"TOUCH MICROBIOME" AS A POTENTIAL TOOL FOR FORENSIC INVESTIGATION: A PILOT STUDY

Abstract

Human skin hosts a variety of microbes that can be transferred to surfaces ("touch microbiome"). These microorganisms could be considered as forensic markers similarly to "touch DNA". With this pilot study, we wanted to evaluate the transferability and persistence of the "touch microbiome" on a surface after the deposition of a fingerprint and its exposure for 30 days at room temperature. Eleven volunteers were enrolled in the study. Skin microbiome samples were collected by swabbing the palm of their hands; additionally, donors were asked to touch a glass microscope slide to deposit their fingerprints, that were then swabbed. Both human and microbial DNA was isolated and quantified. Amelogenin locus and 16 human STRs were amplified, whereas the V4 region of 16S rRNA gene was sequenced using Illumina MiSeq platform. STR profiles were successfully typed for 5 out of 22 "touch DNA" samples, while a microbiome profile was obtained for 20 out of 22 "touch microbiome" samples. Six skin core microbiome taxa were identified, as well as unique donor characterizing taxa. These unique taxa may have relevance for personal identification studies and may be useful to provide forensic intelligence information also when "touch DNA" fails. Additional future studies including greater datasets, additional time points and a greater number of surfaces may clarify the applicability of "touch microbiome" studies to real forensic contexts.

Keywords: microbiome, touch DNA, next-generation sequencing, STR analysis, personal identification, DNA fingerprinting

1. Introduction

Microbes are present all around us and nearly everywhere on Earth, building ubiquitous communities and interacting together in a manner that is closely associated with the surrounding environmental conditions [1]. Microorganisms can be found not only in external environments, but also within specific human districts constituting stable ecological niches [2]. In fact, several studies on the human microbiota have shown a wide diversity of microbial communities both within human hosts and in different body districts and/or biological fluids (e.g., saliva, gut, hair, skin) [3]. Human microbiota represents the totality of microorganisms found within the human body [4]. It varies for each single individual and it is influenced by several factors such as body site [5, 6], sex [5, 7], age [8, 9], geographical provenience of the person [10],

health condition [11, 12] and lifestyle (e.g., diet [13], alcohol consumption [14], physical activity [15], smoking habits [16, 17], etc.).

Despite the great number of studies conducted on the human microbiota for clinical reasons due to their correlation with health/disease states [18–20], they have been lately applied also to the forensic field due to their potential use as auxiliary tool in crime cases [21]. Forensic microbiology is a relatively new science originated from the intersection between microbiology and forensics, whose development is particularly linked to the recent advances in Next Generation Sequencing (NGS) platforms that allow the obtainment of a large amount of data within a single analytical run and the rapid and efficient analysis of whole microbial communities without the need of performing in vitro cultures [21]. NGS reduces both the analytical costs and the time needed for the analysis in comparison to previously Sanger sequencing [21, 22]. In addition, this technology allows the characterization not only of the whole genome of a given microbe, but also the identification and quantification of the species belonging to whole microbial communities, providing an overview of different taxa and bacterial strains associated with a specific sample [23]. From the first applications of forensic microbiology to bioterrorism and bio-crime associated topics [24, 25], the analysis of the microbiome is now an important tool in the forensic field [26, 27] since and it could help to define post-mortem interval (PMI) [28–30], cause of death [31, 32], place of death [33] and personal identification [34–38].

The application of skin microbiome analyses to achieve personal identification for forensic applications is based on the fact that the microbial diversity among different body sites of a specific individual is smaller than the microbial diversity observed among different individuals [39]. Moreover, skin microbiota is highly individual [27], relatively stable over time [40] and easy to be found and collected on the crime scene from the surfaces of objects that have been touched by a potential perpetrator [36, 41, 42]. These skin bacteria may persist on touched surfaces for prolonged periods of time because many of those are highly resistant to environmental stresses, including moisture, temperature, and UV radiation [43, 44]. Recent studies have demonstrated that skin-associated bacterial communities are surprisingly diverse, with a high degree of inter-individual variability [45, 46]. Given that individuals appear to harbor personally unique, temporally stable, and transferable skin-associated bacterial communities, it has been hypothesized that bacteria can be used as “fingerprints” for forensic identification. This microbial fingerprint of the skin could be defined as "touch microbiome" and represents a bacterial signature that may provide forensically relevant information ultimately useful for human identification [47–49], providing not only the confirmation of an association between individuals, objects and places [41, 50], but also information about the hosts’ lifestyle [51], such as with whom they live and if they have pets [52].

Microbiome analyses have been admitted as evidence in court, but several questions remain to be addressed before the analysis of these microbial biosignatures can become routine [53]. In particular, the lack of validation strategies for the laboratory technique and for the collection of metadata currently limits their use [27, 37]. Moreover, to understand the reliability of the data that can be obtained, the differences between bacterial communities on the body and within the environment should be carefully considered, as it could be important to understand what statistical power is needed to have reliable microbiome-derived data [53].

Contrary to this, “touch DNA”, that is the DNA transferred from a donor to a certain object through direct or indirect contact, has been successfully admitted as evidence in forensic cases, since the increased sensitivity in the simultaneous amplification of different Short Tandem Repeats (STRs) enabled forensic geneticists to recover DNA profiles from highly degraded samples and low DNA content evidence [54], as fingerprints [55, 56]. In spite of this, there is still some lack of knowledge, such as the source of this touch DNA, the manner to be transferred and its capacity to resist and persist in time at different environmental conditions [57, 58].

In this context, analysis on “touch microbiome” could be more informative and can integrate "touch DNA" fingerprint analysis when only partial prints and degraded samples are available, therefore when a full human STR profile cannot be obtained [59]. Due to the potential that “touch microbiome” analyses can have in discriminating different individuals [36], also when other identification techniques fail, with this pilot study we wanted to evaluate the possibility to use microbiome analyses as a potential tool in forensic investigation for personal identification purposes, exploring the transferability of the “touch microbiome” on a hard surface and its survival 30 days post-deposition, and evaluating the presence of core microbiomes and specific donor characterizing taxa that may ultimately be good indicators for achieving personal identifications.

2. Materials and Methods

This pilot study was approved by the Ethical Committee “Comitato Etico Interaziendale Novara” (CE 57/20) and a written informed consent has been obtained for each individual enrolled. The datasets generated for this study can be found in the NCBI Sequence Read Archive (SRA-NCBI, https://www.ncbi.nlm.nih.gov/sra) under project accession number PRJNA685984 and BioSample accession numbers SAMN17103338–SAMN17103359.

2.1 *Sampling*

Eleven volunteers of both sexes (five males and six females) of different ages, ranging from 20 to 70 years, were enrolled in the study. The inclusion criteria were living in Italy for at least three generations, being in good general health

87 conditions and not having taken antibiotics and/or antifungals in the 15 days prior to the sampling. Each volunteer filled
88 in an assessment questionnaire that investigated their health and lifestyle (e.g., gender, age, height, weight, working
89 activity, lifestyle habits, diet, use of public transports and health condition with any previous or current pathologies and
90 drug treatments), and information about other intrinsic and extrinsic factors that are known to, or could, influence the
91 skin microbiome, such as the last hand washing (Supplementary Table S1). The volunteers were asked to maintain a
92 normal daily routine in terms of their personal hygiene, diet and exercise.
93 "Skin samples" were collected by sliding two sterile swabs moistened with physiological water over the entire palm
94 surface, including fingers, of the dominant hand for 15 seconds. Swabs were then stored at -20 °C for 30 days, after
95 which DNA (both bacterial and human) was extracted.
96 The same donors were asked to touch two glass microscope slides with all five fingers for about ten seconds in order to
97 deposit their fingerprints all around the surface of the slide, and after 30 days at room temperature the deposited
98 fingerprints were swabbed in order to obtain a "glass fingerprint sample". It has to be noted that this process was not
99 made under sterile conditions to better simulate real forensic scenarios. The idea was to simulate the random release
100 of genetic material (both bacterial and human) on surfaces touched by the subject (called respectively "touch
101 microbiome" and "touch DNA"). Since the focus of the paper is to evaluate the possibility of using microbiome analysis
102 as a potential tool to achieve personal identification, particularly in cases where "touch DNA" analysis fails, we fully
103 reported below only the steps required for the analysis of the touch microbiome and its associated results, whereas the
104 analyses carried out for the "touch DNA" and consequent results are summarized in the Supplementary Material.
105 2.2 *Microbiome Extraction and Analysis*
106 The gold standards suggested from the Earth Microbiome Project to target and sequence the highly variable V4 region
107 of the 16srRNA gene for bacterial identification were followed. Microbial DNA was extracted from skin swabs (A, B, C,
108 D, E, F, G, H, I, L, M-skin) and glass swabs (A, B, C, D, E, F, G, H, I, L, M-glass fingerprint) using the "QIAamp PowerFecal
109 Pro DNA Kit" (QIAGEN, Hilden, Germany), that has been optimized to isolate bacterial DNA from stool and gut samples.
110 We followed the manufacturer's instructions, except that 800 µl of CD1 solution were added to each swab, then the
111 samples were vortexed for 5 seconds, and centrifuged at 5000 rpm for 10 minutes. DNA was eluted for skin swabs in 50
112 µl of C6 Solution, while for glass swabs in 35 µl of the same solution. The extracted DNA was quantified using NanoDrop
113 One Microvolume UV-Vis Spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA) and sent to "NUOmics DNA
114 Sequencing Research Facility" (Northumbria University, Newcastle, UK) for the amplification and sequencing of the
115 hypervariable region V4 of the 16S ribosomal RNA gene using the Illumina Miseq Next Generation Sequencer (Illumina

Inc., San Diego, CA, USA), following the method used by Brabin et al [60]. Briefly, forward (GTGCCAGCMGCCGCGGTAA) and reverse (GGACTACHVGGGTWTCTAAT) primers were used following the standard operating procedures for 16S metagenomic sequencing library preparation for the Illumina MiSeq system[61]. Amplifications were conducted on 96-wells plates, and each plate contained both a positive and a negative control. PCR was run using 1x Accuprime Pfx Supermix, 0.5 µM of each primer and 1 µL template DNA under the following conditions: 95°C 2 minutes, 30 cycles 95°C 20s, 55°C 15s, 72°C 5 minutes with a final extension 72°C 10 minutes. Barcodes were incorporated into the PCR primer construct. PCR products were normalised using SequalPrep™ Normalization kit (Invitrogen, United Kingdom) following manufacturer's protocol and combined into four pools. These four pools were then quantified using fragment size determined by BioAnalyzer (Agilent Technologies) and concentration by Kappa qPCR (Kappa Biosystems) [61], and were combined in equimolar amounts to create a single library further normalised. The library was then denatured using 0.2N NaOH for 5 minutes followed by 2-minute incubation at 96°C. The library was diluted to a final concentration of 3.5 pM and supplemented with 5% PhiX and loaded onto a MiSeq V2 2x250 cartridge. Paired-end reads from each sample were sequenced with forward and reverse reads in separate files by the NUOmics DNA Sequencing Research Facility, and processed by means of the microbiome bioinformatics platform QIIME2 (Quantitative Insights Into Microbial Ecology 2), version 2019.7 [62, 63]. Denoising and quality control, including removal of chimeras, were achieved by means of the DADA2 [63] plugin (qiime dada2 denoise-paired) and to avoid low quality sequences reads were truncated (250 bp for forward, 240 bp for reverse reads). The classifier adopted for the taxonomic assignment was the QIIME release Greengenes (16S rRNA) v.13.8). The short and long md5 hashes for all the ASVs are provided in Supplementary Table S2. We decided to use short md5 hashes in the text and in the figures for ease of reading.

2.3 *Statistical Analysis*

Statistical analyses were performed within the computing environment R (https://www.R-project.org/). All the taxon abundances have been calculated and graphically plotted with the aid of the R package PHYLOSEQ V.1.22.3 [64]. Rarefaction curves have been rendered by means of the function ggrare, provided by the richness.R script from the phyloseq extension package by Mahendra Mariadassou (https://github.com/mahendra-mariadassou/phyloseq-extended).

3. Results

3.1. *Quantitation*

Results for the quantitation carried out on DNA isolated with QIAamp Powerfecal Pro DNA" (for bacterial DNA) are summarised in Table 1.

Table 1. Quantitation results in ng/µL for each sample extracted with "QIAamp PowerFecal Pro DNA" kit.

SAMPLES	"QIAamp PowerFecal Pro DNA" (for bacterial DNA)	
	SKIN (ng/µL)	GLASS-FINGERPRINT (ng/µL)
A	6.4	5.9
B	2.9	6.4
C	5.5	5.2
D	3.8	5.5
E	4.9	5.8
F	3.4	5.0
G	6.1	5.3
H	3.9	5.4
I	2.3	5.4
L	6.2	4.6
M	6.9	4.6

3.2 *Microbiome Analysis*

Microbiome sequencing effort has provided 429 to 82,870 reads for skin swab samples, and 49 to 25,781 for glass fingerprint swab samples. A first sequence quality survey revealed that the samples C-glass fingerprint and E-skin were characterized by a very low read counts (499 and 429 sequences obtained respectively for these two samples) and therefore they have been eliminated in the subsequent analyses. The final dataset was composed by nine samples (A, B, D, F, G, H, I, L, M). After the application of the Qiime2 pipeline, 213,301 high-quality 16S rRNA sequences (out of a total of 342,124 raw sequences) were retained and clustered into 586 amplicon sequence variants (ASVs).

Species richness can only be compared when the species richness as a function of sequence sample size has reached a clear asymptote in rarefaction curves. In the ASV table, all the species present in a sample were well described since the curve ascribed to each sample reached its plateau, even at different sequencing depth (Supplementary Figure S1).

A first round of normalization via rarefaction, run with the minimum sample size value of 1,766, provided an ASV table from which we first removed, for each donor, those ASVs with zero counts on both skin and glass, i.e., those generated by the microbiome other donors, but not found in everyone. In this feature table, we then highlighted ASVs with non-zero counts found only on the glass and not on the donor's skin. Assuming that the presence of taxa only on glass could be random, possibly due to the sensitivity of the sequencing that detected bacteria of environmental origin (since the fingerprinting was not done under sterile condition), these ASVs has to be eliminated from the dataset as, for the

purposes of our work, they were not transferred to the glass from the donor's skin (data not shown). So, we revised the initial ASV table, donor per donor, removing the ASVs not counted on the skin but detected on the glass, reconstructing the dataset and expecting that the ASVs on the glass biome was a subset of skin biome.

The reconstructed dataset consisted of 431 taxa (Supplementary Figure S2), which were then reduced to 329, after rarefaction the minimum sample size value of 1,649. Since we noticed that the rarefaction results were not constant without specifying a random seed used in the permutation functions, we set the random seed value to 25,470 and re-checked and removed cases with zero counts for the skin samples, introduced by the normalization process. This process allowed us to obtain a final ASV table with 328 taxa, with the phylum Proteobacteria as the most represented (37,102 ASVs), followed by Firmicutes (21,745), Actinobacteria (8,721), and Bacteroidetes (2,309). The order distribution among donors is depicted in Figure 1. Although in the initial dataset four taxa attributable to phylum Archaea were counted, they were excluded from the analysis due to the effects of rarefaction and subsequent filters. This is a further confirmation of the specificity of the primers used for phylum Bacteria.

By splitting the dataset into skin and glass subgroups and intersecting the relative taxa names, we showed that all 75 taxa from glass subgroup were shared with the skin subgroup while, on the contrary, 253 taxa were not detected on glass.

The Core Microbiome (CM), defined by those taxa always present in all 18 samples, consists of only two ASVs: 65d43491, an unidentified bacterium belonging to order Bacillales (Firmicutes, Bacilli) and d46e2205, an unidentified bacterium belonging to family Enterobacteriaceae (Proteobacteria, Gammaproteobacteria, Enterobacteriales). The Skin Core Microbiome (SCM), defined by those taxa always present in all nine skin samples, comprises six ASVs, three can be defined at species level: 06f825b5 (*Streptococcus agalactiae*), 394eda29 (*Actinobacillus delphinicola*) and 923f521b (*Anaerosinus glycerini*), two at order level: 65d43491 (Bacillales) and 7d78ed99 (Actinomycetales), and one at family level: d46e2205 (Enterobacteriaceae). The Glass Core Microbiome (GCM), defined as those taxa always present in all nine glass samples, coincides with the CM.

In order to identify the taxa successfully transferred to the glass slide, we removed for each donor all ASVs not found at least once in both swabs and retained only ASVs present on both skin and glass samples. Then we highlighted the DCTs by subtracting the taxa belonging to the SCM (SCM reported in grey font in Supplementary Table S3 and in Figure 2A-C, DCTs reported in black font in Supplementary Table S3 and in Figure 2A-C). The ASVs distribution across the nine donors is plotted in Figure 2A-C, while the detailed list of ASV of DCTs along with abundance and full taxonomic lineage is reported in Supplementary Table S3. We obtained a variable number of DCTs, ranging from four (donor A and H) to 30

(donor B). The most occurring phylum was Proteobacteria (56), followed by Actinobacteria (36), Firmicutes (35) and Bacteroidetes (13), while at order level, Gammaproteobacteria were the most occurring (38), followed by Actinobacteria (36) and Bacilli (26). At genus level, among the identified ASVs, Actinobacillus, Pseudomonas, Streptococcus were the most common with six counts each. In three cases (Donor D, G, M), it was possible to find all six ASVs belonging to the SCM. Among the DCTs transferred to the glass slides, we were additionally able to identify unique taxa (uDCT) belonging specifically to one individual and not being found in any other donor (represented with an asterisk in Supplementary Table S3 and in Figure 2A-C), and they were 0 in samples A, H and I, 2 in samples F and G, 8 in samples D and L, 11 in sample B and 14 in sample M.

Discussion

This pilot study was aimed at investigating the usefulness of the "touch microbiome" analyses for personal identification, comparing different types of samples originated from a skin swab or from a fingerprint swab on a glass slide, in order to understand how informative each of these analyses could be for forensic contexts, especially, as in the case of the present study (see Supplementary Materials for "Touch DNA" results), when the analysis of classical STR polymorphisms fails. Furthermore, we wanted to evaluate the "touch microbiome" transferability and survival on a surface and identify any existing DCTs transferred to the glass surface that may have forensic relevance for identification purposes.

Significantly higher concentrations of genetic material were detected in the extracts obtained using the kit "Chargeswitch® Forensic DNA" (Supplementary Table S4), specifically designed for extracting human DNA, in comparison with the "QIAamp PowerFecal Pro" kit used to extract the microbial DNA. Results showed that the "Chargeswitch® Forensic DNA" kit has been more effective than the "QIAamp PowerFecal Pro" both on skin and on glass slides swabs, probably due to the fact that the former has been specifically developed to extract small amounts of DNA from different forensic samples with very high-performance levels, and particularly also for extracting DNA from swabs, while the latter has been created to extract microbial DNA from different sample types, such as faeces, that are notably richer in DNA than a fingerprint. The strong capability of the latter to remove PCR inhibitors can also have conducted to some "subtraction" of DNA material, overall resulting in decreased concentrations of extracted DNA in comparison with the Chargeswitch® Forensic DNA kit.

Due to the fact that in forensic caseworks it is not infrequent to run across samples containing degraded DNA that partially or totally fail the classical STR typing, such as in the case of this study, it appears clear that skin's microbiome

analysis could integrate human DNA typing eventually providing information regarding someone's identity that can be ultimately extremely beneficial for forensic applications.

Even though we obtained a lower DNA concentration from both skin and glass swabs when using the "QIAamp PowerFecal Pro" kit in comparison with the "Chargeswitch® Forensic DNA" kit, only two samples resulted in being not suitable for microbiome studies, whereas 20 resulted in being idoneous for subsequent analyses. The bacterial DNA concentration obtained from all the skin samples of the volunteers ranged from 2.3 to 6.9 ng/µL, a smaller fluctuation among individuals in comparison with results found for the human DNA extracted with the "Chargeswitch® Forensic DNA" kit from the same skin samples (6.6 to 28.4 ng/µL). Permanova tests (data not shown) did not highlight any significant differences among the bacteria communities of different donors characterised by similar times elapsed since the last washing of their hands, suggesting that this variable did not affect in a significant way our results.

It is interesting to notice that the donors that generated the best STR profiles on the glass slides, namely donors D, L and M (data not shown), generated touch microbiome profiles charaterised by a relatively high number of DCTs (16, 12 and 29 respectively, higher than the average of ASVs found among the nine donors of 12.55 ASVs), but that the donor that was characterised by the highest number of ASVs (individual B, 30) did not allow for the obtainment of a good "touch DNA" sample (less than seven loci successfully typed, data not shown). These results suggests that there could either be a connection among "good shedders" for "touch DNA" and "touch microbiome" samples, as showed by donors D, L and M, either that this correlation may not exist, as showed by donor B. Further analyses with a greater sample size may clarify which of these two hypothesis may be the most correct one.

Our results showed that samples extracted from a glass surface were characterised by a reduced number of ASVs in comparison with their skin swab counterparts, revealing that the transferred microbial fingerprint does not fully represent the cutaneous microbiome [65]. This can be due to the limited transfer and adhesion of specific bacterial species on the glass slide, or by differences in the persistence of the genetic material (human and bacterial) on a substrate different from the skin [65–67]. It should be also noted that different surfaces may generate different fingerprint microbial profiles, and further studies are required to better understand the influence of the type of substrate on these analyses. Degradation and trace loss, both for microbial and human DNA, can also be linked to the method of storage of the glass slides (at room temperature for 30 days), a condition chosen to simulate as much as possible the conditions in which forensic geneticists work, and may occur faster in the absence of continuous deposition from the host [65].

When looking at the profiles obtained from the skin and the glass surface belonging to the same donor, we identified the presence of some taxa not found in skin but identified in the glass swab counterparts [68, 69]. We assumed that these ASVs belonged to the “environmental” signature, and that could have been either present on the glass slides before the deposition of the fingerprints or could have reached the glass slides during the 30 days prior to swab samplings [50, 70]. For this reason, we excluded those ASVs from our dataset. We advise that a “blank” swab from the surface of interest should be taken at the time of the collection of the “touch microbiome” sample in order to exclude taxa not associated with the donor of the trace.

We identified remarkable differences among the bacterial composition of the skin and of traces left on the slide both in terms of presence and absence of specific taxa, and in terms of ASVs abundances. Despite the preliminary nature of this study requires more research to be conducted in order to allow the application of this methodology to real forensic contexts, this finding implies that comparisons between a microbial trace found on the crime scene and the skin microbiome of potential culprit should be performed in a way that allows the obtainment of a similar type of trace to what has been found on the scene (e.g., a fingerprint deposited on a glass slide). This has clear implications for the creation of a forensic microbiome reference database similar to the forensic DNA one, since different microbial signatures might be identified depending on the surface considered in the analysis. Additionally, the high variability of the microbial composition of human skin in time could compromise the forensic capability of connecting touched objects found on the crime scene to subjects who touched them if suspects are identified after a long period of time and if the comparison samples are not obtained soon after the occurring of the criminal event [36, 37, 41, 51].

Some orders were more represented on the the glass slides than on skin samples and the most evident example is the one regarding the family of Enterobacteriales. This is a large group of facultatively anaerobic Gram-negative bacteria [71] that is ubiquitary in several ecological niches. They have been found in soil, water and in association with different living organisms, including humans [71, 72]. Many members of this order have been found pathogenic bacteria for humans, other animals and plants [73]. After their transmission to inanimate environmental sources, they can become secondary reservoirs if they meet the needs of the deposited microorganisms and therefore allow them to survive and grow [74]. For these reasons, we believe that they have managed to survive better than other bacteria, causing a notable increase in glass samples in comparison with their skin counterparts. However, an "addition" effect with bacteria already present on the non-sterile slide may not be excluded; this hypothesis will be verified in future works, when we will test the surface of the material on which the fingerprint will be deposited.

When looking at the CM and SCM, that represents taxa not useful for identification purposes within this particular study, we identified taxa belonging to Firmicutes, Proteobacteria and Actinobacteria phyla (Supplementary Table S3). This is in line with a previous study where Firmicutes, Actinobacteria, Proteobacteria, and Bacteroidetes were reported to be the four main bacterial phyla present on hands [75]. Among them, we identified three taxa at species specific level: *Streptococcus agalactiae*, *Actinobacillus delphinicola* and *Anaerosinus glycerini*. *S. agalactiae* is commonly present in the gastrointestinal, rectal and uro-genital tract of about 30% of healthy individuals, both female and male [76]. *A. delphinicola* is a gram-negative bacterium isolated from various tissues (lungs, cervix, uterus, intestine) in cetaceans [77–79]. *A. glycerini* is a bacterium that has been isolated from freshwater mud and groundwater [80]. None of these specific species has been previously reported in other forensic studies, however they could have been found here as a result of a "contamination" of the skin microbione originated from the contact with water. In fact, these organisms use water as a vector of transmission into the homes, and that they are not confined only to bathrooms and kitchens but can also populate tap water systems and household appliances. Microorganisms are introduced into domestic appliances via water, air, dishware, food, hands and clothes; here, microorganisms do not just persist, but may spread further within the housing, survive wastewater treatments and return to the environment, potentially creating a microbial vicious circle [81, 82]. Additionally, the attempts to improve the energy-efficiency and environmental friendliness of household appliances has resulted, as a side effect, a greater inclination of these to microbial growth; as well as the use of less aggressive detergents and lower washing temperatures [81]. All together, this could explain and justify the presence of these "water associated" taxa on the skin microbiome samples.

Another aim of the study was the identification of DCTs transferred on the glass samples, and particularly of the unique ones per donor that may act as microbial signatures for personal identification. It is interesting to note that the number of detected DCTs (both common and unique among the donors) ranged between 3 and 30. Interestingly, we did not find an obvious relationship between the time elapsed since the last hand washing and the number of identified DCTs on the surface (time elapsed for both B and H <30 mins, however 30 DCTs found in B and only three found in H), nor with specific skin conditions (donor G has psoriasis and dyshidrosis but we found 10 DCTs, a value close to the mediane of the DCTs numbers in the study). This may suggest the existence of good and bad microbiome shedders, as they are already known to exist when dealing with "touch DNA" studies [83], and therefore inter-variability should be taken into consideration for future studies.

Within this specific study we were able to find several taxa that have been transferred to the glass slide and that are associated specifically and uniquely with a sample that we reported with an asterisk in Supplementary Table S3 and

Figure 2A-C for improved clarity (e.g., ASV 69fc8436 for B, 77a920bd for D, 0920dcf0 for F, 0e2d370f for G, a0da905b for L and 455219ee for M). We could not find unique taxa for A, H, I, however specific combinations of DCTs found in these samples may provide collectively a microbial signature for these samples as well. Future studies may include the pairwise comparisons of DCTs among two individuals, to clarify whether or not donor specific taxa or their combinations could be useful for forensic purposes in situations involving the presence of two suspects that left their microbial signature on a surface [70].

Among the uDCTs, we were able to identify some taxa at species level, and in particular we found *Corynebacterium aurimucosum, Prevotella intermedia* and *Neisseria oralis* in individual "D"*, Pelomonas saccharophila* in individual "F"*, Anaerosinus glycerini, Pseudoalteromonas ruthenica* and *Macrococcus brunensis* in individual "L" and *Abiotrophia defectiva, Corynebacterium renale, Cytophaga xylanolytica* and *Prevotella nanceiensis* in individual "M"*. Corynebacterium aurimucosum* is a Gram-positive bacterium isolated from the female urogenital tract and considered to be non-patogenic. *Prevotella intermedia* is a Gram-negative anaerobic pathogen associated with peridontal infections. It has also been isolated from women with bacterial vaginosis. *Neisseria oralis* is a Gram-negative bacteria that has been isolated from healthy gingival plaques [84]. *Pelomonas saccharophila* is a Gram-negative soil bacterium that has been found on the human skin [85] and in the meconium, in the amniotic fluid, in vaginal fluid, in faeces and in saliva samples [86]. *Anaerosinus glycerini* is a Gram-negative anaerobic bacterium that ferments glycerol to propionate that has been isolated from freshwater mud. To date it has not yet been reported in any skin microbiome study. This bacterium also belongs to the core microbiome of the donors considered in this study, as reported previosuly, however this specific ASV is different from the one found in the CM and for this reason it has been identified as a uDCT for "L". Clearly this ASV cannot be considered as a useful biomerker for identification purposes, due to its genetic similarity with an ASV shared among all donors. *Pseudoalteromonas ruthenica* is a bacterium that has been isolated from marine invertebrates [87], and also in this case there are still no studies that have reported its presence on human skin. *Macrococcus brunensis* is a Gram-positive bacterium that has been isolated for the first time from llama skin [88]; other *Macrococcus* species have been found in human clinical material [89] and it has been shown they have virulence potential, but *Macrococcus brunensis* has not yet been reported in human samples. *Abiotrophia defectiva* is a Gram-positive virulent bacterium that can cause bacteremia and infective endocarditis and that is normally found in the human flora (such as in the oropharyngeal, in the gastrointestinal and in the urogenital tracts) [90]. *Corynebacterium renale* is another Gram-positive pathogenic bacterium responsible for genitourinary infections in animals. Despite the presence of other *Corynebacterium* spp. known to be human pathogens, there have been no

reports for *Corynebacterium renale* in humans [91]. *Cytophaga xylanolytica* is a Gram-negative bacterium found in freshwater environments [92] that has not been found either in studies on human tissues. *Prevotella nanceiensis* is a Gram-negative bacterium that belongs to the human oral, urogenital and gastrointestinal flora that is also involved in various infections [93]. Due to the identification of some uDCTs that have not been reported previously in human studies, such as *Pseudoalteromonas ruthenica, Macrococcus brunensis, Corynebacterium renale* and *Cytophaga xylanolytica,* it appears evident their potential usefulness in forensic caseworks, as they could act as biomarkers able to identify the responsible for a criminal act when multiple suspects have been identified and when their skin microbiome has been sampled. The main drawback of this technique is related to the scarce knowledge on the survivability, persistance and stability of these uDCTs on the skin, and on the intrinsic and extrinsic variables able to affect it. Although more research is therefore needed before this methodology could be used routinely in Court, this study shows that there are great potentials for metagenomic studies to provide biomarkers for personal identification.

The identification of DCTs and their analysis together with the collected metadata may be extremely useful for future applications. In fact, specific DCT or combinations of those could be correlated with particular life habits / health conditions (e.g., smokers vs non-smokers, type of diet, presence of disease etc.) and therefore reveal additional intelligence information that could be fundamental to guide the investigations. In this pilot study we only evaluated statistically the effect that the time elapsed since the last washing of hands had on the list of identified ASVs without finding significant results, however future analyses with greater datasers may involve the testing of other metadata and their correlation with the obtained metagenome to evaluate the predictive power that a specific ASV or a combination of multiple ones may have to infer specific features (e.g. diseases, drug intake, etc.) of the dataset.

For this particular study we selected volunteers who did not take antibiotics and/or antifungals in the 15 days before sampling because it has been observed that topical and antibiotic treatment induces skin microbiota changes [94–96] and fungal species have been shown to modulate expression of host molecules involved in changes in epithelial physiology therefore antifungal treatment also could change skin microbiota composition [97, 98]. Despite this choice may be seen as a potential limitation in the study, it is important to highlight that particular bacterial compositions/alterations may be related to specific antibiotic treatments that could provide investigative leads that could help in looking for suspects (e.g., knowing that the individual that left the trace on the scene recently took a specific antibiotic that affected his/her microbiome), so future studies should evaluate the inclusion of subjects that took this medications to evaluate their effect on the skin microbiome and consequently on the DCTs deposited on the surfaces.

Even if this is a pilot study, we are aware of the intrinsic limitations of our work. First of all, we considered only a single time from deposition (30 days), but we are aware that it may be useful to conduct future analyses including more time points to evaluate the persistence of the deposited microbiome on different surfaces, starting from "time 0" samples, analyzed immediately after swabbing the palm of the hand and touching the slide, and sampling at selected time points until reaching the final desidered depositional time. In fact, despite in real contexts it will be impossible to sample a "time zero" deposition, because this will mean sampling a trace at the time in which the crime is committed, this information could provide interesting insights on the persistence and survaivability of the "touch microbiome" over the course of the time.

Another limitation of the study is not to have carried out a "blank sample" of the glass-slides for the evaluation of environmental microbial contaminants. Surely any future protocols for the use of the microbiome as evidence to be taken to a court of law will have to include sampling of the surfaces. It will also be necessary to investigate the persistence in time of fingerprint microbiomes on different types of surfaces with different porosities as well as at different environmental temperatures, to determine which taxa are more stable over time and on which surface/in which condition, since it is expected that the skin microbiome changes frequently over time and this could clearly limit the applications of this methodology to real forensic scenarios [99] .

Ultimately, we are aware of the relatively limited sample size of this study, however this work was intended to represent a stepping stone into the investigation of the survival and extractability of the touch microbiome from glass fingerprint samples, and not an analysis aimed at infering informations on the donors starting from the unique taxa deposited on the surface, for which a bigger sample size will indeed be needed. Increased magnitude of samplings may be used in the future to identify unique microbial features that could overall shine new lights on the use of metabarcoding analyses to assist the process of human identification. In fact, future studies should expand on the number of individuals enrolled, also aiming at analysing the microbial stability in the same individual over the course of time and in the presence of specific life habits / health conditions. Moreover, it should be targeted at involving, for example, volunteers from a wider geographical area, in order to maximise the difference among them and to increase the number of identified DCTs [100].

5. Conclusions

On the basis of the results obtained within this work, it has been possible to show the potential that the microbiome analysis can have in assisting forensic geneticists in performing personal identifications ~~or~~ but especially

investigators in obtaining useful information about the habits or health condition of a suspect. In future, the analysis of the microbiome could become an important tool to support the analysis of the classical DNA polymorphisms in forensic cases, in particular when other identification techniques cannot provide useful information in the same way as DNA Phenotyping and Biogeographic Ancestry [101]. However, further in-depth analyses are required to verify the actual applicability of the study of skin microbiome to human identification in real forensic settings. Despite we are aware of the limitations that this methodology, still in its infancy, can have, we believe that the integration of the microbiome analysis together with STR typing could be more informative than standard DNA analyses when only low template or degraded samples are available and when a complete human STR profile cannot be obtained [36, 59].

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