

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

2 3 **Abstract**

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

17

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

20

21 **1. Introduction**

22 Microbes are present all around us and nearly everywhere on Earth, building ubiquitous communities and interacting
23 together in a manner that is closely associated with the surrounding environmental conditions [1]. Microorganisms can
24 be found not only in external environments, but also within specific human districts constituting stable ecological niches
25 [2]. In fact, several studies on the human microbiota have shown a wide diversity of microbial communities both within
26 human hosts and in different body districts and/or biological fluids (e.g., saliva, gut, hair, skin) [3]. Human microbiota
27 represents the totality of microorganisms found within the human body [4]. It varies for each single individual and it is
28 influenced by several factors such as body site [5, 6], sex [5, 7], age [8, 9], geographical provenience of the person [10],

29 health condition [11, 12] and lifestyle (e.g., diet [13], alcohol consumption [14], physical activity [15], smoking habits
30 [16, 17], etc.).

31 Despite the great number of studies conducted on the human microbiota for clinical reasons due to their correlation
32 with health/disease states [18–20], they have been lately applied also to the forensic field due to their potential use as
33 auxiliary tool in crime cases [21]. Forensic microbiology is a relatively new science originated from the intersection
34 between microbiology and forensics, whose development is particularly linked to the recent advances in Next
35 Generation Sequencing (NGS) platforms that allow the obtainment of a large amount of data within a single analytical
36 run and the rapid and efficient analysis of whole microbial communities without the need of performing in vitro cultures
37 [21]. NGS reduces both the analytical costs and the time needed for the analysis in comparison to previously Sanger
38 sequencing [21, 22]. In addition, this technology allows the characterization not only of the whole genome of a given
39 microbe, but also the identification and quantification of the species belonging to whole microbial communities,
40 providing an overview of different taxa and bacterial strains associated with a specific sample [23]. From the first
41 applications of forensic microbiology to bioterrorism and bio-crime associated topics [24, 25], the analysis of the
42 microbiome is now an important tool in the forensic field [26, 27] since and it could help to define post-mortem interval
43 (PMI) [28–30], cause of death [31, 32], place of death [33] and personal identification [34–38].

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

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

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

70 In this context, analysis on “touch microbiome” could be more informative and can integrate “touch DNA” fingerprint
71 analysis when only partial prints and degraded samples are available, therefore when a full human STR profile cannot
72 be obtained [59]. Due to the potential that “touch microbiome” analyses can have in discriminating different individuals
73 [36], also when other identification techniques fail, with this pilot study we wanted to evaluate the possibility to use
74 microbiome analyses as a potential tool in forensic investigation for personal identification purposes, exploring the
75 transferability of the “touch microbiome” on a hard surface and its survival 30 days post-deposition, and evaluating the
76 presence of core microbiomes and specific donor characterizing taxa that may ultimately be good indicators for
77 achieving personal identifications.

78

79 **2. Materials and Methods**

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

84 *2.1 Sampling*

85 Eleven volunteers of both sexes (five males and six females) of different ages, ranging from 20 to 70 years, were enrolled
86 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 “NUOomics 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

116 Inc., San Diego, CA, USA), following the method used by Brabin et al [60]. Briefly, forward (GTGCCAGCMGCCGCGGTAA)
117 and reverse (GGACTACHVGGGTWTCTAAT) primers were used following the standard operating procedures for 16S
118 metagenomic sequencing library preparation for the Illumina MiSeq system[61]. Amplifications were conducted on 96-
119 wells plates, and each plate contained both a positive and a negative control. PCR was run using 1x Accuprime Pfx
120 Supermix, 0.5 μ M of each primer and 1 μ L template DNA under the following conditions: 95°C 2 minutes, 30 cycles 95°C
121 20s, 55°C 15s, 72°C 5 minutes with a final extension 72°C 10 minutes. Barcodes were incorporated into the PCR primer
122 construct. PCR products were normalised using SequalPrep™ Normalization kit (Invitrogen, United Kingdom) following
123 manufacturer's protocol and combined into four pools. These four pools were then quantified using fragment size
124 determined by BioAnalyzer (Agilent Technologies) and concentration by Kappa qPCR (Kappa Biosystems) [61], and were
125 combined in equimolar amounts to create a single library further normalised. The library was then denatured using 0.2N
126 NaOH for 5 minutes followed by 2-minute incubation at 96°C. The library was diluted to a final concentration of 3.5 pM
127 and supplemented with 5% PhiX and loaded onto a MiSeq V2 2x250 cartridge. Paired-end reads from each sample were
128 sequenced with forward and reverse reads in separate files by the NUOmicS DNA Sequencing Research Facility, and
129 processed by means of the microbiome bioinformatics platform QIIME2 (Quantitative Insights Into Microbial Ecology
130 2), version 2019.7 [62, 63]. Denoising and quality control, including removal of chimeras, were achieved by means of
131 the DADA2 [63] plugin (qiime dada2 denoise-paired) and to avoid low quality sequences reads were truncated (250 bp
132 for forward, 240 bp for reverse reads). The classifier adopted for the taxonomic assignment was the QIIME release
133 Greengenes (16S rRNA) v.13.8). The short and long md5 hashes for all the ASVs are provided in Supplementary Table
134 S2. We decided to use short md5 hashes in the text and in the figures for ease of reading.

135 2.3 Statistical Analysis

136 Statistical analyses were performed within the computing environment R (<https://www.R-project.org/>). All the taxon
137 abundances have been calculated and graphically plotted with the aid of the R package PHYLOSEQ V.1.22.3 [64].
138 Rarefaction curves have been rendered by means of the function ggrare, provided by the richness.R script from the
139 phyloseq extension package by Mahendra Mariadassou (<https://github.com/mahendra-mariadassou/phyloseq-extended>).
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141

142 3. Results

143 3.1. Quantitation

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

146 **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

147

148

149 3.2 Microbiome Analysis

150 Microbiome sequencing effort has provided 429 to 82,870 reads for skin swab samples, and 49 to 25,781 for glass
151 fingerprint swab samples. A first sequence quality survey revealed that the samples C-glass fingerprint and E-skin were
152 characterized by a very low read counts (499 and 429 sequences obtained respectively for these two samples) and
153 therefore they have been eliminated in the subsequent analyses. The final dataset was composed by nine samples (A,
154 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
155 total of 342,124 raw sequences) were retained and clustered into 586 amplicon sequence variants (ASVs).

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

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

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

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

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

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

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

194 (donor B). The most occurring phylum was Proteobacteria (56), followed by Actinobacteria (36), Firmicutes (35) and
195 Bacteroidetes (13), while at order level, Gammaproteobacteria were the most occurring (38), followed by Actinobacteria
196 (36) and Bacilli (26). At genus level, among the identified ASVs, Actinobacillus, Pseudomonas, Streptococcus were the
197 most common with six counts each. In three cases (Donor D, G, M), it was possible to find all six ASVs belonging to the
198 SCM. Among the DCTs transferred to the glass slides, we were additionally able to identify unique taxa (uDCT) belonging
199 specifically to one individual and not being found in any other donor (represented with an asterisk in Supplementary
200 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
201 sample B and 14 in sample M.

202

203 **Discussion**

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

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

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

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

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

233 It is interesting to notice that the donors that generated the best STR profiles on the glass slides, namely donors
234 D, L and M (data not shown), generated touch microbiome profiles charaterised by a relatively high number of DCTs
235 (16, 12 and 29 respectively, higher than the average of ASVs found among the nine donors of 12.55 ASVs), but that the
236 donor that was characterised by the highest number of ASVs (individual B, 30) did not allow for the obtainment of a
237 good "touch DNA" sample (less than seven loci successfully typed, data not shown). These results suggests that there
238 could either be a connection among "good shedders" for "touch DNA" and "touch microbiome" samples, as showed by
239 donors D, L and M, either that this correlation may not exist, as showed by donor B. Further analyses with a greater
240 sample size may clarify which of these two hypothesis may be the most correct one.

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

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

258 We identified remarkable differences among the bacterial composition of the skin and of traces left on the slide
259 both in terms of presence and absence of specific taxa, and in terms of ASVs abundances. Despite the preliminary
260 nature of this study requires more research to be conducted in order to allow the application of this methodology to
261 real forensic contexts, this finding implies that comparisons between a microbial trace found on the crime scene and
262 the skin microbiome of potential culprit should be performed in a way that allows the obtainment of a similar type of
263 trace to what has been found on the scene (e.g., a fingerprint deposited on a glass slide). This has clear implications for
264 the creation of a forensic microbiome reference database similar to the forensic DNA one, since different microbial
265 signatures might be identified depending on the surface considered in the analysis. Additionally, the high variability of
266 the microbial composition of human skin in time could compromise the forensic capability of connecting touched
267 objects found on the crime scene to subjects who touched them if suspects are identified after a long period of time
268 and if the comparison samples are not obtained soon after the occurring of the criminal event [36, 37, 41, 51].
269 Some orders were more represented on the the glass slides than on skin samples and the most evident example is the
270 one regarding the family of Enterobacteriales. This is a large group of facultatively anaerobic Gram-negative bacteria
271 [71] that is ubiquitous in several ecological niches. They have been found in soil, water and in association with different
272 living organisms, including humans [71, 72]. Many members of this order have been found pathogenic bacteria for
273 humans, other animals and plants [73]. After their transmission to inanimate environmental sources, they can become
274 secondary reservoirs if they meet the needs of the deposited microorganisms and therefore allow them to survive and
275 grow [74]. For these reasons, we believe that they have managed to survive better than other bacteria, causing a
276 notable increase in glass samples in comparison with their skin counterparts. However, an "addition" effect with
277 bacteria already present on the non-sterile slide may not be excluded; this hypothesis will be verified in future works,
278 when we will test the surface of the material on which the fingerprint will be deposited.

279 When looking at the CM and SCM, that represents taxa not useful for identification purposes within this particular
280 study, we identified taxa belonging to Firmicutes, Proteobacteria and Actinobacteria phyla (Supplementary Table S3).
281 This is in line with a previous study where Firmicutes, Actinobacteria, Proteobacteria, and Bacteroidetes were reported
282 to be the four main bacterial phyla present on hands [75]. Among them, we identified three taxa at species specific
283 level: *Streptococcus agalactiae*, *Actinobacillus delphinicola* and *Anaerosinus glycerini*. *S. agalactiae* is commonly
284 present in the gastrointestinal, rectal and uro-genital tract of about 30% of healthy individuals, both female and male
285 [76]. *A. delphinicola* is a gram-negative bacterium isolated from various tissues (lungs, cervix, uterus, intestine) in
286 cetaceans [77–79]. *A. glycerini* is a bacterium that has been isolated from freshwater mud and groundwater [80]. None
287 of these specific species has been previously reported in other forensic studies, however they could have been found
288 here as a result of a “contamination” of the skin microbiome originated from the contact with water. In fact, these
289 organisms use water as a vector of transmission into the homes, and that they are not confined only to bathrooms and
290 kitchens but can also populate tap water systems and household appliances. Microorganisms are introduced into
291 domestic appliances via water, air, dishware, food, hands and clothes; here, microorganisms do not just persist, but
292 may spread further within the housing, survive wastewater treatments and return to the environment, potentially
293 creating a microbial vicious circle [81, 82]. Additionally, the attempts to improve the energy-efficiency and
294 environmental friendliness of household appliances has resulted, as a side effect, a greater inclination of these to
295 microbial growth; as well as the use of less aggressive detergents and lower washing temperatures [81]. All together,
296 this could explain and justify the presence of these “water associated” taxa on the skin microbiome samples.

297 Another aim of the study was the identification of DCTs transferred on the glass samples, and particularly of the
298 unique ones per donor that may act as microbial signatures for personal identification. It is interesting to note that the
299 number of detected DCTs (both common and unique among the donors) ranged between 3 and 30. Interestingly, we
300 did not find an obvious relationship between the time elapsed since the last hand washing and the number of identified
301 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),
302 nor with specific skin conditions (donor G has psoriasis and dyshidrosis but we found 10 DCTs, a value close to the
303 median of the DCTs numbers in the study). This may suggest the existence of good and bad microbiome shedders, as
304 they are already known to exist when dealing with “touch DNA” studies [83], and therefore inter-variability should be
305 taken into consideration for future studies.

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

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

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

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

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

356 For this particular study we selected volunteers who did not take antibiotics and/or antifungals in the 15 days
357 before sampling because it has been observed that topical and antibiotic treatment induces skin microbiota changes
358 [94–96] and fungal species have been shown to modulate expression of host molecules involved in changes in epithelial
359 physiology therefore antifungal treatment also could change skin microbiota composition [97, 98]. Despite this choice
360 may be seen as a potential limitation in the study, it is important to highlight that particular bacterial
361 compositions/alterations may be related to specific antibiotic treatments that could provide investigative leads that
362 could help in looking for suspects (e.g., knowing that the individual that left the trace on the scene recently took a
363 specific antibiotic that affected his/her microbiome), so future studies should evaluate the inclusion of subjects that
364 took this medications to evaluate their effect on the skin microbiome and consequently on the DCTs deposited on the
365 surfaces.

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

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

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

391

392 **5. Conclusions**

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

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

403

404 **References**

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