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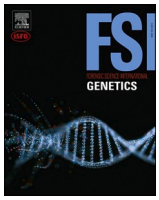

The Oral Microbiome for Geographic Origin: An Italian Study

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ABSTRACT

The human oral microbiome has primarily been studied in clinical settings and for medical purposes. More recently, oral microbial research has been incorporated into other areas of study. In forensics, research has aimed to exploit the variation in composition of the oral microbiome to answer forensic relevant topics, such as human identification and geographical provenience. Several studies have focused on the use of microbiome for continental, national, or ethnic origin evaluations. However, it is not clear how the microbiome varies between similar ethnic populations across different regions in a country. We report here a comparison of the oral microbiomes of individuals living in two regions of Italy – Lombardy and Piedmont. Oral samples were obtained by swabbing the donors' oral mucosa, and the V4 region of the 16S rRNA gene was sequenced from the extracted microbial DNA. Additionally, we compared the oral and the skin microbiome from a subset of these individuals, to provide an understanding of which anatomical region may provide more robust results that can be useful for forensic human identification.

Initial analysis of the oral microbiota revealed the presence of a core oral microbiome, consisting of nine taxa shared across all oral samples, as well as unique donor characterising taxa in 31 out of 50 samples. We also identified a trend between the abundance of Proteobacteria and Bacteroidota and the smoking habits, and of Spirochaetota and Synergistota and the age of the enrolled participants. Whilst no significant differences were observed in the oral microbial diversity of individuals from Lombardy or Piedmont, we identified two bacterial families – Corynebacteriaceae and Actinomycetaceae – that showed abundance trends between the two regions. Comparative analysis of the skin and oral microbiota showed significant differences in the alpha ($p = 0.0011$) and beta ($Pr(>F) = 9.999e-05$) diversities. Analysis of skin and oral samples from the same donor further revealed that the skin microbiome contained more unique donor characterising taxa than the oral one.

Overall, this study demonstrates that whilst the oral microbiome of individuals from the same country and of similar ethnicity are largely similar, there may be donor characterising taxa that might be useful for identification purposes. Furthermore, the bacterial signatures associated with certain lifestyles could provide useful information for investigative purposes. Finally, additional studies are required, the skin microbiome may be a better discriminant for human identification than the oral one.

1. Introduction

The human oral microbiome refers to the total genetic material of all microorganisms in various areas of the oral cavity, including the hard and soft palates, tongue, oral mucosa, and teeth [1]. The oral cavity contains one of the largest and most diverse cohorts of microbial

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communities across the human body [2,3]. Different studies have demonstrated the importance of the oral microbiome in determining and understanding human health and disease states [4,5]. Reduced microbial diversity and increased dominance of certain taxa in saliva or other oral micro-niches has been associated with a host of diseases including chronic obstructive pulmonary disease [6], chronic recurrent multifocal osteomyelitis [7], rheumatoid arthritis [8], cancers [9–11] and even obesity [12]. Similarly, oral dysbiosis – which is the disruption of the equilibrium of oral microbiota [13] – has been noted for diseases localised within the oral cavity, including periodontitis [14,15] and caries [16,17]. Thus, the observation of oral dysbiosis is often studied as a microbial marker for declining oral health and disease [18–20] as there is a consensus concerning the critical role of the oral microbiome in oral health or disease states [21]. Consequently, more efforts continue to be directed towards understanding the oral microbiota, its composition and clinical relevance for health, disease, and medical interventions.

In forensics, studies on the human microbiome are focused on providing alternative methods to address questions or issues related to post-mortem interval (PMI) estimation [22–24], body fluid identification [25–27], sexual assault detection [28], time since deposition of a stain [29], geolocation [30–32] and human identification [33–35]. The Human Microbiome Project [36], and subsequent studies [3,37,38], have identified within the oral microbiome, a core microbiome – which can be found across unrelated individuals – and a variable microbiome that differs between individuals. This diversity in the oral microbial composition is influenced by various factors such as host genetics [39], sex [40,41], biogeographical provenience [42,43] and lifestyle activity such as diet [41], antibiotic-use [44], alcohol intake [45] and smoking habits [46], and documented them as contributors to microbiome variability seen across unrelated individuals, and even among monozygotic twins [47,48]. Other studies have utilised this variability conferred by environmental or genetic factors to demonstrate the potential of the oral microbiome to predict age [49] and parental relationships [50]. Thus, the observation of differences in the oral microbiome among individuals could be a potential tool for forensic investigative purposes.

Studies leveraging on the microbiome variation amongst people present the opportunity to address crucial lines of inquiry on human identification for forensic applications. This is especially relevant in crime investigations involving low quantity or quality human DNA, where it may be crucial to have alternatives that rely on other DNA sources. However, in understanding microbial variation from person-to-person, it is equally important to characterise variation across populations as such information may lend insights on 'group-identity'. Several biogeographical provenance studies [42,51–55] utilising various tissues have been conducted to assess microbiome variability across continents, countries and ethnicities and have shown that the geographic origin of the study participants significantly drives the differences observed in the microbiome. These findings, therefore, present compelling prospects for the integration of the microbiome for biogeographic inference and for forensic human identification purposes. Studies involving participants from countries such as the U.S.A. and Australia [54–56] have attempted to characterise the microbiome of Americans, giving useful insights on the oral microbial diversity across distinct ethnicities and highlighting their variability.

A few studies have focused on the differences in the oral microbiome of individuals from various regions of the same country [57,58], although their focus has largely been on characterising microbial diversity regarding ethnicity or urbanisation and have also not aimed at forensic applications.

In order to incorporate microbiome analysis for the determination of geographical origin in forensics, it is necessary to understand oral microbial diversity among individuals with similar ethnic origins, albeit from different regions within the same country. In this study we report, for the first time to our knowledge, the composition and distribution of the oral microbiome of individuals from two regions with similar urbanisation levels – Lombardy and Piedmont – in Italy. We also attempt to evaluate the influence of certain lifestyle factors on the oral microbiota and discover if distinct microbial communities exist between these two regions in Italy.

Additionally, in exploring the oral microbiome as a potential tool in the forensic analytical 'toolkit', it is important to recognize how it may differ in comparison to a different microbial habitat on the body. Skin microbiomes have famously been investigated for its individualising potential [33,59–61] as skin microbiota are easily and constantly shed onto surfaces and persist for considerable periods. Such studies have reported up to 93 % [59] accuracy in human identification. With such results, this study aimed to discover which microbial habitat – skin or oral – may be more appropriate in providing discriminating information which could be useful for human forensic identification, by comparing oral and skin microbiomes sampled from the same individuals.

2. Materials and methods

2.1. Demographic data

This study was approved by the Northumbria University Ethics Committee, United Kingdom (submission ref. 29218) and by the "Comitato Etico Interaziendale Novara", Italy (submission ref. CE 57/20). Informed consent was requested and obtained from all the participants enrolled.

Fifty healthy individuals (25 males and 25 females), living in different areas of the North of Italy and aged between 20 and 70 years, were included in this study. The donors completed a questionnaire that assessed their lifestyle factors (smoking, alcohol-use, hygiene, transport-mode, occupation, sport activity, travel) and health (BMI, health status, medications, in particular antibiotic-use) that could affect the composition of the microbiome.

2.2. Sampling

Oral samples were obtained by rubbing a sterile swab around the oral mucosa (left and right cheeks) for 15 s in each area, as per standard sampling approaches in forensics (e.g., for human DNA collection). Skin swabs were taken from a subset (11) of the same 50 individuals by sliding two sterile swabs moistened with physiological water over palms and fingers of the dominant hand for 15 s as explained in Procopio et al. [61]. These skin samples were incorporated into this study as they had already been used in a previous study [61]. Both skin and oral swabs were then frozen at −20 °C and kept stored until further processing.

2.3. Microbiome extraction and analysis

Microbial DNA was extracted from all samples using the QIAamp PowerFecal Pro DNA Kit (QIAGEN, Hilden, Germany), according to the protocol detailed in Procopio et al. [61]. Samples were quantified and checked for purity with NanoDrop One Microvolume UV-Vis Spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA), prior to metabarcoding analysis.

Bacterial profiling of the V4 region of the 16S rRNA gene was carried out by NU-OMICS (Northumbria University, Newcastle, UK). Briefly, PCR was carried out using 1x Accuprime Pfx Supermix, 0.5 μM each primer and 1 μL of template DNA under the following conditions 95 °C 2 min, 30 cycles 95 °C 20 s, 55 °C 15 s, 72 °C 5 min with a final extension 72 °C 10 min. One positive and one negative control sample were included in each 96 well plate and carried through to sequencing.

PCR products were normalised using SequalPrep™ Normalization kit (Invitrogen) as described in the manufacturer's instructions and combined into four pools. Each pool was quantified using fragment size determined by BioAnalyzer (Agilent Technologies) and concentration by Qubit (Invitrogen). Pools were combined in equimolar amounts to create

Table 1
Demographic information of 50 study participants including lifestyle or habit information collected, and the number of participants associated with each factor and level.

Demographic	**Number of Participants**									
Factor Level	Age	Birth Region	Residence Region	Smoking	Alcohol use	Sport	Drugs – Medical use	Antibiotic use (3 months)	Medical Condition	Transport mode (to Work)
Lombardy	-	20	22	-	-	-	-	-	-	-
Piedmont	-	22	26	-	-	-	-	-	-	-
*Puglia**	-	2	-	-	-	-	-	-	-	-
*Umbria**	-	2	-	-	-	-	-	-	-	-
*Campania**	-	1	-	-	-	-	-	-	-	-
*Veneto**	-	3	2	-	-	-	-	-	-	-
20–30 (years)	10	-	-	-	-	-	-	-	-	-
31–40 (years)	10	-	-	-	-	-	-	-	-	-
41–50 (years)	10	-	-	-	-	-	-	-	-	-
51–60 (years)	11	-	-	-	-	-	-	-	-	-
61–70 (years)	9	-	-	-	-	-	-	-	-	-
Yes	-	-	-	10	31	24	19	6	20	-
No	-	-	-	25	19	26	31	44	30	-
Past (Former)	-	-	-	15	-	-	-	-	-	-
Private Vehicle	-	-	-	-	-	-	-	-	-	34
Public Transport	-	-	-	-	-	-	-	-	-	8
Foot or Bike	-	-	-	-	-	-	-	-	-	8
TOTAL	**50**	**50**	**50**	**50**	**50**	**50**	**50**	**50**	**50**	**50**

* These regions were excluded from alpha and beta diversity analysis due to under sampling of the areas.

a single library then denatured using 0.2 N NaOH for 5 min and diluted to a final concentration of 5 pM, supplemented with 15 % PhiX and loaded onto a MiSeq V2 500 cycle cartridge (Illumina Inc., San Diego, CA, USA). Targeting and sequencing of the V4 region of the 16S rRNA gene for bacterial identification were done following the gold standards suggested by the Human Microbiome Project [36].

2.4. Bioinformatic and statistical analysis

Paired-end reads from NGS were demultiplexed and analyzed using QIIME2 (version 2021.11). Denoising, as a quality control measure for filtering and to remove chimeric sequences, was achieved using DADA2. Reads were truncated at 240 bp forward and 194 bp reverse reads to avoid low quality sequences. The taxonomic classifier adopted was the QIIME2 release SILVA-138–99 [60,61] database. On plotting a rarefaction curve (Supplementary Fig. S1), one skin sample, SG16B, was excluded from further downstream analysis due to low DNA quantity and/or low sequencing quality. ASV counts were standardized to the median sequencing depth to control for differences in sequencing depth across samples. This was achieved in R using PHYLOSEQ package (ver. 1.40.0) and pre-processing code written by the package's developer and reported here:[2]

total = median(sample_sums(physeq))

*standf = function(x, t=total) round(t * (x / sum(x)))*

ps = transform_sample_counts(physeq, standf)

To evaluate statistical difference between groups, statistical analyses were run in R (https://www.R-project.org/) ver. 4.2.2. Taxon abundances, alpha and beta diversity indices were calculated with PHYLOSEQ package (ver. 1.40.0) and all PERMANOVA was run with vegan (ver. 2.6–4), to test for differences between groups. Seed was set to 22 for all PERMANOVA analysis to ensure reproducibility of results. Differential abundance testing to identify significantly different genera between groups was calculated using the DESeq2 package, (ver. 1.36.0). Taxa were filtered to an abundance (3 times) and a prevalence in at least 50 % of the samples before differential abundance testing.

[2] https://joey711.github.io/phyloseq/preprocess.html#preprocessing

Table 2
Number of participants in relation to frequency of habits – smoking, drinking and exercise.

	Number of Participants		
Frequency	*Light*	*Normal*	*Heavy*
Smoking (Cigarettes per day)	*3*	*6*	*1*
Drinking days (per week)	*23*	*3*	*7*
Sport (per week)	*7*	*14*	*2*

KEY *Smoking (per day)* - Light: < 5; Normal: 5–10; Heavy: > 10 *cigarettes per day.*
Drinking days (per week) - Light: < 3; Normal: 3–5; Heavy: > 5 days.
Sport (per week) - Light: Once a week; Normal: 2–3 times; Heavy: > 3 times a week.

3. Results

3.1. Oral microbiome analysis

6847,595 reads were obtained from all samples – skin and oral – ranging from a minimum sample read of 2968 and a maximum of 399,411 after sequencing. Sequencing also produced an average sample read of 114,005.5. The oral microbiome of the participants was assigned into 20 phyla. Of these, *Firmicutes* (45.8 %), *Proteobacteria* (35.4 %), *Bacteroidota* (7.8 %), *Actinobacteriota* (6.6 %) and *Fusobacteriota* (3 %) were predominant, and every other phylum had a relative abundance of less than 1 %. A total of 775 Amplicon Sequence Variant (ASVs) remained after filtering mitochondria and chloroplasts from the feature table.

Nine ASVs were constantly found in all 50 samples and belonged to taxa *Firmicutes* (genus, *Gemella*; genus, *Streptococcus*; genus, *Veillonella;* genus, *Granulicatella*), *Proteobacteria* (family, *Pasteurellaceae;* genus *Neisseria*), *Actinobacteriota* (genus, *Rothia*; genus, *Actinomyces*) and *Fusobacteriota* (genus, *Fusobacterium*).

The analysis of the microbiome phyla associated with metadata collected in Table 1 showed specific trends connecting the abundance of certain bacteria to specific factors, particularly smoking habits and age of the participants. *Proteobacteria* abundance was the highest among participants who had never smoked and the lowest in current smokers, and vice-versa for the *Bacteroidota* phylum **(**Fig. 1**A)**. Regarding the chronological age, *Spirochaetota* and *Synergistota* abundance increased with increasing age of participants and declined after 41–50 years

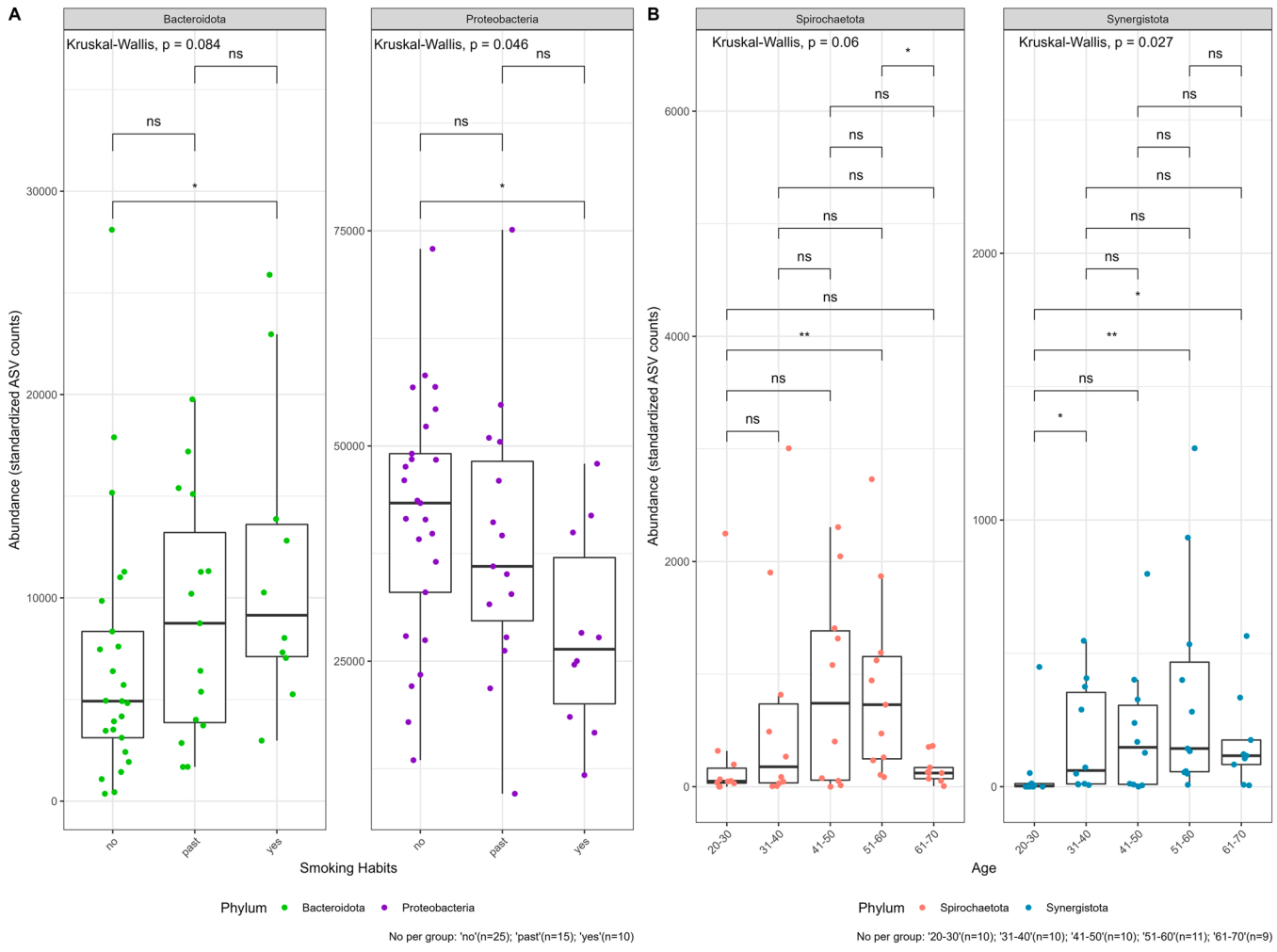

Fig. 1. Bacterial abundance (standardised ASV counts) of different phyla related to **(A)** smoking habits and **(B)** chronological age.

(Fig. 1**B).**

Alpha diversity metrics obtained by measuring the Shannon diversity, showed no statistically significant differences between the microbial community evenness of participants born and residing in the two regions of Lombardy or Piedmont, Italy **(**Fig. 2**A).** Individuals from the other regions (as per Table 1) were excluded from further quantitative analyses due to their limited sample size. However, an abundance analysis – standardised ASV counts – of all samples is given in (Supplementary Fig. S2). Permanova analysis on the PCoA of unweighted Unifrac distance **(**Fig. 2**B)** resulted in a Pr(>F) value of 0.4733, showing that no significant differences in samples were observed between the two regions under consideration.

DESeq2 analysis at FDR 0.01 of differentially abundant taxa identified two taxa (Fig. 3**)** that were differentially abundant between individuals from the two regions. These taxa were represented in the phylum Actinobacteriota (family – Corynebacteriaceae, species – Corynebacterium_matruchotii and family – Actinomycetaceae, genus – F0322).

3.2. *Microbial habitat comparison: skin and oral*

Here, we compared skin and oral microbiome from 10 of the participants – excluding the participant whose skin sample had low read count, SG16B – from which both types of samples (buccal and skin swab) were collected. *Firmicutes* and *Proteobacteria* were the dominant phyla across oral and skin samples, and together represented 80.2 % of the total bacterial population. Alpha diversity, using Shannon index values, showed significant differences ($p = 0.0011$, Pairwise Wilcoxon Rank Sum Test) among the two sites **(**Fig. 4**).**

Skin samples displayed higher species richness and diversity than their oral counterparts. PERMANOVA analysis using unweighted UniFrac distances further confirmed the distinction between oral (buccal swabs) and skin bacterial microbiomes, with a Pr(>F) value of 9.999e-05. The resulting PCoA plot showed a clear clustering of samples based on the tissue type **(**Fig. 4**).** Further analysis revealed 79 differentially abundant taxa (Supplementary Fig. S3) between the oral and skin microbiomes.

In addition, we investigated the existence of donor characterising taxa (DCT), i.e., taxa that are present in a participant's oral and/or skin microbiome and absent in those of other participants. No shared DCTs were found in both the oral and skin microbiome of any participant, however, we found some DCTs unique to either the participant's oral or skin microbiome (Table 3).

Results showed that there were more DCTs in the skin microbiome, with some participants having no DCTs in their oral microbiome. A full taxonomic characterization of the DCTs found are listed in Supplementary Table S1 and S2.

4. Discussion

The oral microbiome, albeit primarily saliva and oral wash samples, has been explored particularly in relation to oral dysbiosis and oral or systemic diseases. It also has been established that the oral microbiome has the potential to be used as a tool for human/personal identification

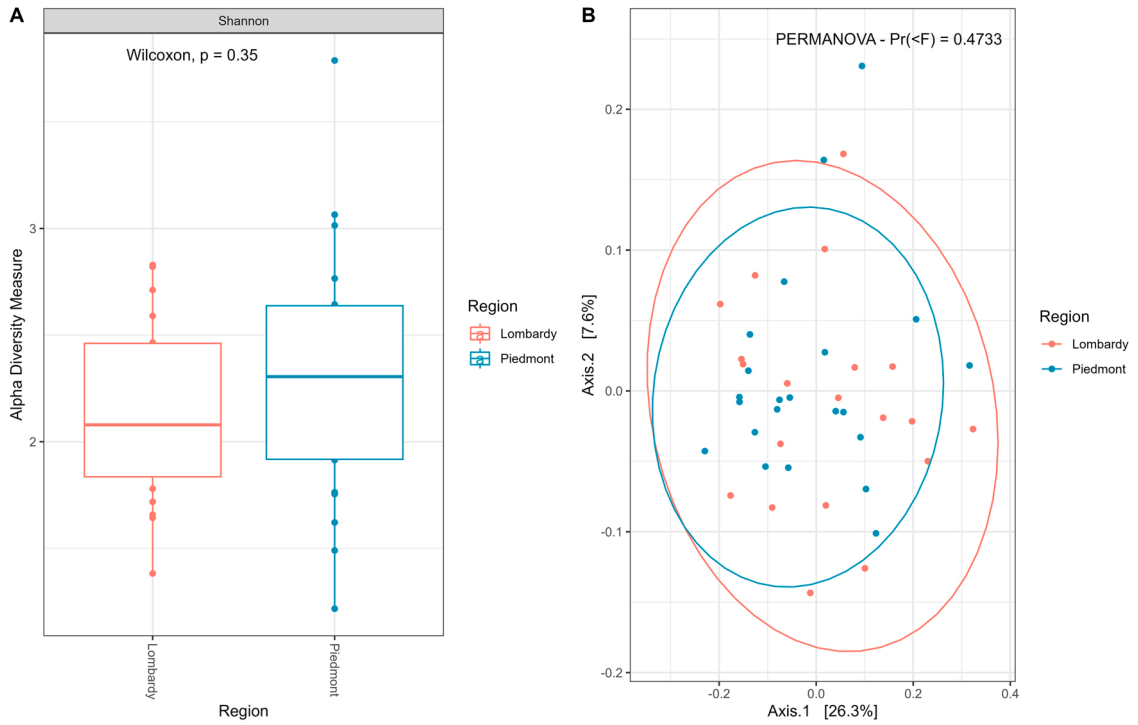

Fig. 2. Oral diversity metrics – alpha (Fig. 2**A**) and beta (Fig. 2**B**) reflecting the unweighted Unifrac distance – of taxa between samples from the regions of Lombardy and Piedmont.

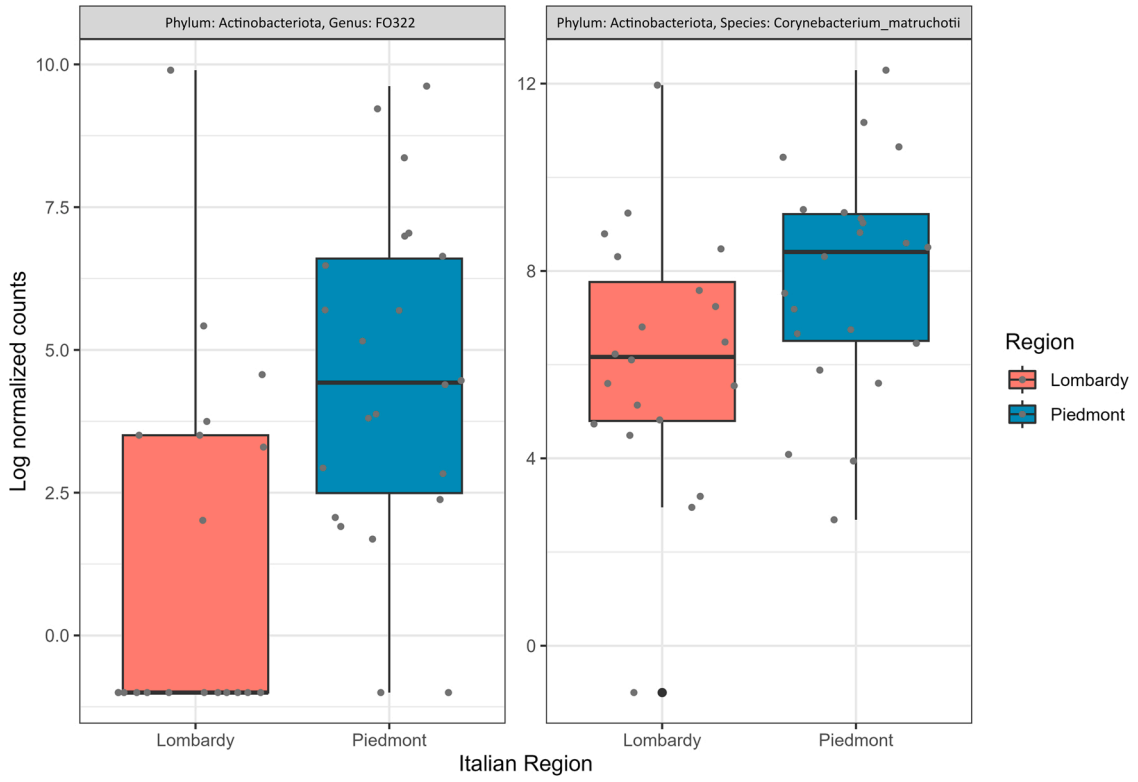

Fig. 3. Differential abundance testing with DESeq2 for significant taxa between Lombardy and Piedmont, Italy.

[35,47,62]. However, in assessing its use for individual-specific identification, it is important to characterise oral microbial variation within similar populations. Therefore, the aim of this study was to evaluate forensically relevant oral microbiome samples, hence buccal swabs, to evaluate oral microbiome diversity between regions in Italy with similar urbanisation, involving participants of the same ethnicity.

Similar studies describing oral microbiota of individuals across regions of the same country are largely designed to assess the impact of disease states [55,63] and extrinsic factors like cultural differences [64], urbanisation [58,65] and even altitude [66]. We investigated bacterial

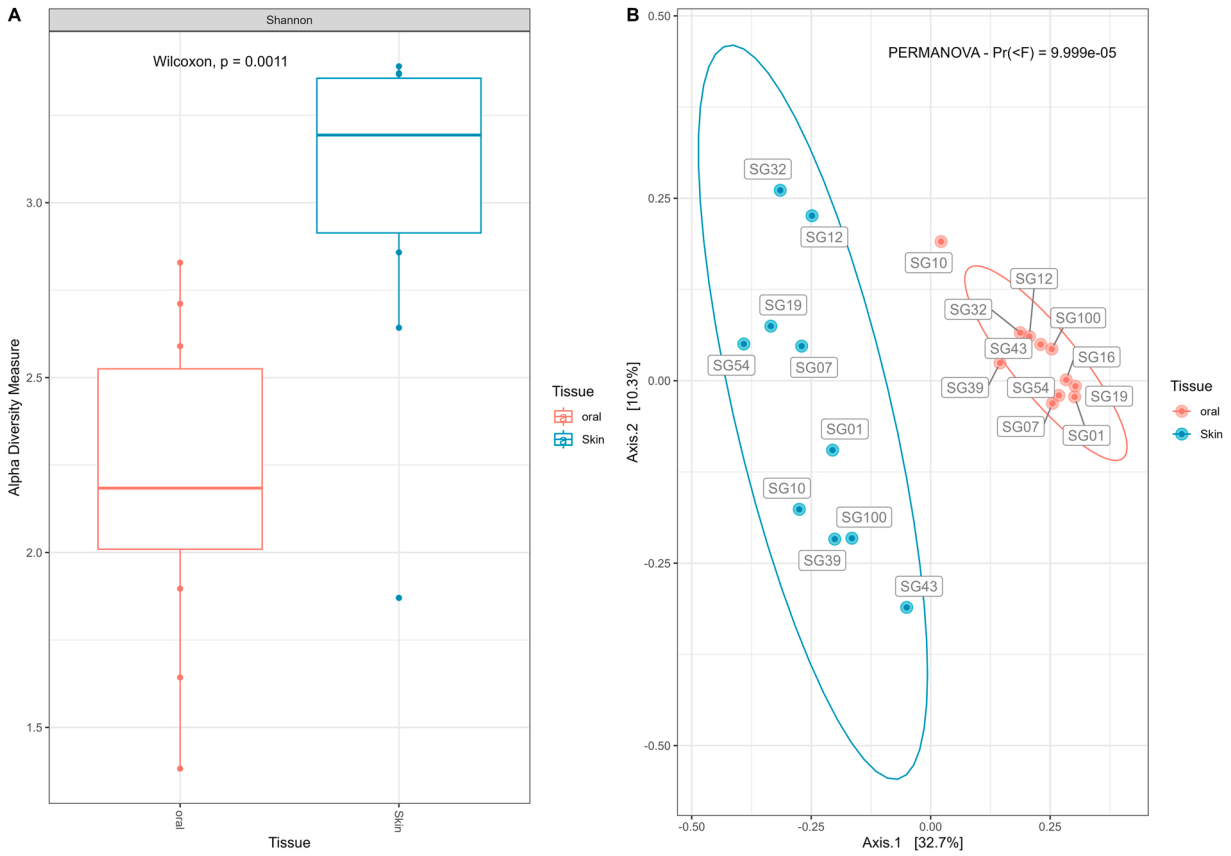

Fig. 4. Diversity analysis comparing oral mucosa (buccal swab) and skin microbiome of 10 participants. **A)** Boxplot showing alpha diversity metrics (observed and Shannon). **B)** Principal component analysis plot of beta diversity using unweighted Unifrac distance.

Table 3
Number of donor-characterising-taxa (DCT) found in oral and skin samples.

Sample ID	Number of DCTs (Oral)	Number of DCTs (Skin)
SG01	1	13
SG07	2	15
SG10	0	42
SG12	1	5
SG19	0	5
SG32	7	4
SG39	0	46
SG43	0	47
SG54	13	47
SG100	0	20

diversity and taxonomic composition in the buccal mucosa of 50 Italian individuals living in Italy. Analyses of the oral microbiome showed the presence of a core microbiome shared amongst people despite their geographical provenience and health status. These findings are consistent with previous reports [3,37,38] showing similar core bacterial taxa with different populations.

Overall, there was no significant difference in diversity between the two regions of Italy assessed. This finding is not unexpected as the population assessed were consistently similar regarding origin country, ethnicity, and culture. However, our finding of two taxa that were differentially abundant in the two regions of Italy, despite the limited number of individuals considered in this preliminary work, highlights the relevance of understanding microbial variation in similar populations for forensic applications. Larger datasets including an extended number of individuals from several regions from the same country may in fact reveal the presence of specific taxa able to characterise the population of specific regions, and this may have significant implications for forensic investigations.

In the assessment of the participants' lifestyle factors, we obtained consistent results with other studies, identifying a significant depletion of *Proteobacteria* in the oral microbiome of never-smokers in comparison with current smokers [66–68]. *Bacteroidota* abundance displayed similar trends, being highest however in current smokers, and lowest in never-smokers. Certain bacterial taxa – including the aerobe, *Haemophilus* (*Proteobacteria*) [67], and anaerobic bacteria *Prevotella* (*Bacteroidota*) [67,68] – have been associated with smoking. These studies and others [69,70] have suggested that cigarette-use facilitates an anaerobic oral environment which favours the depletion of aerobic bacteria and the enrichment of facultative and strict anaerobes. Thus, the observed alterations to the abundance levels of these phyla may be due to oxygen deprivation. Furthermore, in comparison to never-smokers, previous-smokers had lower abundance of *Bacteroidota*, which was higher in comparison to current smokers. A reverse of this phenomenon was observed with *Proteobacteria* and suggests the recovery of oral microbiota following cessation of smoking. Oxygen deprivation may also explain the recovery of the microbiome following smoking cessation, as restoration of oxygen could re-establish original levels of depleted or enriched microbiota. While the exact mechanism remains undetermined and is not the focus of this study, our observations nonetheless show the potential of the oral microbiome as a predictor of smoking habits.

Regarding age, we observed an increasing abundance of phyla *Spirochaetota* and *Synergistota* with increasing age, peaking at the group age of 41–50, after which there was a general decline. Clinical microbiology studies have indicated that oral bacteria belonging to these phyla are usually indicators of periodontal diseases [71–74]. While periodontal disease is primarily caused by poor hygiene, age has been reported to be a risk factor [75]. This may explain the trends observed here, as oral health has been shown to decline with age [76–78], despite the decline

in abundance after the 41–50 age group resulting from causes not yet completely identified. Whilst the reasons behind this trend will require further research, this finding may indicate a future potential use of these taxa as indicators for the estimation of age in unknown individuals.

As an additional aim of the paper, the oral and skin microbiomes of 10 participants in the study were compared to investigate which anatomical location and associated microbiome could potentially provide more useful information for forensic human profiling. Clustering of samples in the PCoA plot (Fig. 4**B**) was based on the sample type (skin or oral swab), rather than on the individuals, as expected, indicating that the microbiome variations between these samples were more related to the sample type than to the donors. PCoA and alpha diversity measures also showed that the skin microbiome is more diverse than the oral microbiome, revealing an increased potential for human identification using the skin microbiome as opposed to using the oral microbiome. The observation of more DCTs in the skin microbiome when compared to the oral microbiome may also suggest greater potential for human identification. However, the skin microbiome has been reported to be more susceptible to changes over time [79,80], and consist of transient microorganisms. Hence, further research on the stability of these DCTs in long term or longitudinal studies would be beneficial for conclusively determining which habitat – skin or oral – may be a more suitable alternative to human DNA for forensic discriminatory purposes.

5. Conclusion

While research on microbial composition of similar populations and ethnic groups is often overlooked, our study confirms the importance for the assessment of such populations. Characterisation of such groups is necessary to advance the integration of the oral microbiome in routine forensic analysis as both group- and individual identification have forensic importance. From a forensic perspective, the skin microbiome may have more potential for individual characterisation than its oral counterpart, due to the presence of multiple DCTs that could be useful for identification purposes. We do acknowledge, however, the forensic challenges that accompany temporal changes of skin microbiota, as such changes could compromise the possibility of using skin microbiomes collected after a long time, for example, from a crime scene to accurately identify individuals or provide investigative information about an individual. On the other hand, the conserved or less diverse nature of the oral microbiome indicates that it could be better suited for body fluid identification – saliva – and that it could reflect certain habits or lifestyle characteristics useful for forensic investigations.

In summary, our findings have provided insights into oral and skin microbiomes of Italians and how they might be utilised in forensic profiling. Results from our study have suggested that the abundance of certain bacterial taxa can provide information on individuals and their lifestyles. There is need however, for further studies involving a larger sample size and similar cohorts within other regions of the same country, as well as comparisons between regions from other countries, for clarifying to which extent these analyses may be used in the future to assist with human identification and forensic investigations.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.fsigen.2023.102841.

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