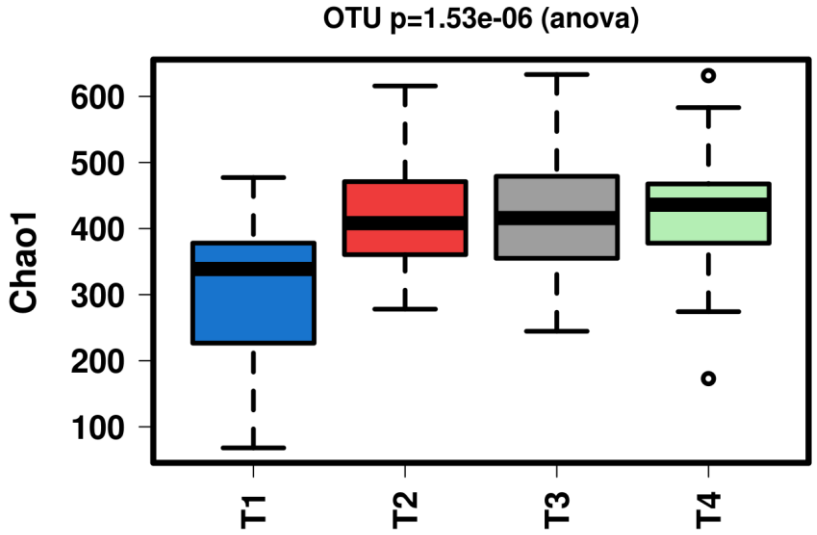


Figure 41: Chao1 indices of the different time points including T4 as the Follow-up sampling timepoint after 6 weeks without product use. This figure shows a significant increase of diversity over all timepoints.

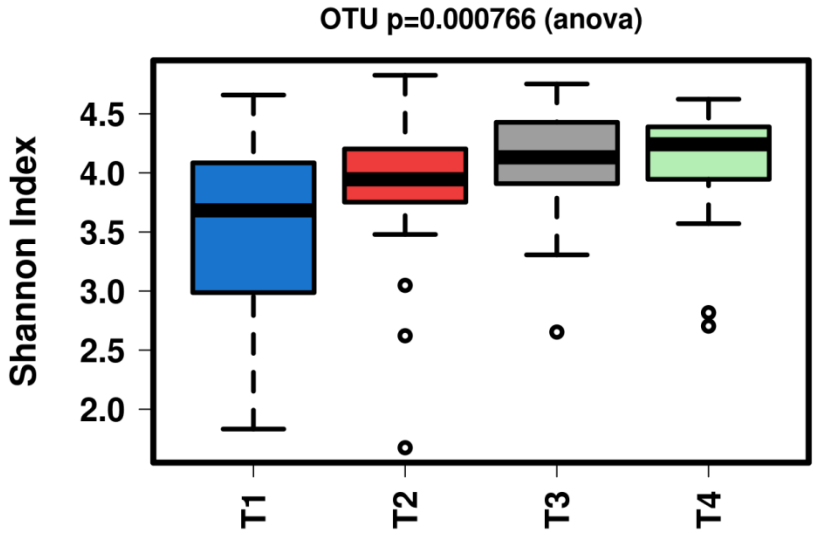


Figure 42: Shannon indices of the different time points including T4 as the Follow-up sampling timepoint after 6 weeks without product use. This figure shows a significant increase of diversity over all timepoints.

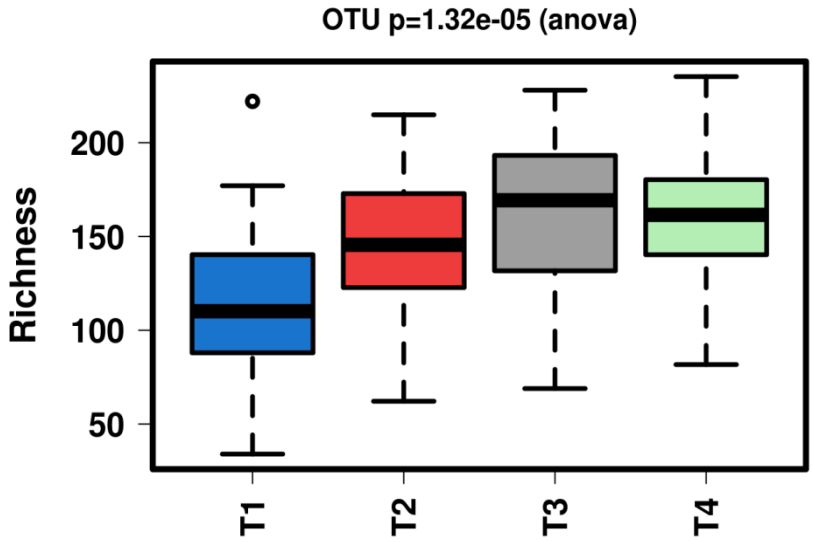


Figure 43: Richness indices of the different time points including T4 as the Follow-up sampling timepoint after 6 weeks without product use. This figure shows a significant increase of diversity over the timepoints T1 to T3, followed by decrease of diversity in T4.

Examining the values of all timepoints and from all groups together, there was a significance between the samples from participants, who used a shower gel only once a day (in the time from T3 to T4) and the ones, who used it twice a day. The Richness indices were decreasing when a shower gel was applied two times a day, as shown below. (Fig. 44)

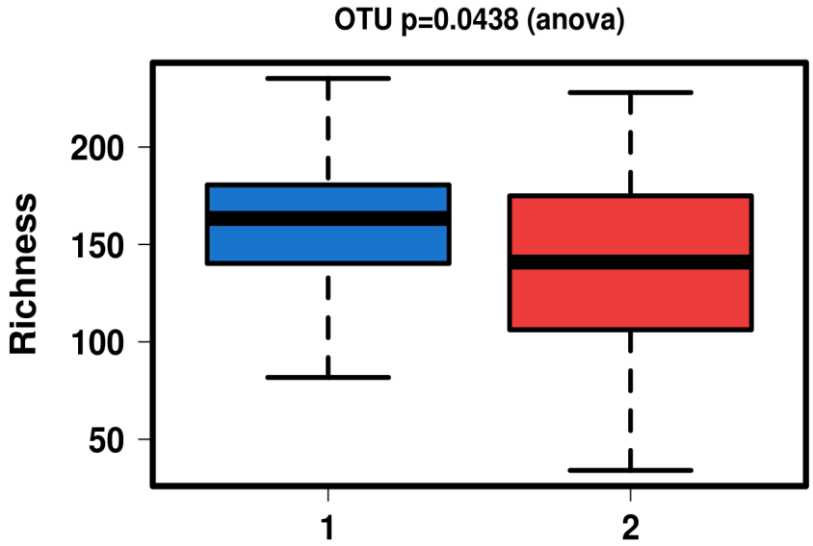


Figure 44: Difference in Richness indices, for participants that used a shower gel only once a day (1) and participants who used it twice a day (2). This figure shows that the use of a shower gel twice a day decreased the Richness index.

The β-diversity of the different time points again did not show any significant grouping in the PCoA plot.

Redundancy analysis (RDA -β-diversity) showed that the parameter "time", "pH", "product change" and "use of shower gel per day" had a significant effect on grouping, what led to the assumption, that these parameters could have had an effect on the skin's microbial community. However, the percentages of axes in RDA analysis were very low (x-and x%) and therefore explaining only a minor share of the total variance of the data set. For the parameter "time" it could be seen that all values of T2 and T3 (during use of the specified products) were grouping in a different way, than values of measurements at T1 (before product use) and again different to the grouping of the values of T4 (6 weeks without product use) (Fig. 45).

In the parameter "product change" group 1 and 2 were grouping together, while group 3 and "different products" grouped differently (Fig. 46).

The RDA for the "pH" and the "use of shower gel per day" are shown in the Appendix, since they both showed only a slightly different grouping of the two identified groups. In terms of pH it were one group high (h) pH and one group low (l) pH, while regarding "use of shower gel per day" the groups were using it once a day (1) and using it twice a day (2) (Appendix Fig. 82 and 83)

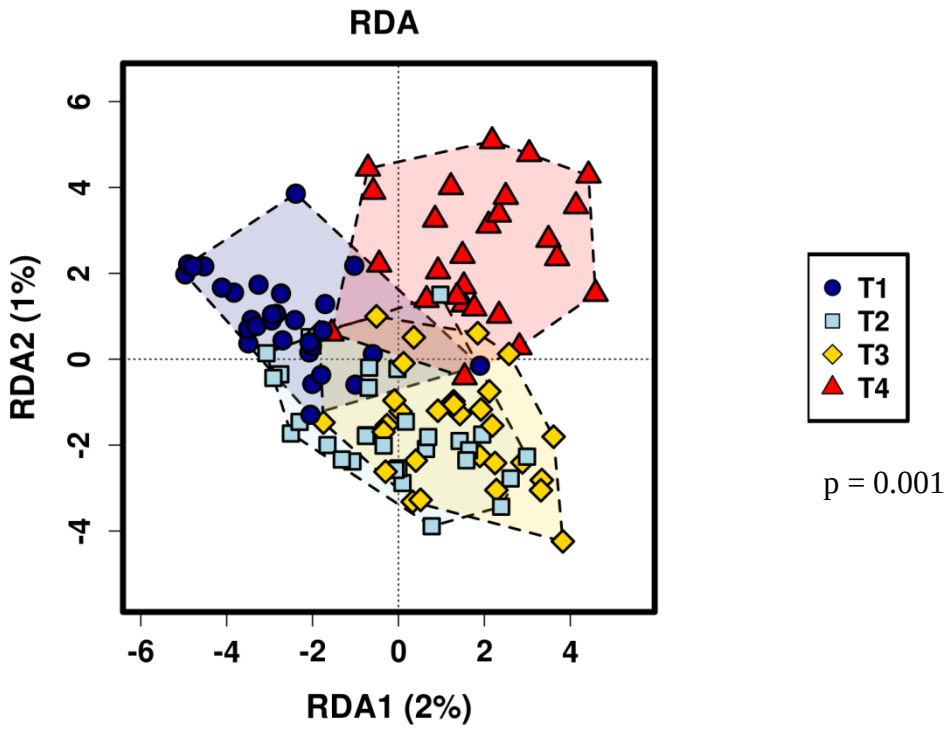


Figure 45: RDA Plot of the different timepoints per group including T4 for the Follow-up sampling after 6 weeks without product use.

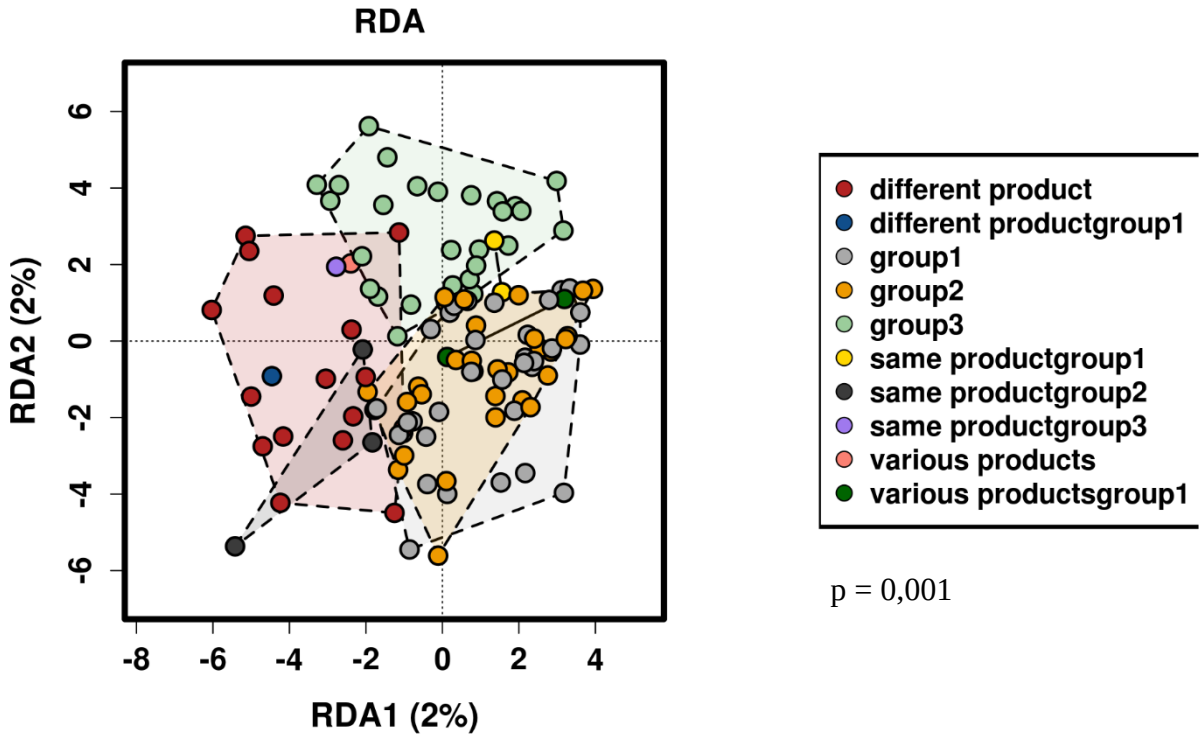


Figure 46: RDA Plot of the different products used during the study and the changes in shower gel during the Follow-up study.

The α and β diversity were also compared for T1/T4, T3/T4 and for the new parameters like "lotion use" and "use of shower gel per day" for each group individually.

When just comparing the values of T1 and T4, there were a lot of significant parameters found, for example in the Chao1 and Richness indices for α diversity and in the RDA for β diversity related to the parameters "time", "use of shower gel per day" and "lotion use", whereas the only significant difference between the timepoints T3 and T4 was found in the RDA of the Tewameter measurements.

Comparing T1 and T4 in terms of the Chao 1 index, we detected an increase of α diversity the more often a lotion was used and the less often a shower gel was used (Fig. 47 and 48). The same result could be found in the Richness indices (Appendix Fig. 84 and 85).

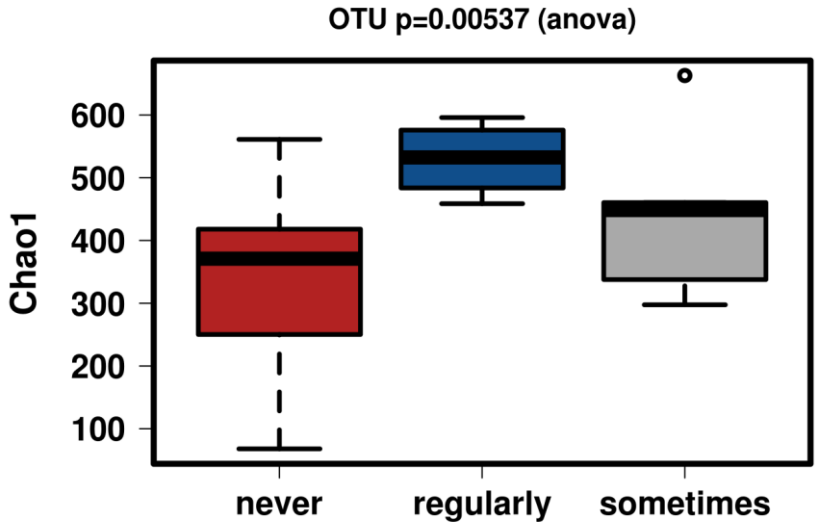


Figure 47: Chao 1 showing the diversity increase correlating with the use of lotions including only datapoints from T1 and T4. This figure shows that the more often a lotion was applied the higher the α diversity became.

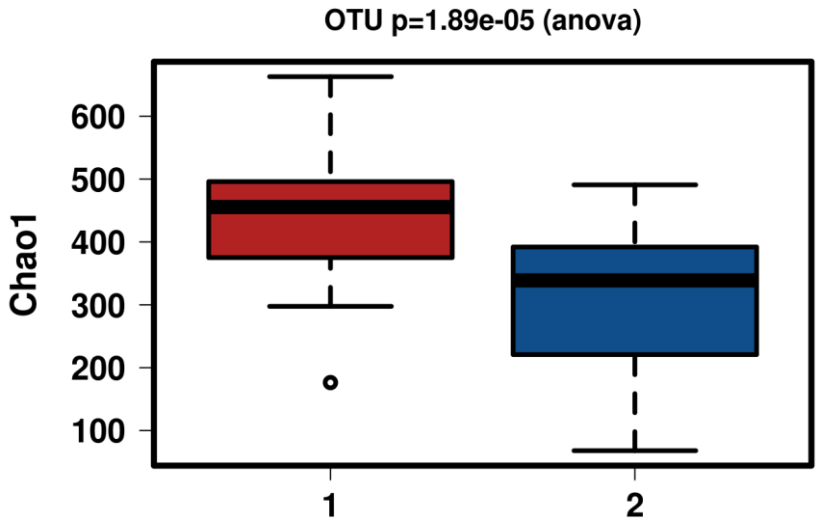


Figure 48: Chao 1 showing the diversity decrease through the use of shower gel including only datapoints from T1 and T4. This figure shows that the more often a shower gel was applied, the lower the α diversity.

When the samples were correlated to the three groups, most of the effects were found to be not significant any longer. Only group 2 and 3 still showed a significant decrease in diversity, related to the frequency of applying shower gel.

3.2.2.2 Abundance Follow-up

No changes in the abundance of the microbial signature on phylum and genus level were evident during the Follow-up compared to the main study, when comparing the abundance plots. Minor differences are obvious, when compared to the abundance plots of the main study. In the

Follow-up *Cyanobacteria* was also one of the five most abundant phyla in addition to *Proteobacteria*, *Firmicutes*, *Actinobacteria* and *Bacteriodetes*, whereas this is not valid for the main study (Fig. 49).

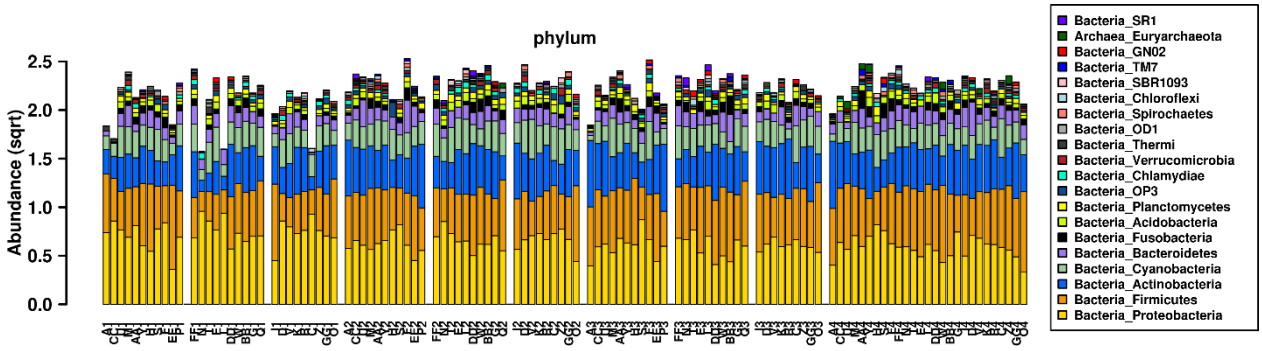


Figure 49: Relative abundance based on phylum level on all samples including the Follow-up, grouped per timepoints and groups.

On genus level *Ralstonia* was quite abundant in the Follow-up, while in the main study all *Ralstonia* were removed (Fig. 50).

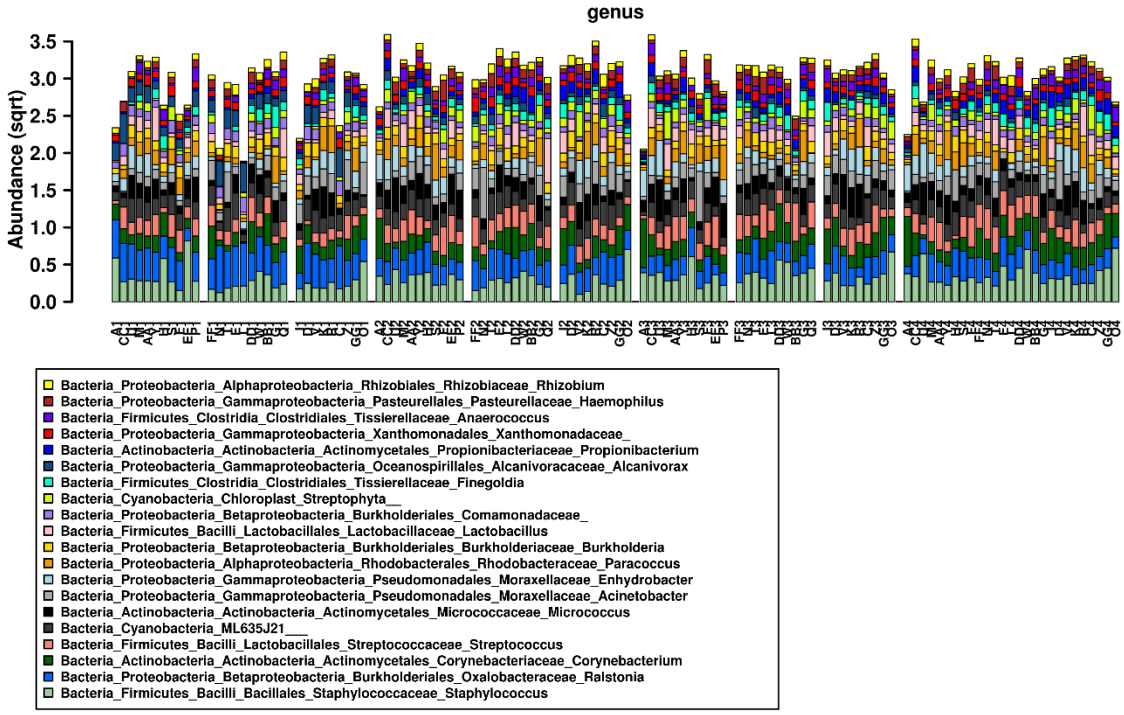


Figure 50: Relative abundance based on the genus level including the Follow-up (T4), grouped per time points and groups.

There were also some changes in the OTUs, which are shown in the Appendix. (Appendix Fig. 86)

At the OTU level some changes over time could be detected, which are shown in the Appendix in Table 14.

The species which were found more abundant in Table 14 were subject to further examination. The chosen OTUs are marked in Table 14. The signatures of *Propionibacterium acnes* OTU, *H. parainfluenza* OTU and *Staphylococcus* OTU increased over time. The signature of *Staphylococcus* was highly positive at time point T3 and T4, but most abundant in T4. The signatures of the *Propionibacterium acnes* OTU was detected at all timepoints, increased over time and was most abundant in T4. The *Streptococcus* OTU was most abundant in T3 and mostly did not show up at any other timepoint. Again one *H. parainfluenza* OTU was significantly increased, but this time only for the timepoint T4 (Fig. 51).

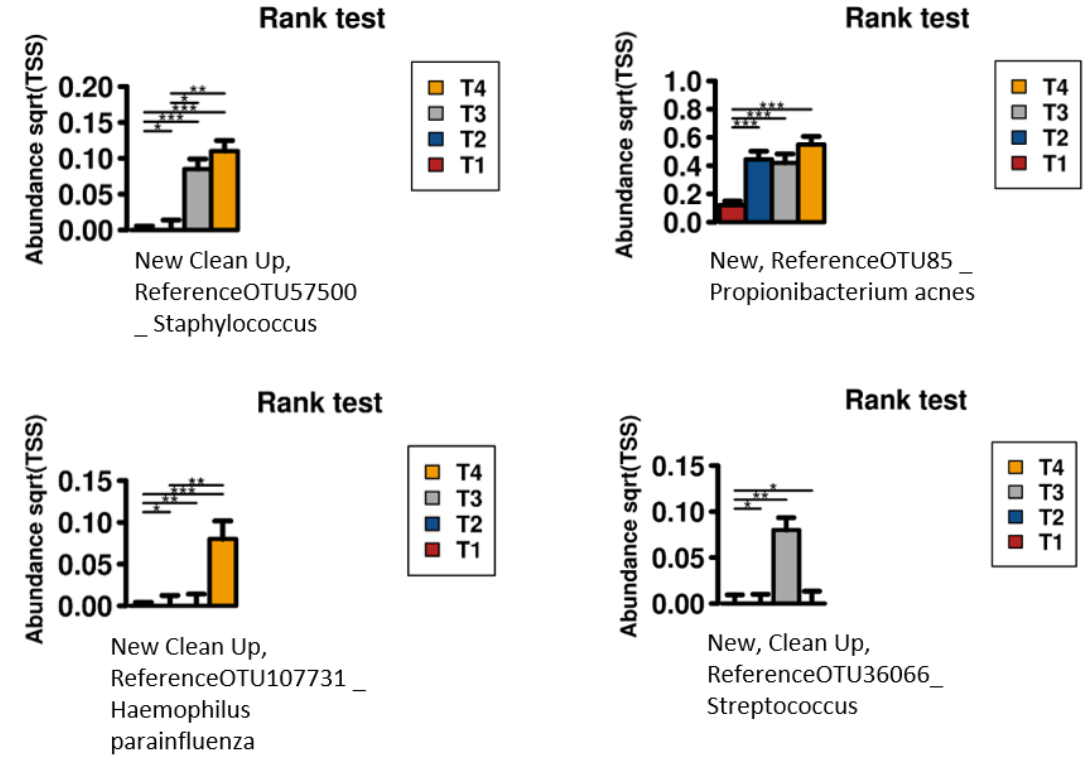


Figure 51: Changes of Abundance over the different time points including the Follow-up for the chosen OTUs.

3.2.2.3 LEfSe Follow-up

The LEfSe analysis revealed again time point specific OTUs. For T1 signatures of a *Sphingobium* OTU and a *Methylobacteriacea* OTU showed significance, while two different *Staphylococcus* OTUs, a *Sphingomonas* OTU and a *Propionibacterium acnes* OTU were significant for T4. For timepoint T3 a *Streptococcus* OTU, a Gemellales OTU, a *Prevotella nancelensis*, two Bacillales OTUs and two *Staphylococcus* OTU showed significance (Fig. 52)

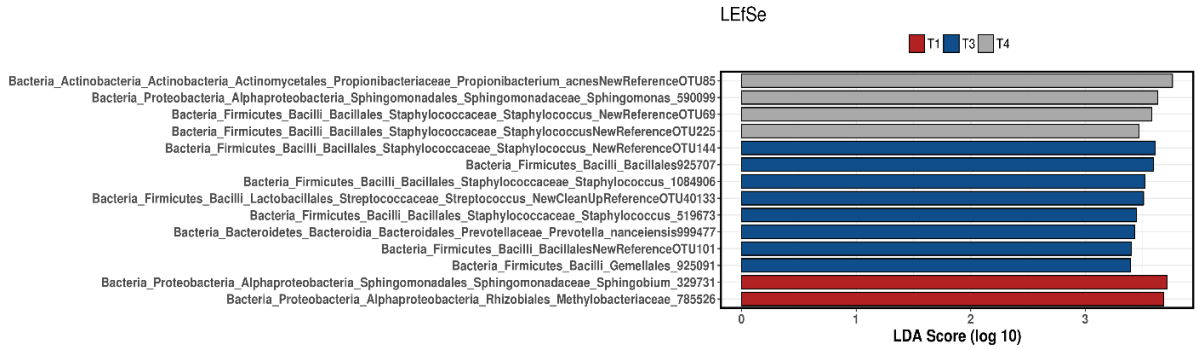


Figure 52: LEfSe analysis, including the top 100 most abundant taxa, showing the associated OTUs, for the different points in time, including the Follow-up (T4).

3.2.2.4 Rarefaction Follow-up

The rarefaction curve in Fig. 53 indicates, that almost all samples still have a high potential of undetected OTUs, which could be detected with deeper sequencing, what was also valid for the Follow-up.

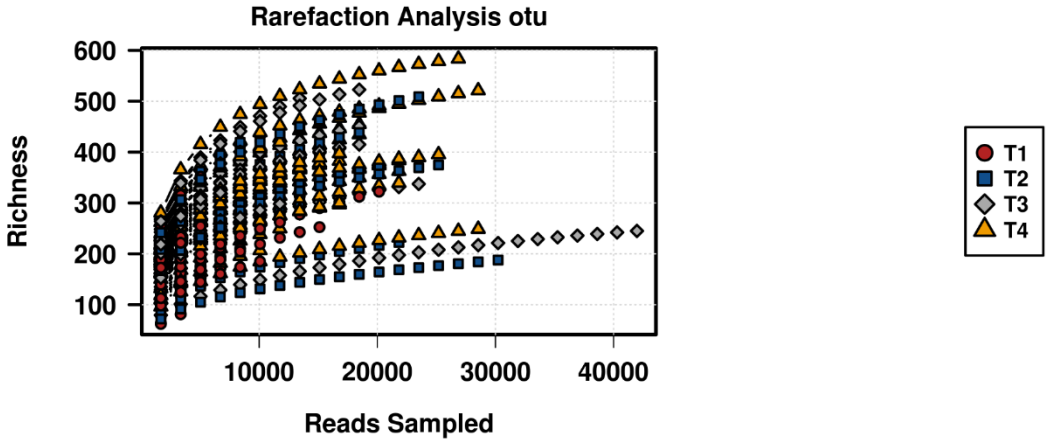


Figure 53: Rarefaction analysis based on OTU level, showing the sequencing potential for the different time points including the Follow-up (T4).

3.2.2.5 Comparison with Silva Database

The data of the Follow-up were also analysed using the Silva database as reference for classification. The phyla were the same as in the abundance plots of the Follow-up with the GreenGenes databank. On genus level there were as well no differences visible. Some changes however could be observed at the OTU level (Appendix Fig. 87).

For a better comparison of the sequencing accuracy, the observed species and the Chao 1 of the data, achieved with the GreenGenes and with the Silva database, are pictured. In all cases except of two the Silva database showed the higher number of detected species. In most samples that was also found valid for the Chao 1 values (Appendix Table 15).

3.2.2.6 PICRUSt

The PICRUSt analysis was used to learn something about the microbial functions most abundant on the skin and was only performed for the data after including the Follow-up data. The most abundant functions were transporters and ABC-transporters, as well as DNA repair and recombination proteins, as well as purine metabolism (Fig. 54).

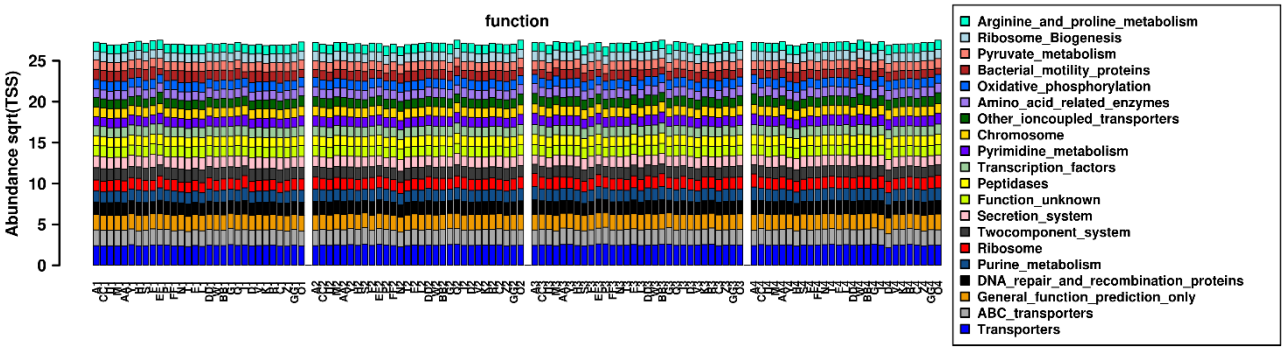


Figure 54: Abundance of PICRUSt predicted functions grouped by the 4 different time points.

Instead of removing the negative controls from the PICRUSt file a PCOA was done to see whether the negative controls group separately, which was the case in this study (Fig. 55).

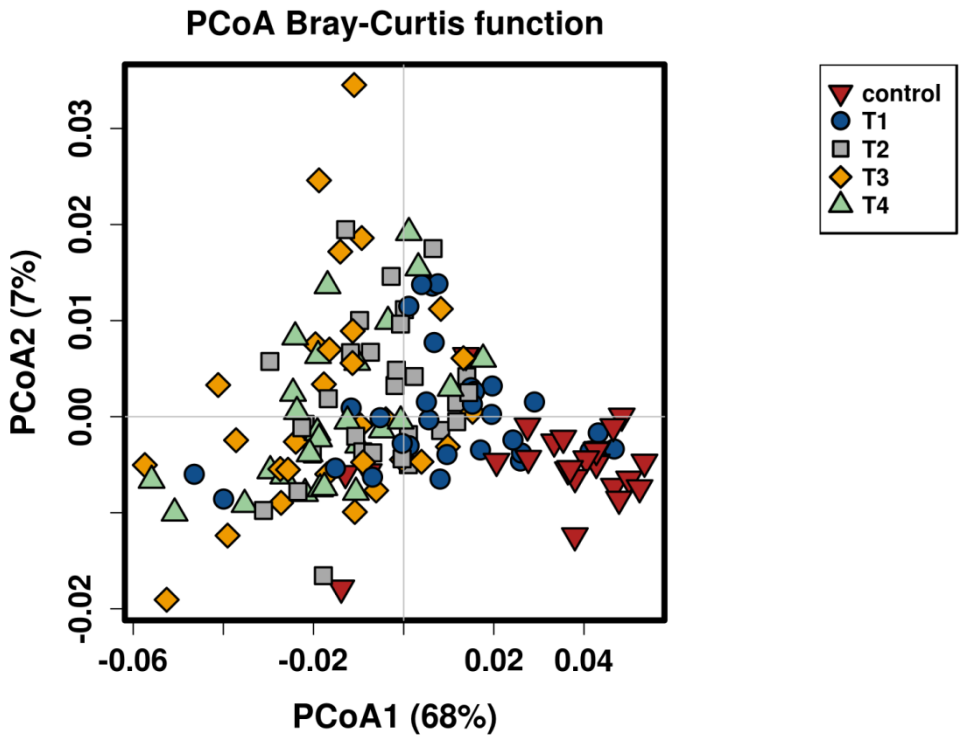


Figure 55: PCoA Plot showing the samples according to time points T1 to T4 and the negative controls.

The LEfSe analysis also showed, that there were specific functions for the timepoints and the controls. The most abundant functions in the controls were, among others, bacterial motility proteins, twocomponent system, bacterial chemotaxis, secretion system and many different metabolism functions. There were no significantly different functions for T1 and only the nicotinate and nicotinamide metabolism for T2. There were many significantly different functions for T3 and T4. For example, starch and sucrose metabolism, fructose and mannose metabolism, photosynthesis proteins and glycolysis/glyconeogenesis were significant for T3, while ribosome, galactose metabolism, amino sugar and nucleotide sugar metabolism, pyrimidin and purin metabolism and DNA repair and recombination proteins were significant for T4 (Fig. 56).

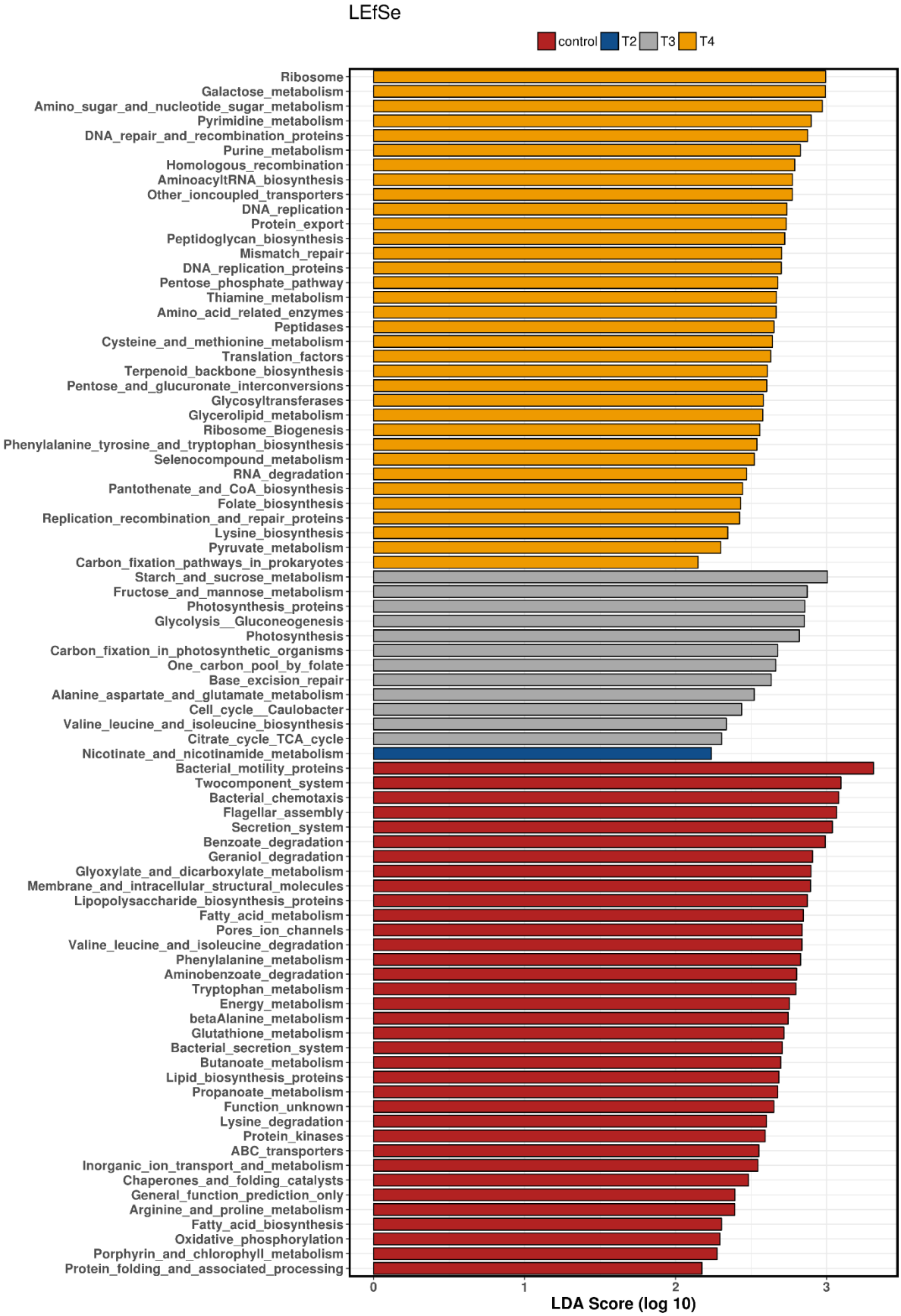


Figure 56: LEfSe analysis, including the top 100 most abundant functions, showing, the different time points and the associated functions.

4 Discussion

The skin is one of our most important organs. It is a kind of shield against environmental stimulus and therefore helps the human body with maintaining its status of homeostasis. Our skin is often exposed to rough environmental conditions, as well as to cosmetic products, which might affect the skin structure and function. In the study of Cheng et al., 2008 the effect of moistening cosmetic products on the skin under simulated harsh environmental conditions were observed.

This study showed, that the use of skin moistening products decreases the transepidermal water loss (TWL) and increases the moisture of the skin (Cheng et al., 2008), what is contradictory to the results obtained in our study. The results of the skin measurements performed in this study showed a decrease in the transepidermal water loss and increasing dryness of the skin after the use of washing products. These results were not product specific and therefore could be subject to different effects during the duration of the study. Since the study was carried out in the winter months, this effect could for example be explained by the changing weather conditions, since this would affect all participants. The use of the products twice a day, or the missing use of additional skin moistening products during the study could also have influence on the results. Based on the data from Cheng et al. 2008 it was expected, that the lack of moistening products, as in our study, should lead to increasing transepidermal water loss and thus to increasing dryness of the skin. In our study only increasing dryness of the skin was found, whereas the TWL was decreasing, contrary to expectations. Accordingly we hypothesize that in our case the changes of the skin were more affected by some unknown external influence, than by the lack of moistening products, or by increasing use of shower gels. The Follow-up, without any specification on product use and no restrictions in using moisteners, showed a further decrease in the transepidermal water loss but just a small increase in moisture of the skin over all participants. This fact also strongly indicates, that at least the transepidermal water loss could be influenced by other factors than by the used products or by the chosen study design. The dryness of the skin anyhow increased again after our main study from time point T3 to T4, which indicates, that the decrease during the main study was based on some unknown factor, caused by our study design. Most likely this could be explained by applying skin washing products twice a day, what also influences the microbiome, as found in this study. This cannot be considered as proof and would ask for advanced research, since a lot of other external factors, like wind and Ultraviolet (UV)- exposure, are known to influence the skin physiology, as also found in Cheng et al., 2008. As the skin physiology measurements in Cheng et al.,2008 were

carried out only under laboratory conditions, they are not applicable to our study, since it was not possible to control external conditions during this study. Only the samplings at the given time points (T1, T2, T3 and T4) were performed under uniform procedure, at clearly defined surrounding conditions.

There are a lot of other influencing factors that should be taken into account. Differences in the values of skin measurements can be for instance based on anatomical region, sex and age (Conti, Schiavi, & Seidenari, 1995). We did our best to minimise such factors, by including only women aged from 20 to 45 and by taking samples only from the volar forearm of the non-dominant arm. Still minor changes related to transepidermal water loss and skin moisture were found, when comparing the values of measurements on neighbouring areas on the volar forearm during one and the same sample drawing. Such aberrance was equalised by measuring always at the same areas and in using the mean value of three measurements at each sampling. Nevertheless, these factors could have influenced the results, as we did not go in for extensive inspection on such.

For taking skin measurements it is recommended to have the participants rest for about 10 minutes and keeping the room temperature constant (Conti, Schiavi & Seidenari, 1995). Applying these recommendations to our sampling procedure, we first took the microbiome samples, to help the participants acclimatise. This was also necessary to make sure that the microbiome samples were not affected by the skin measurements, as the same device was used for all participants and therefore could have led to a microbiome transfer. As the microbiome samples were taken with a pre-moistened swab, this also might have effect on the skin measurements. However, as only changes between the individual samplings were examined, this influence is negligible, because the sampling procedure was always the same.

Results of the skin measurement could be interpreted in different ways, when referring to the classification according to the pie charts or in referencing to the raw data of the statistical analysis. For example, in the Follow-up, the only participant from time point T3 with "dry skin" changed, due to decreasing skin moisture, to "very dry" classification, like all other participants, what would suggest that skin moisture continued to decrease, whereas the raw data by trend saw moisture increasing, even though still all participants being part of class "very dry" skin. Explanation can be found in the fact, that some values were quite close to the boundary values of the pre-defined classes, what means, that even very little changes in real value lead to a change in terms of classification. That’s why our final statements are only based on the raw data of the statistical analysis, using the pie charts are only for additional illustration.

An effect that could only be detected in examining the progression of the average values was a grouping of group 1 and 2, which seemed to be more similar to each other than to group 3. This was evident for the measurements of the transepidermal water loss as well as for skin moisture (Fig. 14 and 19) and was even found in the RDA of the microbiome analysis (Fig. 34). In the Follow-up this similarity became less apparent, especially related to the transepidermal water loss (Fig. 26). In microbiome analysis the class “different products” (which means that some other shower gel was used from time point T3 to T4, than during the main study, what applies to most of the participants during the Follow-up) grouped differently in the RDA (Fig. 46).

The similarity of group 1 and 2 in the main study could find its explanation in the difference of the products. Product 1 and product 2 were strictly made of natural ingredients only, while product 3 contained more artificial, chemical substances. Since this effect was not statistically significant, this can only be seen as a trend, which might suggest that artificial products have a different effect on the skin. If this effect is positive or negative cannot be evaluated based on the available data.

Skin physiology is affected by numerous influences, what has been proven in many previous studies. It was found, that cosmetics may have protective effect on skin physiology, regarding environmental conditions, like wind and UV-exposure. The skin areas treated with cosmetics products showed an increase in the skin moisture levels and at the same time decreasing values of transepidermal water loss. This effect was also found during wind and UV-exposure, where the products helped to maintain a normal skin water content and kept the skin barrier working (Cheng et al., 2008).

The skin microbiome is also influenced by many different factors. Individuality for example may have huge impact on microbial diversity. Comparing same body sites on the right and the left side of one individual showed similar results, while compared to other individuals bigger difference in the skin microbiome were detected.

Moisture level as well seems to affect the microbiome, as it was found, that on dry skin areas, like the palm, the diversity is higher, than on moist areas (Ross, Doxey & Neufeld, 2017). Another study, which analysed the effect of environmental factors on the skin bacteria, tested the influence of temperature and humidity. In this study it was shown that high-temperature together with high-humidity is going along with higher quantity of bacteria (McBride, Duncan & Knox, 1977). In environmental studies it was also shown that acidic environments are associated with lower diversity (Fierer & Jackson, 2006). This could mean, that decreasing pH could also affect diversity of the microbiome of the skin in a negative way. Based on the results, described in the paper mentioned above, increasing diversity was related to a decrease in

moisture, as the driest areas seem to have the most diverse skin microbiome. Also the temperature and humidity could have had an effect in this study, since the samples were taken on the volar forearm during wintertime, when all participants wore clothing that kept the sampling area more or less warm and moist. Since environmental studies showed that low pH was associated with low diversity, it could be suggested that this would also be found valid for the skin microbiome, when products with high pH are applied.

Even if the pH did not show any significant difference in our study, it is apparent, that the skin pH was found to be highest for participants using product 1 (Fig. 24), even though this product was the one with the lowest pH (pH=4.5, while the other two showed pH of 6). It seems, that the application of products with pH even higher than the recommended skin pH, increased the pH of the skin less than products with lower pH value. This could be a protection effect of the skin against acidic conditions, since the pH of the skin should be between 4.5 and 5.5. This probably means that more acidic conditions could affect the skin barrier and the microbial community more drastically, than basic conditions would. In this study there could no effect between acidic conditions and low diversity be found. This could be because of the naturally slightly acid condition of the participants' skin and because of the lack of participants with skin of high acid level.

There are many influence factors that affect the microbiome, but it seemed that they did not affect the main microbiome abundance normally found on the skin. The most abundant phyla in our study were Proteobacteria, Firmicutes, Actinobacter and Bacteroides, which are phyla regularly found on human skin as reported before (Bouslimani et al., 2015; Gao et al., 2007; Staudinger et al., 2011). The most abundant genera in this study were *Staphylococcus*, corynebacteria, *Streptococcus* and *Acinetobacter*. They are also typically found on the human skin, even if other studies found that propionibacteria are normally also one of the most abundant genera (Gao et al., 2007), which was not the case in this study, where propionibacteria was indeed detectable, but only with a very low abundance. In the Follow-up sampling the main phyla stayed the same, even if Cyanobacteria was detected together with the phyla stated up above, as one of the most abundant ones. Also on the genus level there was an additional genus detected in high abundance, namely *Ralstonia*. Since these differences appeared over all groups in the Follow-up, they have to be caused by a slightly different filtering, because of the varying number of samples and controls included in the data processing. *Ralstonia* was also abundant in many negative controls, but could not be excluded, using the same cut-off as in the main study, as in the Follow-up the counts were not high enough compared to the participants' samples. Especially *Ralstonia* is known as a common contamination in buffers and is therefore

most likely caused by an impurity in the sample processing (Kim et al., 2017). In the study from Lee et al., 2017, where they compared the skin microbiome from facial skin with high and low hydration, *Ralstonia* was also found as one of the most abundant genera. They suggested that *Ralstonia* may have the potential role in metabolising cosmetic components since *Ralstonia* increased after cosmetic use, as did the KEGG categories of lipid metabolism and xenobiotics biodegradation and metabolism (Lee et al., 2017). Since we did not test any cosmetics that contained lipids, this study cannot help to verify this statement. Anyhow, since *Ralstonia* is often found as contaminant, it might be difficult to ensure that the results are not based on impurities. Even if there were not found any significant differences in the relative abundance, the increasing diversity is an interesting fact and should be subject to further research. The diversity increased about to the same extent in all groups and therefore it can be assumed, that the different products did not affect the diversity in different ways. As already discussed before, there are many influence factors that could have led to the increase in diversity as shown during our study. Even if it has already been indicated, that cosmetics can lead to an increase in diversity, these studies are mainly based on different moistening lotions and not on shower gels. For example in the study from Lee et al., 2017 it was shown that a set of basic cosmetics, like skin softener, lotion, essence and cream, containing moisturizing compounds, increased the diversity of the facial skin microbiome in high and low hydrated skin types. The study also showed that low hydrated skin has a higher diversity, which could verify the assumption, that the diversity in this study could be based on the decrease of moisture (Lee et al., 2017). Since in our study only different face washes were tested, the findings from Numata et al., 2012, who did research on the effect of face washing on the microbial community, are much more interesting. They found that face washing increased the sebum level over time, but did not change the bacterial counts after face washing with liquid soap. They therefore assumed, that the resident microorganisms are not easily removed, while transient microorganisms can be washed off the human skin more easily. As we do not have any information about the sebum content, since we did not test for it, the results of the study from Numata et al., 2012 may also explain why we only found differences on OTU level and not in highly abundant species. Since the authors only tested with culture and a PCR, they did not analyse the diversity, what may have led to different results than found in our study (Numata et al., 2012).

In the PCoA plot (Appendix Fig.80), through which outliers can be identified, it is shown that only samples from the sampling timepoints T1 and T2 group separately and could be viewed as outliers. These samples grouped more with the rest of the samples after our study at time point T4, meaning the skin of the participants became more similar as before.

In the RDA plots, that show the differences in α diversity, it is obvious, that group 1 and 2 are grouping together, while group 3 and the different products in the Follow-up group differently. This was already discussed before, but with the RDA that also shows that T2 and T3 group together while T1 as well as T4 group separately, it can be concluded, that there might be a difference caused by the product use during the study. Contrary to expectations, T1 and T4 did not show similar values, even though the participants turned back to their previous washing habits. This leads to the assumption, that there could have been two influencing factors leading to the detected changes of the microbiome. On the one hand the product use and on the other hand time related parameters, such as the change of weather. Anyhow the RDA plots show that the detected differences are very small.

Interesting changes were also found in specific OTUs that showed higher relative abundance for certain timepoints and group associated timepoints. The most interesting changes were detected for specific OTUs of the species *Propionibacterium acnes*, *Haemophilus parainfluenza* and the genus *Staphylococcus*. Since, during this study, face washes were used for showering, which did not produce as much foam as other shower gels, it might have affected the cleaning rate of the products and thus led to the increase of transient microorganism. In a study, which compared the microbiota of the forearm and the forehead skin, also potential pathogens like *Haemophilus influenza, Staphylococcus aureus, Neisseria meningitis* and *Streptococcus pneumonia* were found on both sampling areas (Staudinger et al., 2011), what is corresponding to the findings of our study. These organisms are considered as part of the transient human skin microflora, that is capable of producing skin-damaging toxins, that can lead to serious skin diseases (Wilson, 2004). These species are occurring in natural environment and therefore are adapted to rough environmental conditions, like on exposed human skin. Originating from the environment, such opportunistic pathogens may also become resident on the human skin (Dekio et al., 2005).

Propionibacterium acnes (*P. acnes*), which is an important component of the human skin microbiome, as also shown in our study, is said to be related to skin diseases too, like acne. Anyhow it is also likely, that it might play a protective role on the skin, keeping more aggressive pathogens at low level (Leyden, 2001).

Another study suggests that the positive or negative effect of *P. acnes* is depending on the occurring OTUs (Fitz-Gibbon et al., 2013). The sebum and the moisture content were also found influencing the distribution of the specific species. *P. acnes* for example is associated with sebum content, while *Staphylococcus* is more affected by the moisture content of the skin (Costello et al., 2009). Since we have not measured the sebum content and did not have big

differences in the moisture content, we can only assume, that these factors may have influenced the results, finding that especially some specific *Staphylococcus* OTUs were significantly abundant at different points in time.

In addition to the bacterial inhabitants on the skin, we also found Archaea in the study. As described in Probst et al., 2013. The main kind of Archaea found belonged to the class of Thaumarchaeota, but Methanomicrobia, Methanobacteria, Thermoprotei and Halobacteria were detected too. Since the Archaea were co-amplified with the bacterial 16S rRNA primers, which do not perfectly match the archaeal 16S rRNA gene, the Archaea on the skin might be underrepresented in this study (Probst et al., 2013).

Except of the bacterial and archaeal microbiome there are many other compounds that can be found on the human skin, which can be detected with liquid chromatography – mass-spectrometry /mass-spectrometry (LC-MS/MS). With this method the molecules on the human skin can be assigned either to the microbial or to the environmental component. For this reason the LC-MS/MS spectra of the human skin and of the beauty products, used by the researched individuals, were collected and compared in a recently published study (Bouslimani et al., 2015). Thus, it was found, that the majority of the LC-MS/MS spectra were associated with the skin swab samples. About 8% matched beauty products and cosmetic ingredients, 0.5% were associated with cultured keratinocytes and human skin tissues and only around 1% were related to microbial cultures. These results show that beauty products have a lasting impact on the molecular composition of the outermost layer of the skin. Anyhow most of the metabolites on the skin remained uncharacterized. The sources of these molecules may have a wide range, like secreted dietary molecules, modified by environmental factors like light, air and enzymes, and more beauty products that were not included in the analysis. These analysis show, that the skin is a highly complex organ on the molecular level. This is partly due to the influence of the microbiome and second a matter of exposure to environmental conditions (Bouslimani et al., 2015). This paper reveals the need of further research about the human skin and the influencing factors on the human microbiome, as there is still a lack of knowledge in these fields, what could also have influenced the results of our study.

Another factor that influences the microbiome data is the sequencing. To analyse how well the sequencing worked the rarefaction curves are used. In our study this analysis showed that the sequencing was quite deep, even if there would still be potential for deeper sequencing. We used the MiSeq Illumina method, which is especially suitable for small projects, like this one, when having the need of fast processing. The difference to the HiSeq sequencing is especially the scale, which results in different fields of use, where deeper sequencing is needed and more

time is available. (Caporaso et al., 2012). This effect as well as the chosen primer pair and the database for the data analysis might have affected the results in this study, especially in terms of diversity. We used the primers for the V4 region, which are the normally used primers. However, it is shown that the V1-V3 region have more similarity to the whole metagenomic shotgun (WMS), than the V4 region. This could have led to an underrepresentation of skin bacteria, like *Propionibacterium*, in the data, analysed with the V4 region (Meisel et al., 2016). The data classification with the Silva database generates more reads per sample, as the same analysis with the GreenGenes database, and therefore might have been the better choice. Even if the use of the Silva database could have resulted in more detailed observation of the individual samples, when comparing the microbiome changes, there were no large differences to be detected. This finding together with the fact that PICRUSt, which is used to predict the microbial functions, can only be performed, when GreenGenes is used, led to the decision to use GreenGenes for this study.

The metabolic functions, predicted with PICRUSt, that were most abundant in our skin samples, were mostly transporters, DNA repair and recombination proteins and purine metabolism. A big part of the data was grouped, just being predicted as general function. Important to mention is that the negative controls grouped differently in the PCoA Plots, and therefore could be neglected in the further analysis. In the study from Kong & Segre, 2015 the most abundant functions were enzyme families membrane transport, as well as replication & repair, what is concordant with our study. The differences between this study and ours can be explained by the different sequencing, data processing and the use of a function predicting method in comparison to the metagenome analysis that was used in the study from Kong & Segre, 2015.

Another study, that used the SEED annotation for comparing the metagenome functions of the skin of two different individuals, detected functions related to carbohydrates, amino acids and derivates, cofactors, vitamins, prosthetic groups, pigments and protein metabolism as most abundant. Anyhow, this study showed DNA metabolism and membrane transport as abundant functions of the skin microbiome as well (Mathieu et al., 2013). Even though there were many differences between the used methods for the metagenome analysis, at least some similarities could be found.

Predictions of functions, by using PICRUSt, were found to have high consistency in the abundance for metagenome samples all over the whole body. It could also been shown, that the accuracy of PICRUSt is influenced by the metagenome and 16s rRNA gene sequencing depth. This means, that samples with a low sequencing depth may be poor proxies for the community's

true metagenome and therefore have an impact on the PICRUSt predictions (Langille et al., 2013).

4.1 Study Limitations

It has to be taken into account, that during the study the DNA extraction protocol had to be changed in terms of the applied beating process, because of a broken instrument, what could have an impact on the amount of obtained DNA.

For future studies of the skin microbiome, the protocol should stay the same for all samples, maybe implementing some amendments, that could raise efficiency and accuracy. The skin measurements for example, could be more accurate, when keeping a longer break between the skin swab sampling and the measurements, what would also lead to a longer recovery period for the participants, minimising potential influence on the sampling by previous physical activity. Further improvements could be achieved by using another data processing or other metagenomic predicting methods as described in some of the cited papers, in accordance with changes in the starting basis, like higher numbers of participants, required advanced data analysis, or higher project budget made available.

In addition more Archaea sensitive primers could be used, in order to do research on this important part of the human skin too.

As a matter of form, it has to be mentioned, that the Follow-up was not part of the preliminary set-up of the study, but was later on implemented to improve the quality of the conclusions drawn by this study, what leads to the distinction between results of the main study and the Follow-up, as the sequencing for the Follow-up was run separately. It may have improved the results of our study, if even the samples from the Follow-up could have been integrated in the first sequencing run and such provide one single analysis for all common data.

Still this pilot study leads to some interesting findings, which should though be regarded with suspicion, because of the small number of participants and some minor inconsistences in the study design.

To prove the drawn conclusions of this study, it would be necessary to repeat it with a higher number of participants and running it over an extended time, in order to homogenise the basic conditions for all individuals. In this regard a pre-study would be of utmost importance, carried out without the use of any products or determining the use of one specified product for all participants, leading to some well-founded, common baseline, what will improve the set-up of any following study considerably.

During the period of the Follow-up, it also would have been important to make all individuals change back to the product, they were using in the pre-study, or to have them all change to using a completely new product. When conducting a study with larger numbers of participants, even investigations on additional influencing factors, like the use of lotions, or the frequency of using shower gel per day, could be implemented by supplementary grouping.
If the purpose of some future study is a research on a one-to-one correspondence between the changes in the microbiome and the skin parameters and some specific cosmetic product, it will be helpful to evaluate the molecules on the skin and in the selected product, by using LS-MS/MS analysis.

5 Conclusion

Through this study, time dependent skin changes as well in skin parameters as in the microbiome could be detected. A statistical difference was observed in the epidermal water loss, which grew less over time and in the dryness of the skin which became drier. These changes did not seem to have a product specific effect, even if group 1 was the only group, which didn't show a loss of moisture over time, when looked at it separately.
The microbiome showed an increase in α-diversity over time in all groups, which could be also detected in each product group with the Chao 1 index. Richness nevertheless showed no increase of α-diversity in group 1. Interestingly group 1 and 2, where the participants used natural products, showed more similar results to each other than compared to group 3, where a product containing artificial chemicals was used.
There were a lot of changes on the different classification levels concerning low abundant skin bacteria, but the most abundant phyla found on the skin where not affected during our study. The Follow-up study additionally showed that the use of a body lotion and the applying frequency of shower gels also had an impact on the obtained results.

6 Appendix

6.1 Ad. 4.1 Statistical Analysis of the Skin Measurements

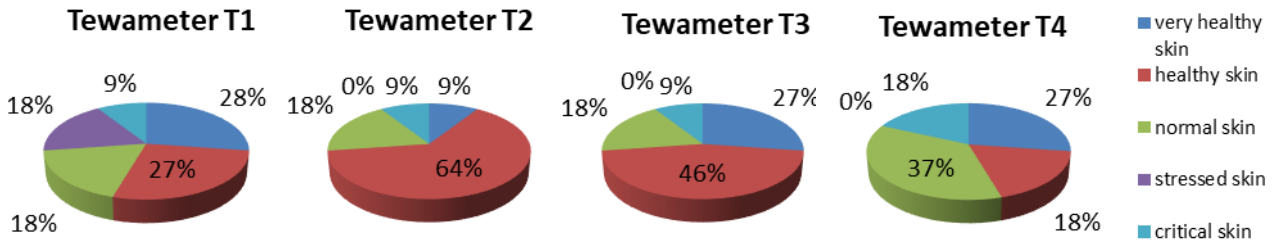


Figure 57: Comparison with respect to the different skin types throughout group 1 participants based on the epidermal water loss measured with a Tewameter before product use (=T1), after 2 weeks of product use (=T2), after 4 weeks of product use (=T3) and after further 6 weeks of individual, personal hygiene (=T4).

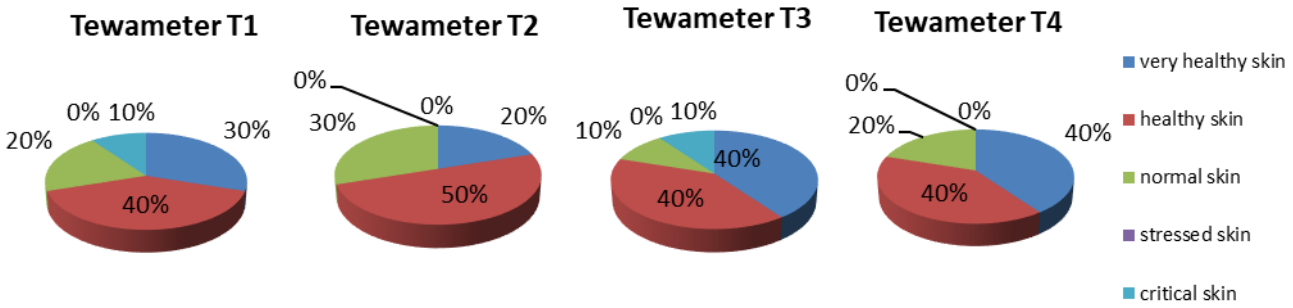


Figure 58: Comparison with respect to the different skin types throughout group 2 participants based on the epidermal water loss measured with a Tewameter before product use (=T1), after 2 weeks of product use (=T2), after 4 weeks of product use (=T3) and after further 6 weeks of individual, personal hygiene (=T4).

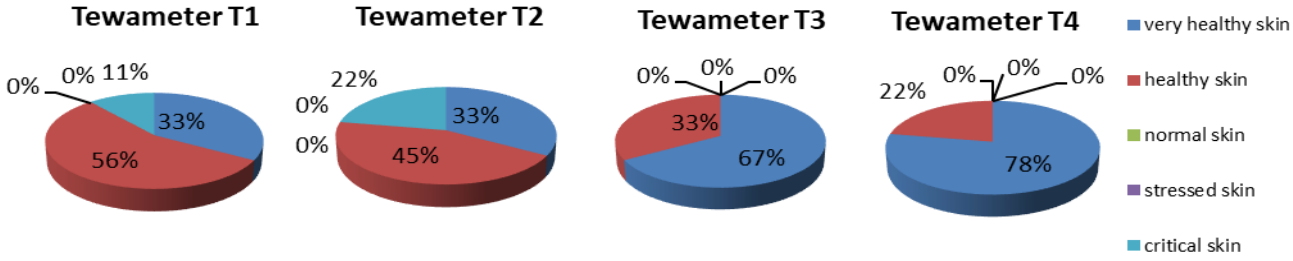


Figure 59: Comparison with respect to the different skin types throughout group 3 participants based on the epidermal water loss measured with a Tewameter before product use (=T1), after 2 weeks of product use (=T2), after 4 weeks of product use (=T3) and after further 6 weeks of individual, personal hygiene (=T4).

1.1 Ad. 4.2 Microbiome Data of the Skin Samples

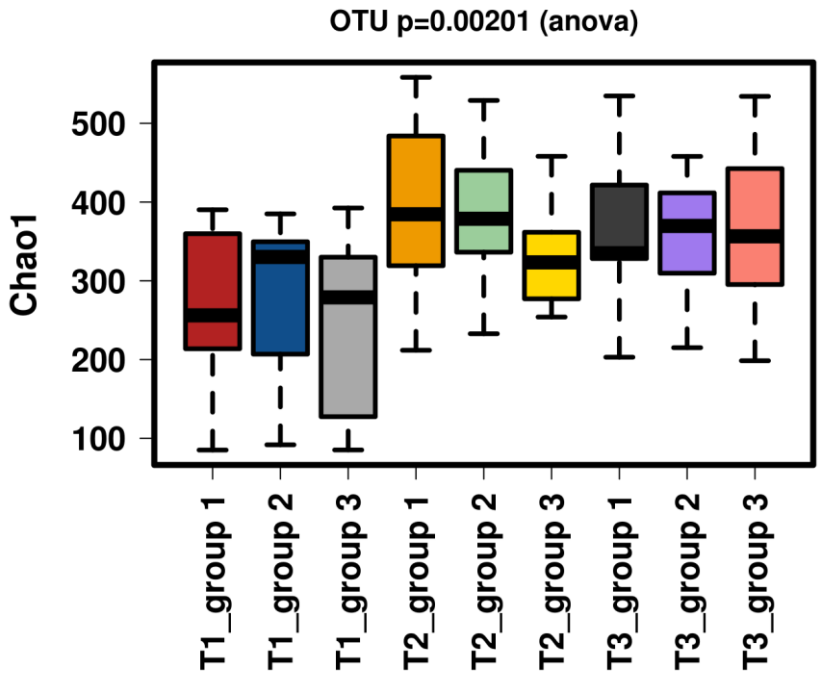


Figure 60: Chao1 index of the different time points per group. This figure shows a significant increase of diversity in all groups over time.

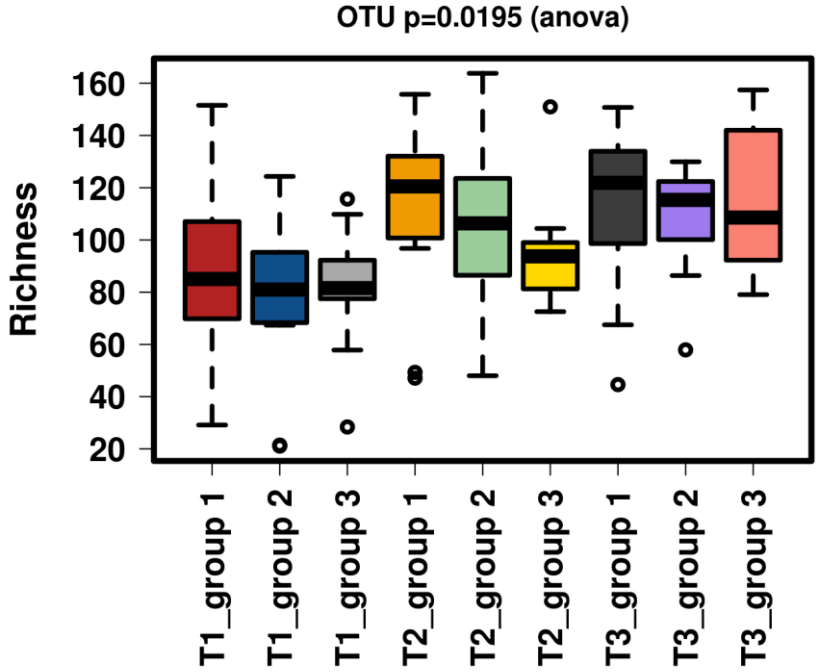


Figure 61: Richness index of the different time points per group. This figure shows a significant increase of richness.

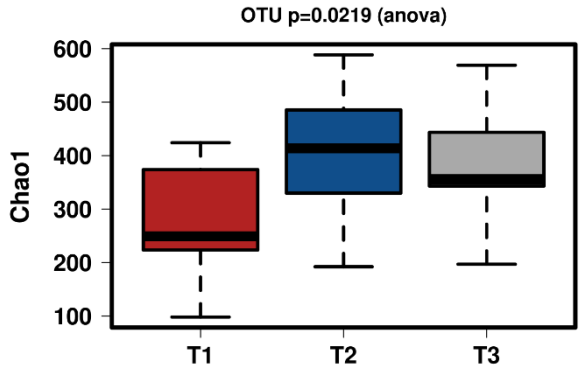


Figure 62: Chao1 index of all time points of the group 1 samples, with a significant increase in diversity.

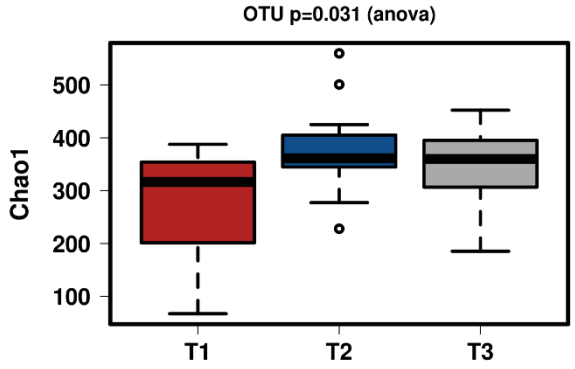


Figure 63: Chao1 index of all time points of the group 2 samples, with a significant increase in diversity.

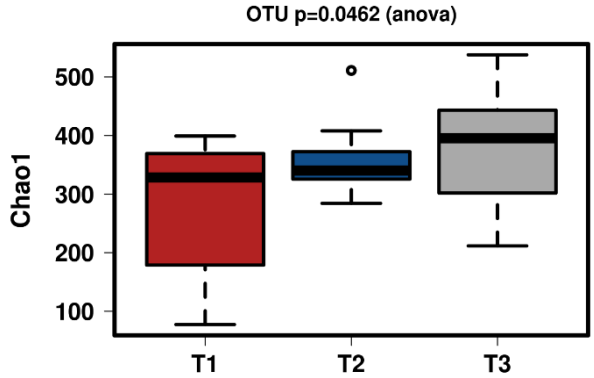


Figure 64: Chao1 index of all time points of the group 3 samples, with a significant increase in diversity.

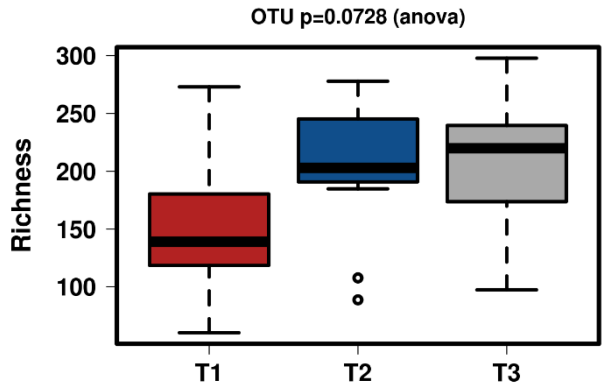


Figure 65: Richness index of all time points of the group 1 samples, with no significant change.

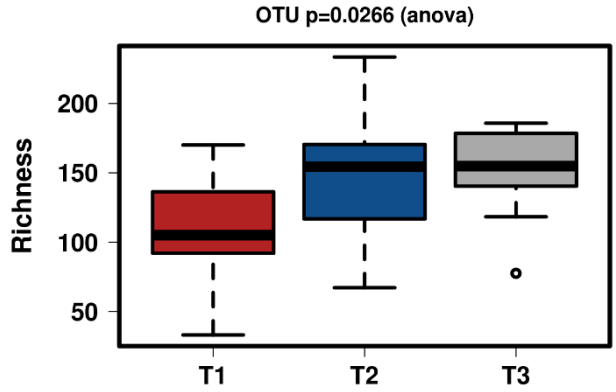


Figure 66: Richness index of all time points of the group 2 samples, with a significant increase over time.

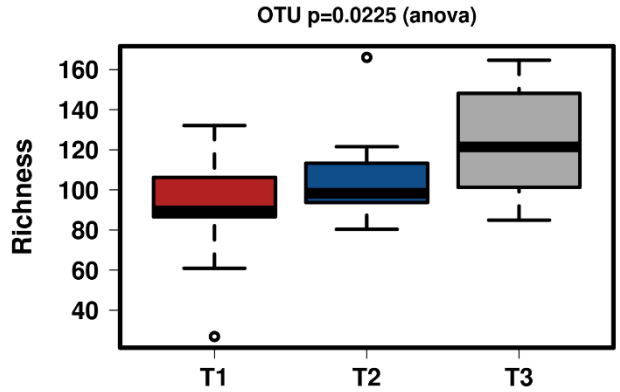


Figure 67: Richness index of all time points of the group 3 samples, with a significant increase over time.

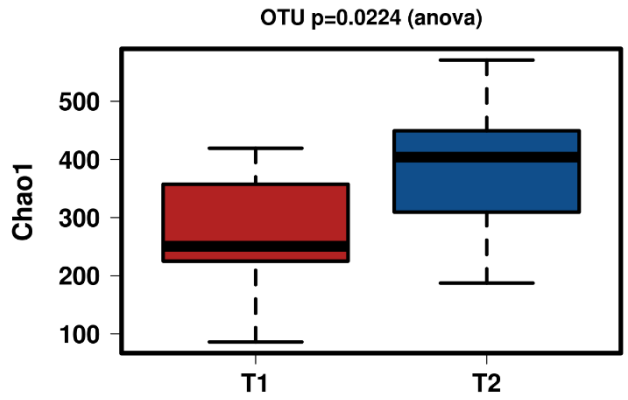


Figure 68: Chao1 index of T1 and T2 of the group 1 samples, with a significant increase over time.

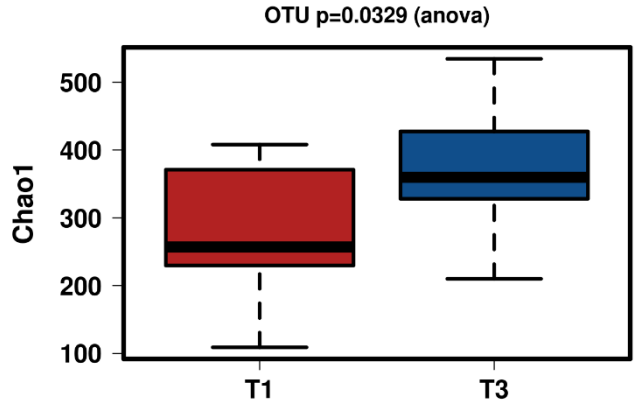


Figure 69: Chao1 index of T1 and T3 of the group 1 samples, with a significant increase over time.

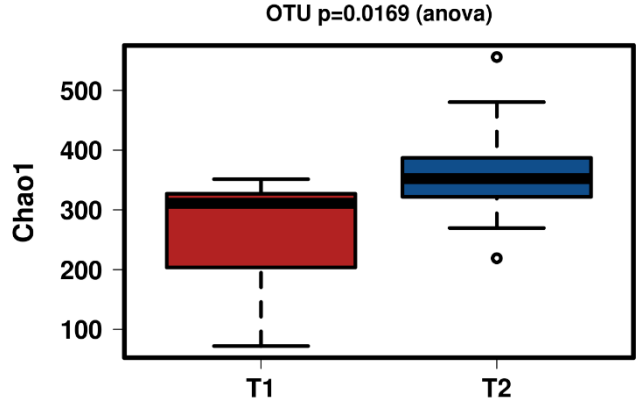


Figure 70: Chao1 index of T1 and T2 of the group 2 samples, with a significant increase over time.

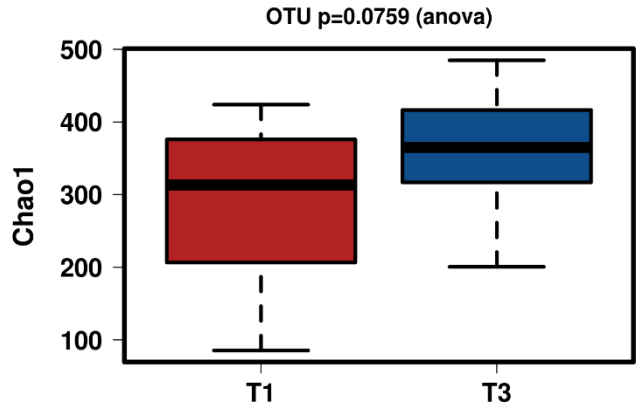


Figure 71: Chao1 index of T1 and T3 of the group 2 samples, with no significant increase over time.

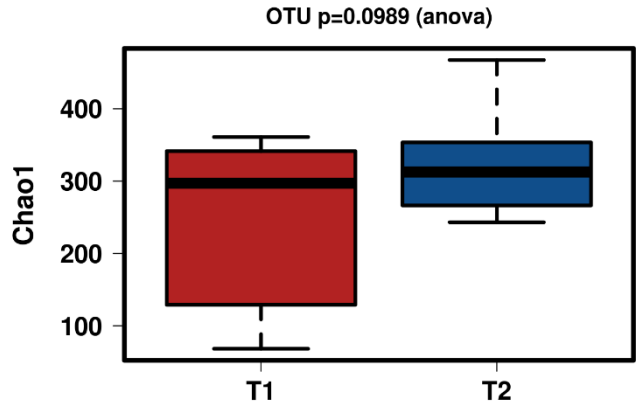


Figure 72 Chao1 index of T1 and T2 of the group 3 samples, with no significant increase over time.

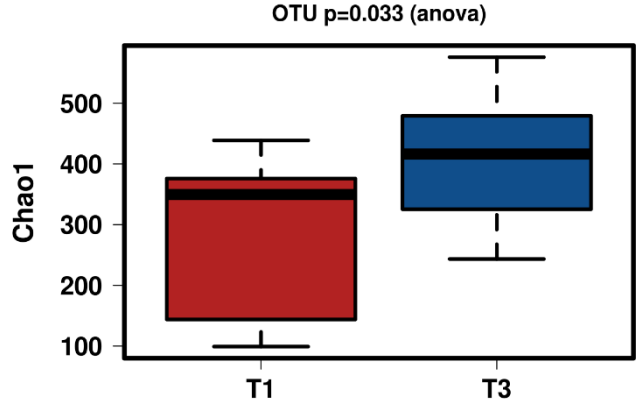


Figure 73 Chao1 index of T1 and T3 of the group 3 samples, with a significant increase over time.

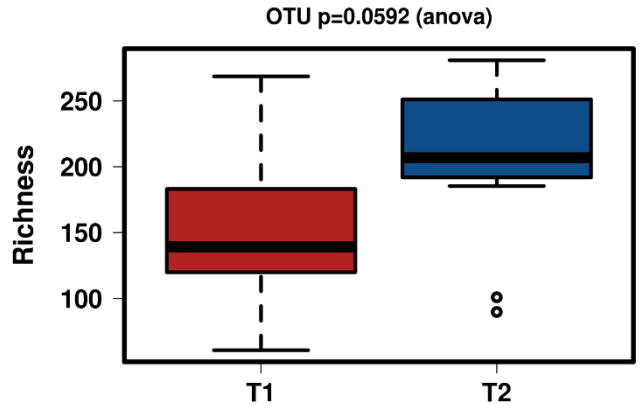


Figure 74: Richness index of T1 and T2 of the group 1 samples, with no significant increase over time.

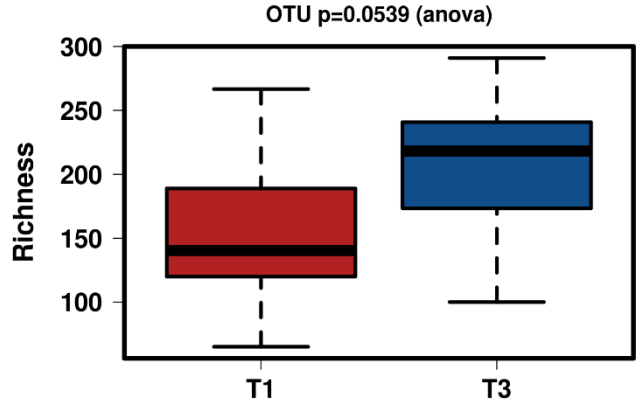


Figure 75: Richness index of T1 and T3 of the group 1 samples, with no significant increase over time.

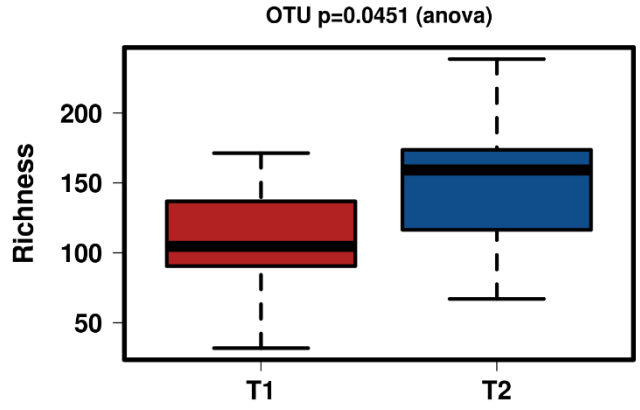


Figure 76: Richness index of T1 and T2 of the group 2 samples, with a significant increase over time.

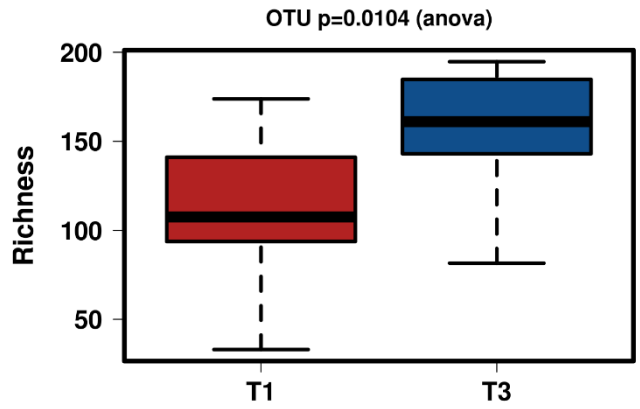


Figure 77: Richness index of T1 and T3 of the group 2 samples, with a significant increase over time.

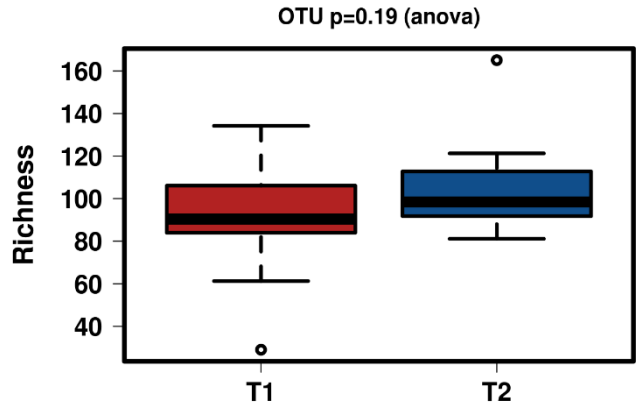


Figure 78: Richness index of T1 and T2 of the group 3 samples, with no significant increase over time.

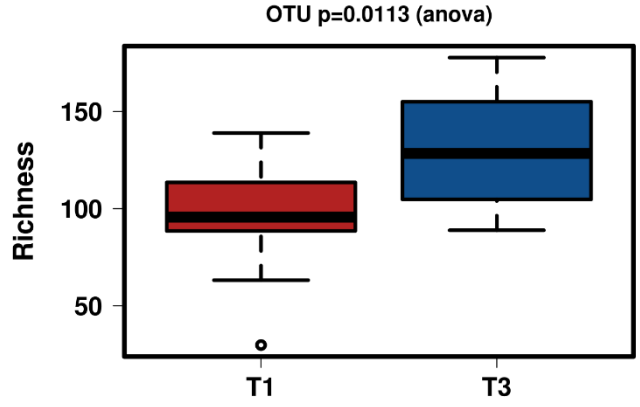


Figure 79: Richness index of T1 and T3 of the group 3 samples, with a significant increase over time.

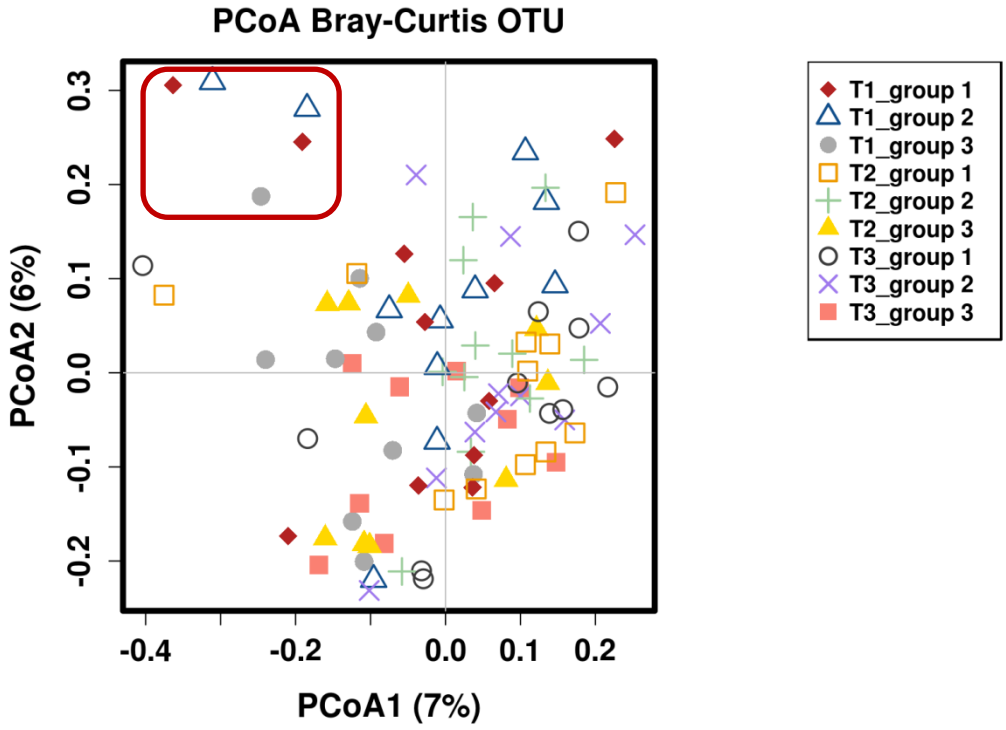


Figure 80: PCoA Plot of the different time points per group. Each point refers to one microbiome measurement. Shapes and colours indicate the different time points and the product group (see legend). Red framed T1 points seem to move towards the rest during product use.

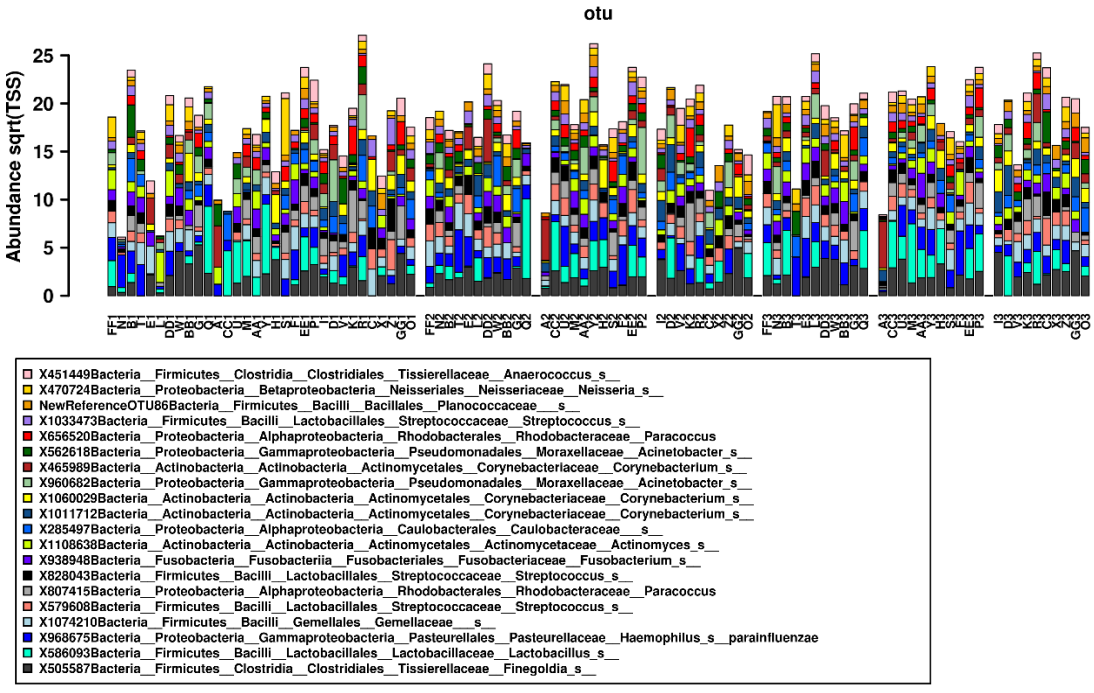


Figure 81: Abundance based on OUT level in all samples, grouped per time points and product use.

Table 13: 20 most significant OTUs that changed over the time of the product use and during the Follow-up 6 weeks further with individual, personal hygiene. Yellow marked OTUs were used for further analysis.

Taxa	P (Anova)	Adjusted P (Bonferroni)	T1 mean	T2_group 2 mean	T2_group 1 mean	T2_group 3 mean	T3_group 2 mean	T3_group 1 mean	T3_group 3 mean
New,ReferenceOTU77Bacteria__Firmicutes__Bacilli__Bacillales__Bacillaceae__Bacillus_s__	0,00000064	0,00084	0,037	0,2	0,14	0,22	0,28	0,12	0,25
New,ReferenceOTU235Bacteria__Firmicutes__Bacilli__Bacillales__Staphylococcaceae__Staphylococcus_s__	0,000029	0,038	0,046	0,095	0,11	0,058	0,19	0,16	0,18
New,ReferenceOTU53Bacteria__Actinobacteria__Actinobacteria__Actinomycetales__Propionibacteriaceae__Propionibacterium_s__acnes	0,000043	0,056	0,047	0,21	0,19	0,25	0,23	0,19	0,29
New,CleanUp,ReferenceOTU54692Bacteria__Proteobacteria__Bproteobacteria__Burkholderiales__Comamonadaceae___s__	0,000052	0,068	0	0	0,016	0,13	0,031	0,0082	0,015
New,ReferenceOTU239Bacteria__Firmicutes__Bacilli__Bacillales__Staphylococcaceae__Staphylococcus_s__	0,000065	0,085	0,27	0,31	0,46	0,38	0,49	0,59	0,6
X897161Bacteria__Actinobacteria__Actinobacteria__Actinomycetales__Corynebacteriaceae__Corynebacterium_s__durum	0,00019	0,25	0,018	0,045	0,056	0,008	0,13	0,06	0,031
X1082607Bacteria__Actinobacteria__Actinobacteria__Actinomycetales__Corynebacteriaceae__Corynebacterium_s__	0,00002	0,26	0,0072	0,085	0,056	0,12	0,12	0,037	0,14
New,CleanUp,ReferenceOTU142608Bacteria__Firmicutes__Bacilli__Lactobacillales__Streptococcaceae__Streptococcus_s__	0,00023	0,3	0,039	0,13	0,15	0,063	0,097	0,17	0,16
New,ReferenceOTU325Bacteria__Actinobacteria__Actinobacteria__Actinomycetales__Micrococcaceae__Micrococcus_s__	0,00034	0,45	0,097	0,24	0,31	0,25	0,24	0,29	0,27
New,CleanUp,ReferenceOTU47475Bacteria__Proteobacteria__Gammaproteobacteria__Pasteurellales__Pasteurellaceae__Haemophilus_s__parainfluenzae	0,00036	0,47	0,018	0,03	0,089	0	0,086	0,03	0,018
New,CleanUp,ReferenceOTU31640Bacteria__Firmicutes__Bacilli__Bacillales__Planococcaceae___s__	0,0004	0,52	0,12	0,16	0,24	0,16	0,32	0,27	0,3
X519673Bacteria__Firmicutes__Bacilli__Bacillales__Staphylococcaceae__Staphylococcus_s__	0,00041	0,54	0,14	0,19	0,32	0,3	0,32	0,4	0,51
X4309764Bacteria__Firmicutes__Bacilli__Lactobacillales__Streptococcaceae__Streptococcus_s__	0,00044	0,58	0,00091	0,048	0,097	0,04	0,025	0,043	0,072
New,ReferenceOTU285Bacteria__Firmicutes__Bacilli__Bacillales	0,00045	0,59	0,22	0,26	0,44	0,3	0,38	0,5	0,58
X851935Bacteria__Bacteroidetes__Bacteroidia__Bacteroidales__Prevotellaceae__Prevotella_s__	0,00047	0,62	0	0	0,051	0	0,13	0	0,01
New,CleanUp,ReferenceOTU56001Bacteria__Firmicutes__Bacilli__Lactobacillales__Streptococcaceae___s__	0,00051	0,67	0,022	0,091	0,075	0,034	0,13	0,095	0,13
X1068572Bacteria__Actinobacteria__Actinobacteria__Actinomycetales__Corynebacteriaceae__Corynebacterium_s__durum	0,00057	0,75	0,27	0,59	0,51	0,13	0,89	0,4	0,35
New,CleanUp,ReferenceOTU84374Bacteria__Firmicutes__Bacilli__Lactobacillales__Streptococcaceae__Streptococcus_s__	0,00007	0,92	0,029	0,12	0,11	0,048	0,11	0,11	0,1
New,CleanUp,ReferenceOTU72701Bacteria__Firmicutes__Bacilli__Bacillales__Bacillaceae__Bacillus_s__	0,00085	1	0,024	0,013	0,038	0,018	0,095	0,042	0,14
X386088Bacteria__Actinobacteria__Actinobacteria__Actinomycetales__Propionibacteriaceae__Propionibacterium	0,00087	1	0,00047	0,094	0,072	0,059	0,11	0,068	0,082

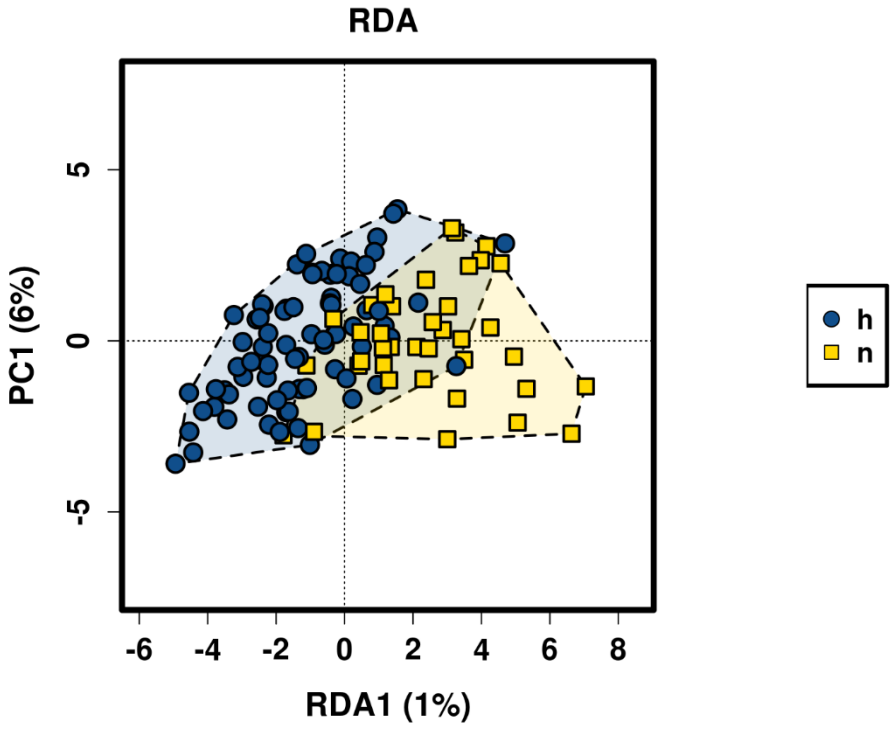


Figure 82: RDA Plot of the different pH ranges h for high pH and n for normal pH including T4 for the Follow-up sampling after further 6 weeks without specific product use.

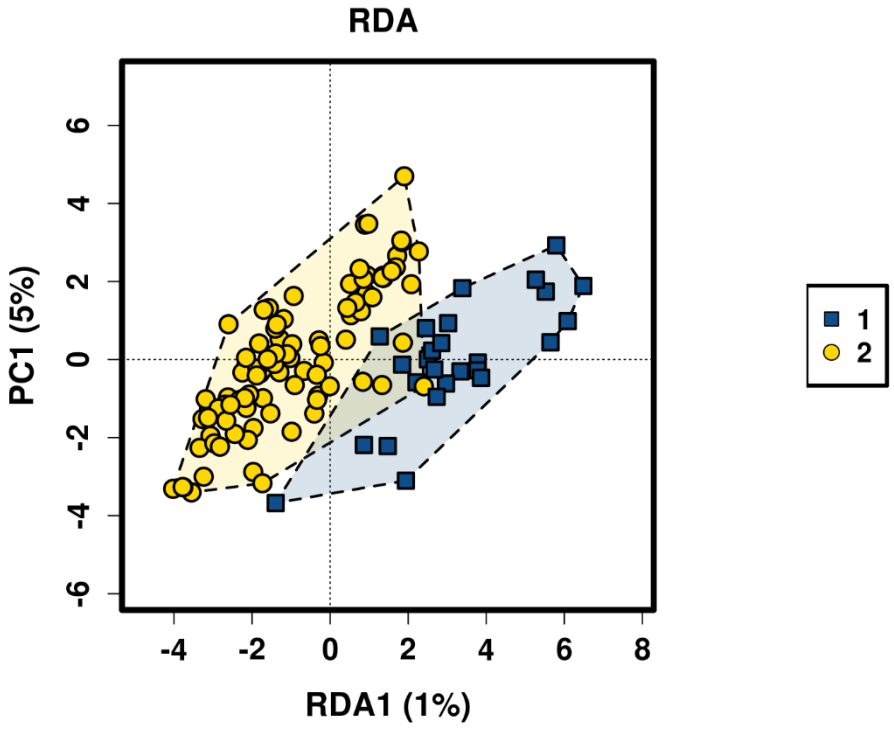


Figure 83: RDA Plot of the different use of shower gel per day (1 for applying shower gel once a day and 2 for twice a day) including T4 for the Follow-up sampling after further 6 weeks without specific product use.

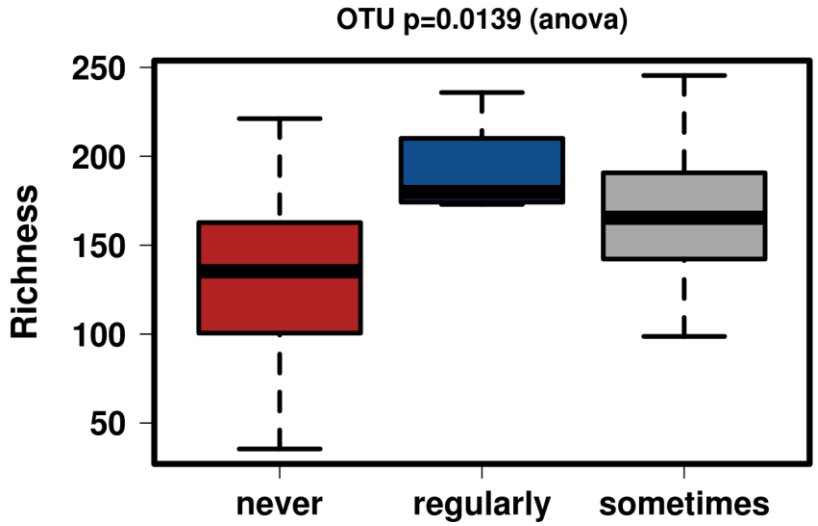


Figure 84: Richness boxplot showing the increase of richness through the use of lotions including only datapoints from T1 and T4. This figure shows that the more often a lotion is applied the higher the richness grows.

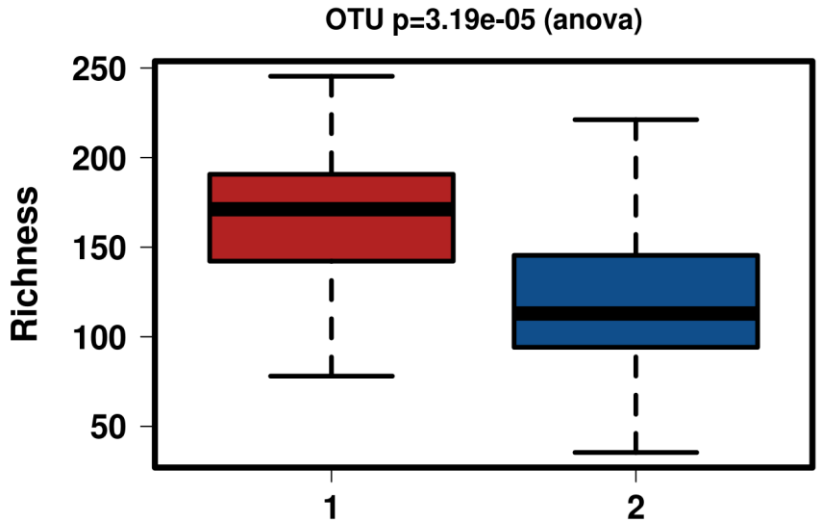


Figure 85: Richness boxplot showing the decrease of richness through the more often use of shower gel per day including only datapoints from T1 and T4. This figure shows that the more often a shower gel is applied the lower the richness grows.

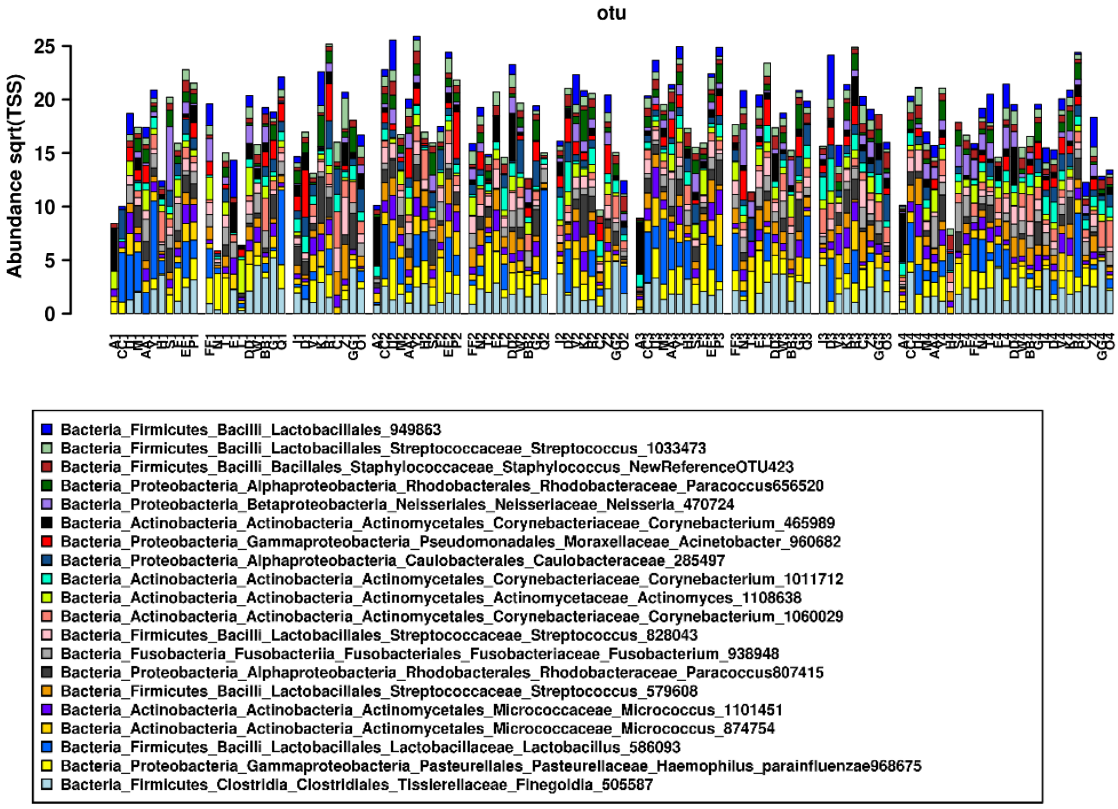


Figure 86: Abundance based on the OTU level including the Follow-up, grouped per time points and groups.

Table 14: 20 most significant OTUs that changed over the time of the product use and during the Follow-up 6 weeks further without product use. Yellow marked OTUs were used for further analysis.

Taxa	P (Anova)	Adjusted P (Bonferroni)	T1_group1 mean	T1_group2 mean	T1_group3 mean	T2_group1 mean	T2_group2 mean	T2_group3 mean	T3_group1 mean	T3_group2 mean	T3_group3 mean	T4 mean
Bacteria_Firmicutes_Bacilli_BacillalesNewReferenceOTU318	0,000002	0,00027	0,036	0,009	0,032	0,11	0,078	0,096	0,089	0,14	0,14	0,15
Bacteria_Firmicutes_Bacilli_Bacillales_Staphylococcaceae_StaphylococcusNewCleanUpReferenceOTU57500	0,000034	0,00045	0,018	0	0,0089	0,085	0,045	0,01	0,072	0,13	0,051	0,1
Bacteria_Actinobacteria_Actinobacteria_Actinomycetales_Propionibacteriaceae_Propionibacterium_acnesNewReferenceOTU85	0,000035	0,00047	0,089	0,15	0,19	0,41	0,5	0,65	0,44	0,56	0,65	0,56
Bacteria_Firmicutes_Bacilli_Bacillales_Staphylococcaceae_Staphylococcus_aureusNewReferenceOTU5	0,000067	0,00089	0,036	0,01	0,043	0,14	0,2	0,22	0,16	0,28	0,25	0,26
Bacteria_Firmicutes_Bacilli_Bacillales925707	0,000088	0,012	0,31	0,22	0,079	0,34	0,32	0,29	0,43	0,47	0,47	0,4
Bacteria_Cyanobacteria_ML635J21_NewCleanUpReferenceOTU82019	0,000019	0,025	0,032	0,02	0,046	0,075	0,032	0,081	0,045	0,098	0,1	0,19
Bacteria_Proteobacteria_Gammaproteobacteria_Pasteurellales_Pasteurellaceae_Haemophilus_parainfluenzaeNewCleanUpReferenceOTU107731	0,000046	0,061	0,0082	0	0,01	0,062	0,046	0	0,046	0,11	0,01	0,11
Bacteria_Cyanobacteria_ML635J21_NewCleanUpReferenceOTU50634	0,00017	0,23	0,013	0,063	0,09	0,14	0,18	0,14	0,1	0,096	0,16	0,13
Bacteria_Firmicutes_Bacilli_Bacillales_Staphylococcaceae_Staphylococcus_NewReferenceOTU144	0,00018	0,24	0,28	0,15	0,22	0,44	0,26	0,3	0,51	0,4	0,45	0,42
Bacteria_Firmicutes_Bacilli_Bacillales_Staphylococcaceae_Staphylococcus_NewCleanUpReferenceOTU5006	0,002	0,27	0,044	0	0,012	0,023	0	0,031	0,049	0,072	0,061	0,12
Bacteria_Firmicutes_Bacilli_Lactobacillales_Streptococcaceae_Streptococcus_NewCleanUpReferenceOTU36066	0,00027	0,36	0,012	0,043	0,0089	0,07	0,034	0,026	0,071	0,13	0,018	0,052
Bacteria_Firmicutes_Bacilli_Bacillales_Staphylococcaceae_Staphylococcus_1084906	0,00035	0,47	0,37	0,27	0,26	0,55	0,37	0,36	0,59	0,53	0,52	0,53
Bacteria_Cyanobacteria_ML635J21_NewCleanUpReferenceOTU88573	0,00049	0,65	0	0	0,012	0,036	0,01	0,064	0,012	0,063	0,036	0,072
Bacteria_Firmicutes_Bacilli_Bacillales_Staphylococcaceae_Staphylococcus_NewReferenceOTU69	0,005	0,67	0,19	0,12	0,17	0,28	0,24	0,29	0,37	0,38	0,45	0,38
Bacteria_Fusobacteria_Fusobacteriia_Fusobacteriales_Fusobacteriaceae_Fusobacterium_NewCleanUpReferenceOTU19935	0,00067	0,89	0,0082	0	0	0,029	0,061	0,021	0,027	0,025	0,017	0,084
Bacteria_Proteobacteria_Bproteobacteria_ASSO13_550484	0,00068	0,91	0	0,19	0	0,047	0	0	0	0	0	0
Bacteria_Proteobacteria_Gammaproteobacteria_Pseudomonadales_Moraxellaceae_Enhydrobacter_NewCleanUpReferenceOTU4406	0,00071	0,95	0,021	0	0,0089	0,014	0,007	0,038	0,037	0,01	0,044	0,087
Bacteria_Firmicutes_Bacilli_Bacillales_Staphylococcaceae_Staphylococcus_NewReferenceOTU33	0,00077	1	0,099	0,08	0,11	0,18	0,1	0,15	0,21	0,21	0,22	0,23
Bacteria_Firmicutes_Bacilli_Lactobacillales_Streptococcaceae_Streptococcus_NewCleanUpReferenceOTU18882	0,00079	1	0,029	0,038	0	0,05	0,087	0,053	0,08	0,14	0,098	0,095
Bacteria_Actinobacteria_Actinobacteria_Actinomycetales_Corynebacteriaceae_Corynebacterium_446403	0,00044	0,59	0,018	0,034	0,21	0,037	0,007	0,35	0,032	0,12	0,37	0,19

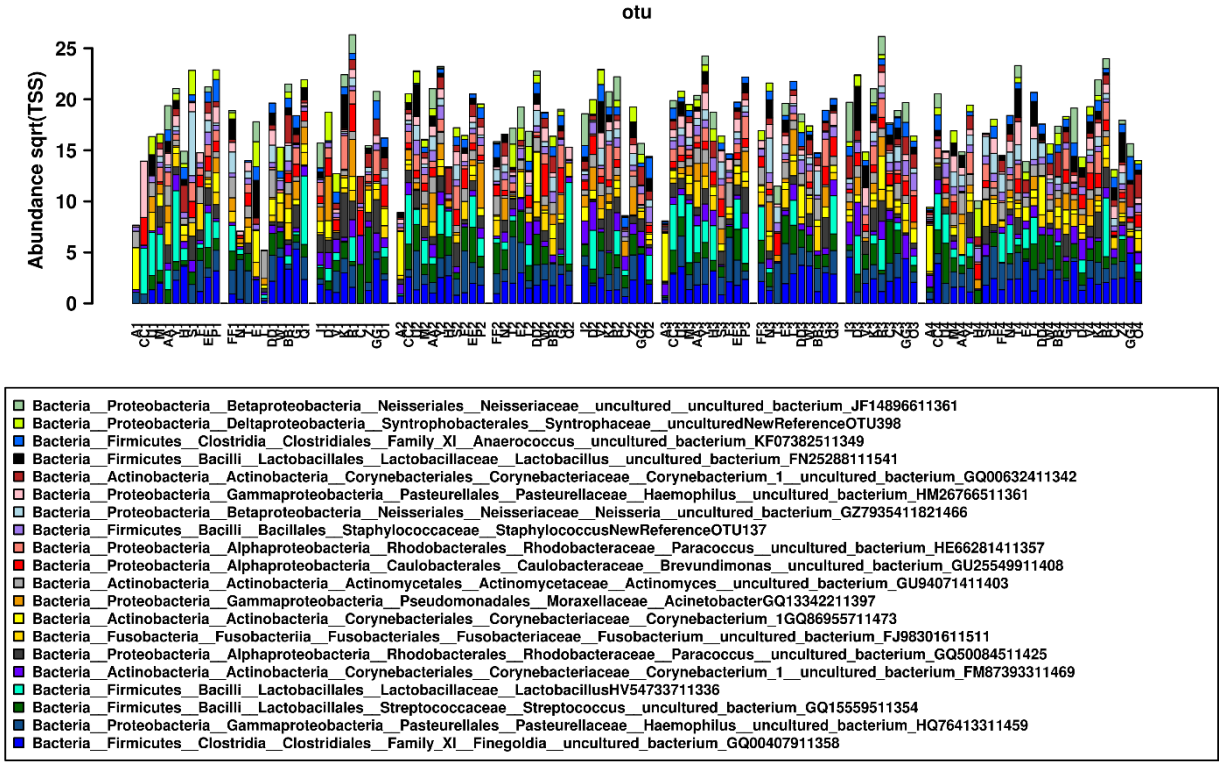


Figure 87: Abundance based on the OTU level created with the Silva databank including the Follow-up, grouped per time points and groups.

Table 15: Comparison of the Chao 1 index and observed species between samples processed with GreenGenes Databank and with Silva Databank.

	chao1	observed_species	chao 1 silva	observed_species silva
BJFU	3490,762846	1856	3895,99074	2144
DF3	3953,058594	1927	4275,88772	2085
KP2	2827,05618	1385	3328,95	1546
DF2	4713,841202	2034	4738,48571	2288
LWFU	5479,016949	2661	5432,89006	2989
KPFU	3096,9	1734	3621,85294	2022
JW3	4277,692308	1951	4620,39871	2249
PL1	4001	1636	4391,60444	1864
IKFU	3730,496689	2116	4112,57368	2380
MPFU	3697,168317	2088	4083,20912	2367
IDG3	4127,981818	2087	4771,37455	2229
CK3	2638,44	1128	2831,61184	1245
JM2	3522,980132	1417	3362,96429	1535
BJ1	2315,7	1083	2749,14	1201
BJ3	2923,112903	1409	3163,72527	1460
SC2	4679,812057	2133	5219,02432	2439
MV2	4545,047619	2318	5462,18713	2611
CME2	3927,472081	1711	4159,28049	1882
JHFU	5929,859589	3457	6432,50609	3579
SR3	3685,119149	1835	4476,87952	2067
MP3	3327,8375	1720	4049,93776	1902
SP2	4195,620209	2101	4973,73333	2367
KP3	3723,727273	1676	3937,88115	1833
JH2	3852,414634	1672	4491,17045	2006
DF1	2087,457143	861	2301,25175	1032
SG2	5105,826087	2282	5572,99038	2510
CMEFU	4206,204969	2274	4814,0027	2553
TB2	2651,14966	1203	2813,3662	1427
CKFU	3824,479751	2180	4601,26849	2554
JWFU	6648,151515	3972	6987,97379	4239
SR2	3593,104651	1559	4011,22439	1747
CME3	4719,170455	2247	4956,55161	2402
SC1	3565,157609	1581	4103,36818	1801
ADFU	4265,318421	2350	4916,36957	2598
NC6	2171,679389	1015	2443,02685	1180
LW2	4583,376518	2078	5178,13953	2334
CK2	2938,75	1357	3273,85973	1583
JM3	5011,214286	2373	5671,68862	2651
DFFU	4535,482315	2295	5030,9403	2664

AD3	5136,574468	2252	5308,44615	2465
TB1	3540,569832	1539	3974,48523	1786
IDG2	4437,25	2213	5194,19408	2421
SC3	4173,723141	1898	4313,00356	2071
AD2	3655,309701	1869	4548,22182	2108
PLFU	5299,271605	2986	5394,50584	3089
MB2	4998,070122	2479	5542,89041	2724
MP2	4608,796226	2107	4798,8625	2332
PL3	4616,092105	2013	5052,33721	2250
IK3	4595,126482	2029	5122,64087	2341
IDGFU	5278,602305	2670	5947,44262	2888
SR1	3069,065359	1382	3383,75	1559
KK3	3889,142857	1759	3614,04292	1768
LW1	2721,753165	1337	3266,3883	1502
TS2	5771,752294	2669	6204,83562	2869
TB3	3317,181818	1539	3889,74242	1708
CKa2	4801,987382	2406	5985,6716	2752
MB1	3368,448276	1550	3604,34211	1730
TS1	3085,719577	1507	3499,17526	1648
PL2	4953,546512	2193	5451,25914	2444
VC2	3038,561798	1431	3175,01463	1545
SG1	2858,8	1297	3279,26705	1436
SRFU	3692,229965	2105	4155,47962	2312
TS3	4858,507463	2472	5019,01977	2597
SP3	4105,987179	1872	4731,81206	2185
LW3	3988,857988	1648	4055,64348	1849
SCFU	4700,258721	2431	4784,96172	2677
MV3	4480,379699	2133	5021,69388	2358
MB3	2667,388235	1353	3162,30159	1530
IK2	3691,318182	1681	4144,5875	1863
CMEugd1	3579,384615	1596	3671,64348	1720
JM1	3666,927152	1405	3736,04972	1570
AD1	2275,982249	1202	2583,96373	1338
KKFU	3041,248649	1474	3256,18779	1641
CKa1	2278,64375	1163	2961,24309	1397
BP2	3558,73301	1634	3907,71959	1990
MVFU	4261,758621	2478	4761,57895	2727
NC3	2105,352941	595	1651,69697	733
CKa3	3076,076142	1478	3386,07317	1601
SPFU	4179,119363	2334	4601,70631	2546
JW2	3082,623596	1402	3913,0567	1643
BPFU	3744,836364	1979	4926,47603	2338
NDFU	5140,034483	2837	5254,7006	3079
IDG1	4483,5	1906	4498,44048	2056
PZ3	3010,19375	1292	3322,00535	1460

NC1FU	1692,858696	741	1852,05	906
NC5	1656,923077	722	1670,648	848
SD1	2972,561497	1418	3090,31707	1627
JH3	3131,421384	1327	3403,87701	1515
AH1	2337,405405	997	2452,39855	1102
SP1	2947,283237	1405	3133,61927	1582
SD3	2834,315271	1421	3328,56604	1570
KK2	3956,466926	1962	4440,79537	2090
SDFU	4079,172872	2319	4568,84211	2586
Kgroup2	2697,592593	989	2092,73333	1061
VC3	3190,532995	1556	3162,12444	1622
SD2	2318,194444	1136	2319,95775	1117
SG3	3894,818182	1644	3758,97468	1776
KK1	2983,674074	1228	3082,20652	1397
nEK5	2550,694737	909	2459,8777	1057
SGFU	3635,460526	1936	4107,39404	2084
nKswabs3	2518,193182	846	2189,70833	927
CMEgd1	3161,258621	1235	2847,74534	1302
nEK4	332,96875	172	472,541667	180
MP1	3498,264516	1426	3793,9734	1594
ND1	3216,26699	1610	3739,18672	1849
JS1	1373,649351	634	1431,32184	705
PZFU	2528,329114	1424	2797,04098	1523
JS2	3005,904762	1419	3342,98592	1556
AH3	4655,533333	2033	5158,24242	2289
MV1	2906,868966	1258	3154,03049	1400
VC1	3119,608696	1306	2908,16667	1385
BP3	2811,43949	1293	3314,05882	1492
AH2	3669,626866	1630	4142,81974	1864
PZ2	3244,885417	1068	3218,85256	1321
IK1	2302,024	968	2691,28099	1059
BL1	2481,034091	912	2822,38393	1098
MBFU	3363,595455	1708	4048,7193	2042
CK1	1780,697917	747	2001,53043	864
JS3	2278,141791	999	2635,21094	1102
BL2	2580,75	1238	2734,33333	1361
JMFU	2109,073171	1012	2428,02439	1239
ND3	3902,598425	1983	4179,27961	2209
BLFU	2016,59375	1213	2209,07692	1319
AHFU	2448,105263	1355	2745,97321	1522
JH1	3092,203252	1182	3318,13548	1348
PZ1	3013,322981	1419	3006,33	1549
ND2	3241,162791	1641	3724,47126	1894
JW1	2582,877698	1180	2728,47561	1300
nKswabs1	2054,598361	940	2332,86131	1086

Kgroup3	38	10	56	11
nKSwabs2	2536,25	843	2261,68103	922
BL3	2098,977186	1367	2318,57551	1439
BJ2	3336,079602	1562	3712,04082	1770
BP1	3312,057143	1504	3617,93925	1681
nEk1	1912,082474	771	2050,49254	932
KP1	2206,709677	822	2233,128	972
NC2	2695,78022	920	2412,68345	1072
nEK2	2761	1053	3078,30496	1186
NC1	1834,229508	828	2479,58333	1002
nSKFU	2066,206612	951	2037,77483	1074
NC3FU	687,1176471	294	714,352941	377
nEK2FU	1820,2	781	1832,22222	935
NC2FU	1461,375	727	1685,21538	915
nEK3	1853,978495	758	2003,04839	898
NC4	2089,06383	565	1642,6	657
NC7	2233,358696	817	2198,7913	938
KJgroup1	37	15	48	15
nEK1FU	278,3333333	44	228	41
nEK6	55	16	76,4545455	58

1 Index of Abbreviations

Bovine Serum Albumin	BSA
Center for Medical Research	ZMF
Corneocytes	SC cells
Corneometer Unit	CU
Deoxyribonucleic Acid	DNA
Deoxyribonucleoside Triphosphates	dNTPs
Ethylenediaminetetraacetic Acid	EDTA
Linear discriminant analysis Effect Size	LEfSe
Lipoteichoic Acid	LTA
Liquid Chromatography – Mass-Spectrometry /Mass-Spectrometry	LC-MS/MS
L2 Normalisation	LSE
Next Generation Sequencing	NGS
Operational Taxonomic Units	OTUs
Phylogenetic Investigation of Communities by Reconstruction of Unobserved States	PICRUST
Polymerase Chain Reaction	PCR
Principal Coordinates Analysis	PCoA
Quantitative Insights Into Microbial Ecology	QIIME
Redundancy Analysis	RDA
ribosomal Ribonucleic Acid	rRNA
Stratum Corneum	SC
Transepidermal Water Loss	TEWL
Tris-acetate-EDTA Buffer	TAE
Total Sum Scaling	TSS
Ultraviolet	UV

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