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Beyond DNA: Human Microbiome Signatures in Forensic Science—Current Advances, Challenges, and Future Perspectives

*Vishal Dhayal**

Department of Forensic Science, Vivekananda Global University, Jaipur, Rajasthan, India

Abstract

Background: Forensic microbiomics is an emerging discipline that investigates the use of human-associated and environmental microbial communities as complementary sources of forensic evidence. The recent development of next-generation sequencing, metagenomics, bioinformatics and computational biology has expanded the possibility to use microbiome analysis for forensic investigations, providing valuable biological information beyond conventional DNA profiling.

Methods: A thorough literature review was performed by searching the major scientific databases including PubMed, Scopus, Web of Science, ScienceDirect, SpringerLink, Google Scholar, and IEEE Xplore. A critical review was performed on relevant peer-reviewed articles on forensic microbiomics, microbial profiling, sequencing technologies, bioinformatics and forensic applications, against pre-defined inclusion and exclusion criteria. The selected studies were synthesized to provide an overview of the current developments, methodological advances, forensic applications, existing challenges and future research directions.

Results: The literature surveyed shows that microbiome analysis has a great potential in a number of forensic applications such as personal identification, reconstruction of crime scenes, postmortem interval estimation, inference of geographical origin, sexual assault investigations, lifestyle reconstruction and identification of disaster victims. Advances in sequencing technologies, artificial intelligence and bioinformatics have made possible improved microbial characterization and predictive analyses. However, several limitations still exist, including methodological variability, environmental contamination, limited reference database, challenges of interpreting complex microbial data, and the requirement for standardized analytical protocols and large-scale validation prior to routine forensic application.

Conclusion: Forensic microbiomics is a promising complementary approach to conventional forensic DNA profiling by providing contextual biological information that cannot be obtained from human genetic markers only. Continuous developments in sequencing technologies, standard methods, world microbiome databases, artificial intelligence, and interdisciplinary collaboration will enhance its scientific credibility and practical utility. Microbiome analysis is unlikely to replace traditional DNA profiling, but its integration with established forensic methods can enhance evidence interpretation and help evolve modern forensic science.

Keywords: Human Microbiome; Forensic Microbiomics; Human Identification; Metagenomics; Microbial Signatures; Next-Generation Sequencing

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Correspondence:
Vishal Dhayal, Department of Forensic Science, Vivekananda Global University, Jaipur, Rajasthan, India, Tel: 7690000901;
E-mail: 25bsc3fs036@vgu.ac.in/
ORCID: https://orcid.org/0009-0003-8770-7550

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Introduction

The Human Microbiome and the Evolution of Microbiome Research

Humans are the habitat of a large and diverse community of microorganisms including bacteria, archaea, fungi, viruses, and protozoa collectively called the human microbiota [1,2]. Not only these microorganisms define the human microbiome, but also their collective genetic material and the intricate interactions they establish with the host and the surrounding environment [1]. These microbial communities colonize different anatomical sites including the skin, oral cavity, gastrointestinal tract, respiratory tract and genitourinary tract, where they help maintain normal physiological functions and overall host homeostasis [2–4].

Many microorganisms are not culturable by conventional laboratory methods, so for many

years research on human-associated microbes was largely based on culture-based methods that underestimated microbial diversity [1, 3]. The revolution in microbiome research was brought about by the development of culture-independent molecular methods like whole-metagenome sequencing, 16S ribosomal RNA gene sequencing and high-throughput bioinformatics, which provided for thorough characterization of microbial communities and their functional potential [1, 4]. A milestone was achieved in 2007 with the launch of the Human Microbiome Project (HMP), which generated reference datasets characterizing the diversity and composition of microbial communities in healthy individuals and significantly advanced our understanding of the relationship between humans and their resident microorganisms [1].

The rapid advance of microbiome research has shown that microbial communities display site-specific features and high inter-individual variation while protecting key functional abilities [1, 4]. These findings have expanded the scope of the human microbiome from health and disease research, arousing curiosity in its utilization in various scientific areas, such as forensic science. Microbial communities' ability to reflect personal and environmental characteristics has provided a biological rationale for the emerging field of forensic microbiomics, which is being explored as an adjunct method to existing forensic identification techniques.

Forensic Significance of the Human Microbiome

DNA profiling has long been the gold standard for human identification in conventional forensic identification due to its high discriminatory power and reliability. However, some forensic scenarios such as highly degraded biological samples, low-template DNA, mixed biological evidence and environmentally compromised specimens, may jeopardize the value of DNA-based analyses, creating a need for complementary biological markers rather than replacements for established methods [5, 6].

Recent advances in microbiome research have shown that the microbial communities associated with humans have features that may be advantageous in forensic investigations. Although microbial composition differs across anatomical sites and between individuals, many microbial signatures are sufficiently individual and temporally persistent to discriminate between individuals under certain conditions [7, 8]. Furthermore, microorganisms can be transported between humans, objects, and the surrounding environment, which can provide additional biological information that may be useful in reconstructing human activity and interaction [9].

These results have given rise to forensic microbiomics, a multidisciplinary field that examines the application of microbial evidence in legal and criminal investigations. Forensic genetics is contrasted with forensic microbiomics, which studies the composition and dynamics of microbial communities to generate evidence that can supplement traditional evidence. Current research has shown its potential for personal identification, postmortem interval estimation, geolocation inference, and linking individuals to objects or crime scenes. Nevertheless, these promising advances also raise important challenges related to methodological standardization, reproducibility, database development and legal validation that must be addressed before microbiome-based evidence can be routinely incorporated into forensic practice [8–10].

Aim and Scope of the Review

The increasing evidence for the forensic importance of human-associated microbial communities underscores the need for a comprehensive evaluation of their potential in forensic investigations. The knowledge on the composition, dynamics and functional characteristics of the human microbiome has increased considerably, but its implementation into routine forensic practice is still hampered by methodological, technical and legal challenges.

This review critically discusses the current state of knowledge on human microbiome signatures and their application in forensic identification. The biological basis of microbial individuality, the analytical approaches used in forensic microbiomics, and new applications in personal identification, postmortem interval estimation, geolocation inference and crime scene reconstruction are discussed in particular. Moreover, this review highlights the key limitations, ethical considerations, and priorities for future research that need to be addressed before microbiome-based evidence can be reliably incorporated into forensic practice. This review integrates seminal and current articles to provide a resource for researchers, forensic practitioners and policy makers and to highlight areas for future research.

Literature Search Strategy and Study Selection Methodology

Databases Used

A comprehensive literature search was undertaken to identify relevant studies on forensic microbiomics and its applications in forensic science. A systematic search of electronic databases, including PubMed, Scopus, Web of Science, ScienceDirect, SpringerLink, Google Scholar and IEEE Xplore were conducted to retrieve peer-reviewed research articles, review papers and book chapters published in English. To maximize coverage of the available literature, reference lists of selected articles were manually reviewed to identify additional relevant publications.

The search was focused on high quality publications on microbiome profiling, microbial forensics, forensic genetics, metagenomics, bioinformatics, next generation sequencing and emerging forensic applications. Preference was given to recent peer-reviewed studies, but landmark publications that have contributed greatly to the development of forensic microbiomics were also included. The critical analysis and synthesis presented in this review were based on the final collection of literature.

Search Terms and Keywords

During literature search, combinations of predefined keywords and Boolean operators (AND, OR) were used to maximize retrieval of relevant publications. The main search terms were forensic microbiomics, forensic microbiology, human microbiome, microbiome profiling, microbial fingerprinting, microbial forensics, DNA profiling, next-generation sequencing, metagenomics, 16S rRNA sequencing, forensic identification, postmortem interval, thanatomicrobiome, geolocation, crime scene investigation, microbial transfer, and forensic genetics. Different combinations of these terms were used based on the search ability of each database.

Where available, other filters were applied to improve the relevance of retrieved studies such as publication type, language and subject area. Synonymous terms and related keywords were included during the search process to ensure comprehensive coverage of the rapidly evolving field of forensic microbiomics. The search strategy was refined iteratively to identify the most relevant and scientifically robust literature to include in this review.

Inclusion Criteria

Eligible for inclusion were studies published in peer-reviewed scientific journals that focused on the application of microbiome analysis in forensic science or closely related disciplines. Original research articles, systematic reviews, narrative reviews, and relevant book chapters were included when they provided substantive information on forensic microbiomics, microbial profiling, next-generation sequencing, metagenomics, bioinformatics, forensic genetics, postmortem microbiology, or other emerging forensic applications.

Priority was given to publications in English and to studies that presented scientifically sound methodologies, validated analytical approaches and significant contributions to the advancement of forensic microbiomics. To provide a balanced view of the historical development, current state, and future prospects of microbiome-based forensic investigations, recent publications and landmark studies were both included.

Exclusion Criteria

Publications not directly relevant to the objectives of this review or lacking in sufficient scientific rigor were excluded. Conference abstracts, editorials, letters to the editor, opinion articles, magazine publications, non-peer-reviewed reports, dissertations, preprints without peer review, and duplicate records were excluded from the final analysis. Studies restricted to clinical microbiology, veterinary microbiology, environmental microbiology or any other non-forensic applications without clear relevance to forensic microbiomics were also excluded.

To ensure the scientific quality of the review, articles published in a language other than English, studies that did not provide sufficient methodological detail and publications reporting incomplete, inconsistent or unreliable findings were excluded. Where findings from the same study population overlapped across multiple publications, the final literature synthesis included the most comprehensive and methodologically robust publication.

Study Selection and Data Extraction

The studies identified in the literature search were systematically screened in several stages to ensure they were relevant to the aims of this review. Duplicates were removed and titles and abstracts were screened to exclude publications clearly not relevant. The remaining articles were then read in full to assess eligibility against the pre-determined inclusion and exclusion criteria. Only those publications that fulfilled these criteria were included in the final synthesis of the literature.

In order to ensure uniformity in the review process, relevant information was extracted from each of the selected studies in a structured manner. Extracted data included bibliographic information, purpose of study, type of sample, analytical methods, sequencing technologies, bioinformatic approaches, main findings, limitations and reported forensic applications. The data collected were critically analyzed and synthesized to provide a comprehensive overview of the current state of knowledge, emerging trends, existing challenges, and future directions in the field of forensic microbiomics.

Fundamentals of the Human Microbiome

Composition and Diversity of the Human Microbiome

The human microbiome is a complex consortium of microorganisms, including bacteria, archaea, fungus, viruses and protozoa that are present on virtually all surfaces and cavities of the human body [1, 2]. “Human microbiota” refers to the microorganisms per se, while “human microbiome” refers to the microbial communities, their collective genomes, functional attributes, and their interactions with the host and the environment. This complex microbial ecology is an integral component of human biology and participates in many physiological processes essential to host homeostasis [2, 4].

The distribution of microbial communities in the human body is heterogeneous and exhibits striking diversity across anatomical sites [3]. Different physicochemical conditions are provided by each habitat, with differences in pH, oxygen availability, moisture, temperature and nutrient availability, which select for microbial colonization and community composition [3, 4]. As a result, the gastrointestinal tract presents the highest microbial biomass and diversity, and the skin, oral cavity, respiratory tract and genitourinary tract present specialized microbial communities adapted to their ecological niches [3, 4].

There is much inter-individual variation in microbial composition, but several microbial functions are conserved between healthy individuals [1, 4]. This phenomenon, often called functional redundancy, enables different microbial taxa to perform similar biological functions despite taxonomic differences, thereby maintaining important metabolic and immunological functions [4]. These observations suggest that the biological properties of the human microbiome are determined by both the composition and the functional potential of the microbial community.

Knowledge of microbial diversity has been greatly improved by the application of culture-independent molecular methodologies. High-throughput sequencing methods, in particular 16S ribosomal RNA (16S rRNA) gene sequencing and whole-metagenome sequencing, have allowed the comprehensive characterisation of microbial communities previously not possible by traditional culture-based methods [1, 4]. These technologies have provided a scientific basis for exploring microbial diversity in biomedical and forensic studies.

The composition and diversity of the human microbiome need to be understood in order to assess the forensic potential of the microbiome. The ecological complexity, body-site specificity and inter-individual variability of microbial communities offer the biological basis to explore microbial signatures as complementary sources of forensic information. The following subsections discuss individual body habitat characteristics and factors controlling microbial community structure.

Human Microbial Niches

The human body is home to several anatomical habitats, which have specific physicochemical properties, influencing the composition, diversity and functional features of resident microbial communities [3, 4]. Temperature, moisture, availability of oxygen, nutrient composition and secretions from the host create unique ecological niches that select for specific microorganisms [3]. Each body site is thus characterized by a microbiome that contributes to physiological homeostasis but varies considerably between individuals.

Skin Microbiome: The skin is the largest organ of the human body and is the first interface between the host and the outer environment. Despite constant exposure to changing environmental conditions, the skin harbors diverse microbial communities adapted to sebaceous,

moist, and dry regions [3, 11]. Members of the genera Cutibacterium, Staphylococcus, Corynebacterium, and Micrococcus are among the most abundant bacterial inhabitants of healthy skin, although their relative abundance varies based on the anatomical location and local environmental conditions [11, 12]. These microorganisms play a role in the maintenance of the skin barrier, modulation of immune responses and inhibition of pathogenic colonization by competitive interactions and production of antimicrobial metabolites [2, 11].

Oral Microbiome: The oral cavity is one of the most complex microbial ecosystems in the human body and is composed of microorganisms colonizing the teeth, tongue, saliva, gingival crevices and oral mucosa [13]. Dietary components, varying oxygen tension and salivary secretions are constantly available and create many ecological niches that can sustain highly specialized microbial biofilms. The main bacterial genera detected are Streptococcus, Veillonella, Actinomyces, Neisseria, Fusobacterium and Prevotella, which together contribute to the maintenance of oral health and ecological balance [13, 14]. Changes in these microbial communities may disrupt oral homeostasis and increase the risk of oral diseases.

Gastrointestinal Microbiome: The gastrointestinal tract is the largest reservoir of microbial biomass and diversity in the human body, with the colon containing trillions of microorganisms representing hundreds of bacterial species [4, 15]. The healthy adult gut microbiome is dominated by members of the phyla Firmicutes and Bacteroidota, with representatives of Actinobacteriota, Proteobacteria, and Verrucomicrobiota [4, 15]. The gut microbiome is one of the best studied microbial ecosystems as these microbial communities are involved in digestion, production of short chain fatty acids, vitamin synthesis, immune regulation and maintenance of intestinal barrier integrity.

Respiratory Microbiome: The respiratory tract was long thought to be sterile, but culture-independent sequencing studies have shown the presence of diverse microbial communities throughout the upper and lower respiratory tract [16]. Respiratory microbial communities contribute to immune homeostasis and protection against respiratory pathogens, although they are less dense than those in the gastrointestinal tract. Common genera are Streptococcus, Haemophilus, Neisseria, Moraxella and Corynebacterium with the composition of the community being influenced by environmental exposure, age, and underlying health conditions [16].

Genitourinary Microbiome: The genitourinary tract possesses a highly specialized microbiome that differs greatly between males and females. Lactobacillus species are usually predominant in the vaginal microbiome of healthy women in the reproductive age and produce lactic acid to maintain an acidic environment and prevent colonisation by opportunistic pathogens [17]. Microbial composition, however, may vary with age, hormonal status, ethnicity, reproductive stage and environmental factors [17, 18]. These dynamic microbial communities are important for reproductive health and represent another important aspect of the human microbiome.

The diverse microbial communities in the different anatomical sites taken together suggest high compartmentalization of the human microbiome and local ecological shaping. Understanding the properties of these microbial niches provides a vital basis for understanding differences in microbial composition and for assessing variables that impact microbial community structure, which are discussed in the next section.

Factors Influencing Microbiome Composition

Human microbiome composition is influenced by a complex interplay of host-related, environmental and lifestyle factors, all of which shape microbial diversity across the life span [4, 15]. Although some microbial communities are relatively stable under healthy conditions, the host–environment interface is responsible for dynamic changes in the composition and function of the microbiota. Understanding these determinants is key for the interpretation of inter-individual variability and the biological complexity of the human microbiome.

Host factors such as age, sex, genetics, hormonal status and immune function, play a fundamental role in the development and maintenance of microbial communities [19, 20]. Microbial colonization begins at birth, undergoes a rapid succession in infancy and is then stabilized gradually in adulthood. Other physiological changes associated with ageing, hormonal changes, pregnancy and immunosenescence also affect the microbial diversity in different body sites [20]. Furthermore, variations in host genetics might affect the susceptibility to colonization by particular microorganisms through differences in immune regulation, epithelial physiology, and metabolic activity [19].

Environmental and life-style factors also play a strong role in the human microbiome. The composition and function of the microbiota can be heavily influenced by factors such as diet, geography, sanitation, occupation, physical activity, antibiotic exposure, smoking, alcohol consumption, and contact with animals or the natural environment [15, 21]. Among these, antibiotic treatment is one of the most dramatic perturbations, with a frequently diminished diversity and a prolonged disturbance of community structure [22]. Similar to this, differences in diet, climate, environmental exposure, and socio-economic conditions result in substantial variation in microbial communities due to geographical and cultural differences [15].

Health status also influences the composition of microbial communities. Changes in microbial diversity and composition referred to as dysbiosis [2, 4] have been linked to infectious diseases, chronic inflammatory diseases, metabolic diseases, autoimmune diseases, and other pathological conditions. Many of the microbial changes associated with disease are still under investigation but there is a growing body of evidence that suggests that disruption of normal microbial equilibrium may influence host physiology and contribute to disease progression. Microbial profiles should, therefore, be interpreted in the greater biological and clinical context, and not as fixed properties.

All of these factors taken together form evidence that the human microbiome is a dynamic biological system, rather than a static entity. Although individual microbial communities are highly diverse and flexible, many cores microbial functions are relatively conserved between healthy populations. This understanding of the biological determinants of microbiome variability provides a critical framework for understanding temporal stability and succession, which we discuss in the next section.

Stability and Temporal Dynamics of the Human Microbiome

The human microbiome is a dynamic ecosystem, which is constantly responding to intrinsic and extrinsic stimuli throughout the life of an individual. Although microbial communities can respond rapidly to changes in environment and physiology, several longitudinal studies have shown stability of the microbial profile in

healthy individuals over time, particularly at higher taxonomical and functional levels [4, 23]. This balance of stability and variability is a hallmark of the human microbiome and it is the foundation of its biological resilience.

Anatomical sites differ markedly in their microbial stability. In general, the gut microbiome has been shown to be more stable in the long-term in healthy adults than the microbial communities associated with the skin or oral cavity, which are more affected by environmental exposure and daily activities [3, 15]. Even in very dynamic body sites, a core group of resident microorganisms is maintained, which contributes to the maintenance of microbial homeostasis despite transient fluctuations in community composition [24]. These observations have raised the concept of a core microbiome, i.e. a set of microbial taxa and functional features consistently associated with a given body habitat.

Temporal changes of microbial community are affected by many biological and environmental factors. Changes in diet, antibiotic exposure, infections, hormonal changes, age, travelling, seasonal changes and lifestyle changes may all lead to measurable changes in microbial composition [19, 22]. However, in many cases, microbial communities are resilient and slowly recover to their pre-disturbance state following removal of the impacting factor, although the degree of recovery varies among individuals and body sites [22, 25].

The microbial succession is another important characteristic of the microbiome dynamics. Colonization starts immediately after birth and is followed by gradual changes in infancy and childhood before relatively stable adult communities are established [20]. Other transitions occur in pregnancy, aging, disease, and other major physiological events, representing the constant interplay of host biology and environmental exposures. The human microbiome should therefore be viewed as a dynamic ecosystem, and not as a static biological entity.

The balance between microbial stability and temporal variation is fundamental to the interpretation of information derived from the microbiome. Microbial communities show quantifiable temporal variation; however, robustness of central microbial features and functional profiles provide a biological basis for the exploration of microbial individuality. The next sub-section addresses the mechanisms underlying individual-specific microbial signatures.

Biological Basis for Individual Microbial Signatures

The individual variation in human microbiome is a reflection of the continuous interaction of host genetics, physiology, immune responses, lifestyle, environmental exposure, and stochastic microbial colonization events [19, 21]. While many microbial taxa are common, the relative abundance, diversity and functional properties of these microorganisms differ considerably between individuals, leading to highly personalized microbial communities [1, 4]. This individuality has been consistently demonstrated in several body habitats including skin, oral cavity, gastrointestinal tract and genitourinary tract, suggesting that each individual has a characteristic microbial profile shaped by both intrinsic and extrinsic factors [3, 11, 13].

Longitudinal studies also support the concept of microbial individuality, by showing that many individuals maintain quite stable microbial characteristics over long periods of time, despite natural fluctuations over time [23, 25]. Microbial communities are not static but are in a state of dynamic equilibrium, preserving core members and functional attributes, while less abundant taxa respond to environmental or physiological influences [24]. This tension between persistence and adaptability distinguishes the human microbiome from a static biological marker and encapsulates its ecological complexity.

Host specific microbial signatures are maintained by selective pressures from the characteristics of the anatomical site, immune regulation, nutrient availability, and interactions between the resident microorganisms [2, 4]. These ecological processes lead to the establishment of relatively unique microbial ecosystems that differ not only between individuals but also between body sites within the same individual [3]. Therefore, microbial community composition should be considered as a biological continuum influenced by multiple determinants, rather than a static feature.

Growing evidence of microbial individuality has prompted researchers to look to the human microbiome as another source of biological information. However, the validity of microbial signatures depends on their reproducibility, temporal stability, methodological standardization and resistance to environmental perturbations. These factors are still under active investigation and need further validation before microbiome-derived evidence can be routinely incorporated into forensic practice. However, there is a well-established biological rationale from existing research to consider microbial signatures as informative biomarkers that could augment traditional forensic evidence.

In this section, we discuss the biological principles that provide the conceptual foundation for forensic microbiomics throughout. With this in mind, the following section discusses the analytical methods currently employed to characterize microbial communities and produce microbiome-derived data for forensic investigation (Table 1).

Analytical Approaches in Forensic Microbiomics

Sample Collection and Preservation

The quality of sample collection and preservation is critical for the reliability of microbiome-based forensic investigations. Unlike conventional forensic DNA analysis, microbial evidence is highly sensitive to environmental exposure, contamination, storage conditions and handling procedures. Thus, standardized sampling protocols are required to ensure that the microbial profiles mirror the original biological sample, rather than changes acquired during collection or laboratory processing [28, 29].

Microbial evidence can be recovered from a variety of forensic substrates including skin swabs, saliva, blood, vaginal and rectal swabs, hair, fingernails, soil associated with human remains, personal effects and environmental surfaces [7]. The choice of an appropriate sampling strategy depends on the forensic question asked, the type of evidence present and the expected microbial biomass. To minimize contamination and to preserve the integrity of microbial communities, sterile swabs, DNA-free collection materials and aseptic handling procedures are recommended [29, 30].

After collection, samples should be transported and stored under conditions that minimize DNA degradation and microbial growth. Immediate refrigeration or freezing at appropriate temperatures is generally advised but repeated freeze–thaw cycles should be avoided as they can alter the microbial composition and decrease the quality of DNA [29, 31]. Moreover, the preservation of the evidential integrity

Table 1: Major Human Microbiomes and Their Forensic Significance.

Body Site	Dominant Microorganisms	Key Characteristics	Potential Forensic Applications
Skin	*Staphylococcus spp.*, *Corynebacterium spp.*, *Cutibacterium acnes*	Highly individualized; influenced by skin physiology, environmental exposure, and personal habits	Personal identification, microbial transfer analysis, linking individuals to objects, crime scene reconstruction
Oral Cavity	*Streptococcus spp.*, *Veillonella spp.*, *Neisseria spp.*, *Prevotella spp.*	High microbial diversity; influenced by oral hygiene, diet, and health status	Saliva identification, bite mark investigations, personal identification
Gastrointestinal Tract	*Bacteroides spp.*, *Faecalibacterium spp.*, *Bifidobacterium spp.*, *Escherichia coli*	Largest and most diverse microbial community; relatively stable over time	Lifestyle reconstruction, dietary inference, postmortem studies, individual differentiation
Respiratory Tract	*Streptococcus spp.*, *Haemophilus spp.*, *Corynebacterium spp.*	Varies with anatomical location, respiratory health, and environmental exposure	Respiratory specimen analysis, disease-associated forensic investigations
Genitourinary Tract	*Lactobacillus spp.* (female), *Corynebacterium spp.*, *Staphylococcus spp.*	Strong body-site specificity; influenced by age, hormonal status, and sexual activity	Sexual assault investigations, microbial transfer studies, personal identification
Thanatomicrobiome	Mixed postmortem bacterial communities (e.g., *Clostridium spp.*, *Enterococcus spp.*, *Proteobacteria*)	Predictable microbial succession during decomposition	Postmortem interval estimation, decomposition studies, disaster victim identification

Abbreviations: *spp.* = species; PMI = Postmortem Interval.

and reproducibility of microbiome analyses requires detailed documentation of collection procedures, environmental conditions, storage duration, and chain-of-custody records.

Quality assurance measures should be included at all stages of the sampling process. Field blanks, extraction controls, reagent blanks and negative controls are used to identify possible contamination from the personnel, laboratory reagents or surrounding environment [30, 32]. Contamination can have a large effect on downstream analyses and result in incorrect biological interpretation, considering the often-low abundance of microbial DNA. Therefore, stringent contamination control and standardized sampling protocols are fundamental to successful use of microbiome evidence in forensic investigations.

The reproducibility of microbiome studies has been greatly improved by advances in sampling technologies and standardized preservation strategies. However, a major challenge for the routine application of forensic microbiomics is the lack of universally accepted forensic sampling guidelines. The next crucial step after successful collection and preservation of samples is efficient extraction and quality assessment of the microbial DNA for accurate downstream sequencing and bioinformatic analysis.

DNA Extraction and Quality Assessment

Successful characterization of microbial communities depends on the extraction of high-quality DNA that is representative of the microbial composition of the original sample. Microbiome studies differ from traditional forensic DNA analysis in those complex biological matrices containing microbial DNA, host DNA, environmental contaminants, and potential PCR inhibitors are often used. DNA extraction is, therefore, one of the most important steps in forensic microbiomics, as differences in extraction procedures can heavily influence microbial community profiles and downstream analytical results [28, 29].

While there are numerous commercial extraction kits or laboratory protocols available for microbiome analysis, there is no one method that is universally applicable to all forensic sample types. Efficient extraction requires effective disruption of microbial cell walls with minimal degradation and contamination of DNA. Extraction bias can affect the relative abundance of microbial taxa and the reproducibility of microbiome analysis because the cell wall structure and the lysis efficiency vary among different microorganisms [28, 30].

After the isolation of DNA, it is important to do both quantitative and qualitative assessment before sequencing. The DNA concentration is generally assessed by fluorometric methods. Purity is evaluated by spectrophotometry and electrophoretic analysis to determine protein contamination, degradation or other impurities [31]. In addition to positive controls, other quality-control steps, including extraction blanks and negative controls, should be part of all analytical workflows to identify laboratory-derived contamination, particularly when analyzing low-biomass forensic samples [30, 32].

Another serious analytical problem is the host DNA presence. Human DNA is often a dominant component of the total DNA present in forensic samples, which lowers the sequencing efficiency for microbial targets and may mask low-abundance microorganisms. Several laboratory and computational approaches have been developed to improve microbial DNA recovery and downstream analysis accuracy, including host DNA depletion methods and bioinformatic filtering [33].

Standardization of DNA extraction and quality assessment protocols is still critical for reproducibility between laboratories. Variability in microbiome datasets can be substantial, owing to differences in extraction methods, sample processing, reagent choice and quality-control procedures, making direct comparison among studies difficult. Thus, standardized laboratory procedures and strict quality assurance measures are essential requirements for the trustworthy use of microbiome evidence in forensic investigations. The extracted microbial DNA is then sequenced using advanced molecular technologies, which are discussed in the following section.

Sequencing Technologies for Microbiome Analysis

The field of microbiome research has been revolutionised by the rapid development of high throughput sequencing methods that allow the complete characterisation of microbial communities without the limitations of traditional culture-based techniques [1, 28]. These approaches provide taxonomic and functional information that forms the basis for microbiome research in biological and forensic sciences. The choice of an appropriate sequencing strategy is determined by the study purpose, sample type, sequencing depth, analytical resolution and resources available.

Of the available methods, 16S ribosomal RNA (16S rRNA) gene sequencing remains the most common method for profiling bacterial communities due to its relatively low cost, established analytical workflows, and capacity to identify bacterial taxa through conserved and hypervariable regions of the 16S rRNA gene [34]. This

approach allows for efficient comparison of microbial diversity across large numbers of samples and has greatly contributed to current knowledge on human-associated microbial communities. However, its application in studies requiring high-resolution microbial characterization is limited by low taxonomic resolution, inability to reliably differentiate closely related species, and absence of direct functional information [35].

Shotgun metagenomic sequencing has gained increasing importance in microbiome research to overcome these limitations. Unlike targeted 16S rRNA sequencing, shotgun metagenomics sequences all genetic material present within a sample, enabling simultaneous identification of bacteria, archaea, fungi, viruses, and functional genes [36]. This broad approach allows for higher taxonomic resolution and the study of microbial metabolic pathways, antimicrobial resistance genes, and strain-level variation. However, higher costs of sequencing, higher computational requirements and the abundance of host DNA are still analytical challenges, especially for forensic samples with low microbial biomass [33, 36].

The emergence of third-generation sequencing (i.e., nanopore sequencing and single-molecule real-time sequencing) has significantly extended the length of sequence reads and subsequently expanded the scope of microbiome studies [37]. Long read sequencing increases the quality of genome assembly, improves species level identification and enables characterization of structurally complex genomic regions that are difficult to resolve using short-read platforms. Despite the ongoing improvements in sequencing accuracy and accessibility of these technologies, higher error rates and lack of standardization currently limit their widespread implementation in routine forensic microbiome analysis [37].

Each of the sequencing strategies has their own strengths and limitations and there is no single platform that is best for all forensic applications. The selection of an appropriate sequencing technology should therefore be guided by the forensic objective, the required taxonomic resolution, the quality of the sample, cost considerations, and downstream analytical needs. Further advances in sequencing chemistry, bioinformatic approaches and computational power are likely to improve the accuracy, reproducibility and forensic utility of microbiome-based analysis.

Bioinformatics and Data Analysis

Robust bioinformatic pipelines are required for accurate processing, interpretation and biological inference of the large volume of sequencing data generated during microbiome analysis. Before meaningful analysis can be performed, raw sequencing reads need to be preprocessed in several steps, including quality assessment, removal of low-quality sequences, adapter trimming, chimera detection, and elimination of contaminating host DNA [28, 36]. These quality-control procedures improve the reliability of downstream analyses and reduce technical bias that could influence microbiome profiles.

After data preparation, microbial sequences are assigned to taxonomic groups based on curated reference libraries and computational classification methods [38, 39]. Depending on the sequencing approach taken, taxonomic profiling can be done at different levels of resolution (i.e., phylum, species or strain level). Besides taxonomic identification, shotgun metagenomic sequencing allows for functional annotation, i.e., the identification of genes related to metabolic pathways, antimicrobial resistance, and microbial virulence, which provides insight into the composition and functional potential of microbial communities [36, 40].

Another important part of studying the microbiome is measuring microbial diversity. Alpha diversity refers to the diversity within a single microbial community, and is defined by species richness and evenness, whereas beta diversity is the difference in microbial composition between samples or groups [41]. These ecological metrics allow investigators to compare microbial communities between individuals, body sites, environmental samples, or forensic evidence, and are most commonly paired with multivariate statistical analysis to identify biologically relevant trends.

Furthermore, appropriate statistical and computational methods are also important for the accurate interpretation of the microbiome datasets. Dimensionality reduction methods, clustering algorithms, differential abundance analysis, and network-based analyses are widely used to identify relationships among microbial taxa and to assess associations between microbial communities and biological or environmental variables [36, 40]. However, differences in sequencing platforms, analytical pipelines, reference databases and statistical methodologies may lead to inconsistent results across studies, highlighting the importance of standardized bioinformatic workflows and transparent reporting practices.

With the increasing size and complexity of microbiome datasets, the demand for sophisticated computational approaches to extract biologically relevant information is growing. Artificial intelligence and machine learning techniques have further extended the scope of analytics by automating pattern recognition, predictive modelling and high dimensional data classification. In the following subsection, the emerging approaches are discussed.

Artificial Intelligence and Machine Learning in Forensic Microbiomics

The rapid growth of microbiome sequencing has generated large, multidimensional datasets that often outstrip the analytical capacity of traditional statistical approaches. Thus, artificial intelligence (AI) and machine learning (ML) have been useful computational tools in detecting complex microbial patterns, classifying biological samples, and developing predictive models from high-dimensional microbiome data [42, 43]. These approaches allow for the simultaneous analysis of thousands of microbial features while accounting for complex relationships between taxa that may not be obvious with traditional analytical approaches.

Supervised machine learning algorithms like Random Forest, Support Vector Machine (SVM) and Gradient Boosting are frequently applied for microbiome classification tasks because of their ability to distinguish microbial communities associated with different individuals, body sites, environmental conditions or disease states [42, 44]. These models are trained on labelled datasets and then used to predict the classification of previously unseen samples. In contrast, unsupervised learning methods such as hierarchical clustering and principal coordinate analysis are mainly used to search for natural relationships in microbiome datasets and to search for the underlying community structures without prior sample categories [39, 41].

Recent advances in deep learning have further extended microbiome research by the ability to automatically extract features from complex sequencing data. Neural network-based models have shown promising performance for microbial classification, functional prediction and biomarker discovery, especially when large

Table 2: Comparison of Major Analytical Techniques Used in Forensic Microbiomics.

Analytical Technique	Principle	Major Advantages	Limitations	Common Forensic Applications
16S rRNA Gene Sequencing	Amplification and sequencing of conserved regions of the bacterial 16S rRNA gene for taxonomic identification	Cost-effective, widely used, suitable for bacterial community profiling	Limited species-level resolution; does not detect fungi or viruses effectively	Personal identification, postmortem interval estimation, microbial community profiling
Shotgun Metagenomic Sequencing	Direct sequencing of all genetic material present in a sample	High taxonomic resolution; detects bacteria, fungi, viruses, and functional genes	Expensive, computationally intensive, requires high-quality DNA	Crime scene investigation, microbial source tracking, functional microbiome analysis
Whole Genome Sequencing (WGS)	Complete sequencing of microbial genomes	Highest genomic resolution; strain-level discrimination	High cost, complex data analysis, longer processing time	Strain identification, outbreak investigation, microbial forensics
Metatranscriptomics	Sequencing of microbial RNA to evaluate actively expressed genes	Provides information on microbial activity and gene expression	RNA is unstable; technically demanding; expensive	Functional microbial analysis, decomposition studies
Quantitative PCR (qPCR)	Quantification of specific microbial DNA targets using fluorescent amplification	Rapid, sensitive, highly specific, suitable for targeted analysis	Limited to predefined targets; lower community coverage	Targeted microbial detection, validation studies, forensic screening
Nanopore Sequencing	Real-time sequencing of DNA molecules using nanopore technology	Portable, rapid sequencing, long-read capability, field deployment potential	Lower accuracy than short-read platforms; requires continuous optimization	On-site microbial profiling, rapid forensic investigations, disaster response

Abbreviations: 16S rRNA = 16S ribosomal RNA; WGS = Whole Genome Sequencing; qPCR = Quantitative Polymerase Chain Reaction.

well-annotated datasets are available [43]. The use of ML in forensic microbiomics is however hindered by the relatively small size of forensic datasets, interpretability issues and the lack of standardized validation frameworks for legal applications.

Approaches based on AI and ML have a lot of potential but should be interpreted with caution. Model performance is highly dependent on data quality, sequencing methodology, feature selection, class imbalance and external validation. Overfitting, poor reproducibility, and lack of diversity in training datasets could reduce the generalizability of predictive models and limit their tangible implementation in forensic investigations [42, 45]. Thus, transparent model development, thorough validation and standardization of analytical pipelines still remain prerequisites for the reliable integration of AI into forensic microbiomics.

The growing integration of high-throughput sequencing, bioinformatics, and artificial intelligence is expected to enhance the accuracy and efficiency of microbiome-based analyses. However, before AI-assisted microbiome profiling can be routinely integrated as a reliable part of forensic practice, methodological harmonization and extensive validation studies are still warranted (Table 2).

Strengths and Limitations of Current Analytical Approaches

Recent improvements in sequencing technology, bioinformatics and computational analysis have advanced the characterisation of complex microbial communities providing unprecedented opportunities for microbiome-based forensic investigations. Comprehensive taxonomic and functional profiling of microbial communities can be achieved with high-throughput sequencing technology, standardized laboratory workflows, and improved analytical pipelines that offer greater sensitivity and resolution than traditional culture-based methods [28, 36]. These technological advances have vastly expanded the potential applications of forensic microbiomics, especially in cases where conventional forensic evidence is limited.

These advances notwithstanding, there are still several methodological challenges that prevent the routine implementation of microbiome analysis in forensic practice. Differences in sample collection, DNA extraction procedures, sequencing platforms, reference databases and bioinformatic pipelines may lead to substantial variations in microbial profiles across laboratories, limiting reproducibility and comparability across studies [28, 29]. Moreover, contamination, host DNA interference, low microbial biomass, and differences in sequencing depth may introduce analytical bias that affects downstream interpretation of microbiome data [30, 31]. These methodological inconsistencies remain among the major obstacles to the development of universally accepted forensic microbiome protocols.

Additional challenges exist in interpreting microbiome data. The composition of microbial communities in humans is affected by numerous biological and environmental factors, e.g. age, diet, geography, medications, health status and lifestyle, all of which contribute to inter-individual variation. Hence, robust statistical models, harmonized analytical frameworks, and large, well-characterized reference data sets are necessary to distinguish between normal biological variation and forensically relevant microbial signatures [42, 44]. Moreover, the lack of internationally harmonized laboratory guidelines complicates direct comparison of microbiome data produced by different forensic laboratories.

Economic and practical considerations are also part of the adoption of forensic microbiomics. Sequencing costs have fallen dramatically in the last decade but shotgun metagenomics, long-read sequencing and sophisticated computational analyses are still relatively resource-intensive compared to traditional forensic DNA profiling. Moreover, routine implementation requires specialized laboratory infrastructure, bioinformatic expertise, validated quality control procedures, and comprehensive reference databases, all of which may limit accessibility in resource-constrained forensic laboratories [36, 37].

However, continued advances in sequencing chemistry, computational biology, artificial intelligence and international standardization efforts are likely to improve the reliability and reproducibility of microbiome analyses. With further development of analytical methodologies, microbiome-based evidence is likely to become an increasingly valuable complement to conventional forensic techniques rather than a replacement for established DNA-based methods. The next section reviews the existing application of these analytical approaches in various forensic investigation fields.

Human Microbiome Signatures in Forensic Identification

Concept of Human Microbial Signatures

As microbial communities vary measurably between individuals, while maintaining characteristic patterns within the same individual over time [1, 23], the human microbiome has emerged as a promising source of biological information. These unique microbial profiles, often termed human microbial signatures [19, 21], are generated by the combination of host genetics, physiology, immune status, environmental exposure, lifestyle, diet and stochastic microbial colonization events. Microbial signatures do not refer to the presence or absence of single microorganisms but to the whole community composition, relative abundance, diversity and functional characteristics [4, 26].

Human microbial signatures are strongly location-dependent. Different microbial communities colonize the skin, oral cavity, gastrointestinal tract, respiratory tract, and genitourinary tract, each habitat providing unique ecological conditions that shape microbial composition [3, 11, 13, 15]. Large inter-individual variation has been consistently reported in addition to body-site specificity, and individuals can be distinguished based on the structure of the microbial community despite shared core microbial taxa [1, 27]. It is this blend of body-site specificity and individual variation which provides the biological rationale for studying microbial communities as complementary forensic biomarkers.

Microbial signatures, unlike traditional genetic markers, are dynamic and can be influenced by environmental, physiological and behavioural factors [22, 25]. However, a number of longitudinal studies have shown that many people harbor relatively stable core microbial communities over long periods of time, especially when in a healthy state [23, 24]. Such a balance of temporal stability and ecological adaptability suggests that microbial signatures possess features that can be useful in forensic inference, but also require careful interpretation with respect to the sample type, environmental exposure, and time since deposition.

Current evidence indicates that microbial signatures should not be considered a replacement for traditional forensic DNA profiling, but rather as an additional source of biological information that can be used to supplement existing forensic methods [7, 8]. They are most useful in situations where the composition of the microbial community can provide contextual information beyond host DNA, such as evidence of biological origin, environmental association or temporal change. However, methodological standardization, population-scale validation and legal evaluation are still needed before microbial signatures can be routinely incorporated into forensic casework.

The major forensic applications of human microbial signatures are discussed in the following subsections, as well as the current evidence for their use in forensic identification.

Stability, Persistence, and Variability of Human Microbial Signatures

The forensic value of human microbial signatures is largely dependent on their stability (so that they can be interpreted meaningfully) and distinctiveness (so that one person may be distinguished from another). Unlike traditional genetic markers, microbial communities are living systems and are constantly adapting to internal and external factors. Thus, the reliability of microbiome-based forensic evidence is governed by the balance between temporal stability and stochastic biological variation rather than absolute microbial stability [23, 25].

Longitudinal studies have shown that many people have relatively stable microbial community structures over time, especially in the gut and in some skin-associated microbial habitats, despite normal daily fluctuations [23, 26]. The persistence is mainly attributed to the presence of a core microbiome that remains relatively stable under healthy conditions, while allowing less abundant microbial taxa to respond to environmental and physiological changes [24]. Such observations support the concept that microbial signatures may be sufficiently consistent to contribute to forensic investigations if samples are collected and interpreted appropriately.

However, many biological and environmental factors influence microbial signatures and could limit their forensic applicability. Microbial community composition can be affected to various degrees by age, diet, medication use, antibiotic exposure, health status, personal hygiene, occupation, as well as travel and environmental contact [19, 21, 22]. Further complicating interpretation are technical differences in sample type, collection time, storage conditions, sequencing methodology, and bioinformatic analysis. These factors underline the need for standardization of analytical protocols and the importance of considering contextual information in forensic evaluation.

Another crucial issue is the difference between intraindividual and interindividual variation. Within an individual, microbial communities tend to be more similar over time than between unrelated individuals, but the degree of variation varies by body site and may be altered by large physiological or environmental disruptions [23, 27]. Therefore, forensic interpretation should be based on probabilistic assessment of microbial similarity, not on the assumption of absolute microbial uniqueness.

Current data suggest that microbial profiles are sufficiently biologically stable to warrant further exploration as additional forensic biomarkers. However, their evidential value is contingent on rigorous laboratory standardization, appropriate statistical interpretation, and validation in populations diverse in geographic and demographic terms. More research, using longitudinal studies and multicentre investigations, is needed to assess the reliability, reproducibility and evidential weight of microbiome-based comparisons in forensic practice.

Forensic Value of Human Microbial Signatures

There is increasing interest in forensic microbiomics because the microbial signatures of humans have characteristics that can provide important forensic information not available from traditional biological evidence. Their body-site specificity, inter-individual variability and measurable temporal persistence support their investigation as complementary biomarkers that can assist forensic interpretation in the right circumstances [7, 23, 26]. While microbial signatures may not replace traditional forensic methods, they can provide additional biological information that can aid in the interpretation of complex forensic evidence.

One of the key benefits of microbial signatures is that they are capable of providing information in cases where conventional forensic evidence is sparse or lacking. Microbial communities can live on human tissues, personal items and environmental surfaces and

produce biological traces which can help in forensic reconstruction even if host DNA is degraded, present in low quantities or absent [7, 9]. Moreover, the progress in high throughput sequencing, bioinformatics and machine learning have significantly enhanced the resolution and interpretation of microbiome data, creating new opportunities for forensic investigation [36, 42].

However, despite these promising developments, some scientific and practical challenges still influence the evidential value of microbiome-based analyses. In addition to geographical differences, microbial community composition may depend on variability associated with environmental exposure, individual lifestyle, health status, and use of antimicrobials [28, 30]. Differences in laboratory protocols, sequencing platforms, and bioinformatic pipelines may influence analytical reproducibility. Therefore, standardized protocols, rigorous quality control and proper statistical approaches are still required for the valid interpretation of microbial data.

Another issue is the legal admissibility of microbiome-based evidence in forensic practice. To facilitate routine use of microbial signatures in the judicial process, multicentre validation studies are required to determine scientific validity, reproducibility and error rates, and international harmonization of analytical standards. In addition, objective interpretation and judicial assessment of microbiome-based evidence will necessitate comprehensive population databases and transparent reporting practices.

In general, the present evidence indicates that microbial signatures in humans have the potential to be a promising addition to, rather than a replacement for, traditional forensic methods such as forensic DNA profiling. Ongoing technological development, methodological standardization and interdisciplinary research are expected to strengthen the science base of forensic microbiomics and to expand its practical applicability. The next section details the major forensic applications of human microbiome analysis and critically appraises the current evidence for their use in forensic investigations.

Forensic Applications of the Human Microbiome

Personal Identification

Forensic science is mainly concerned with personal identification. This is traditionally achieved by well-established techniques such as DNA profiling, fingerprint analysis, dental records and other biometric identifiers. These methods are highly discriminative, but their effectiveness can be limited in forensic cases with degraded biological material, low-template DNA, mixed samples, or evidence compromised by environmental conditions. Thus, the human microbiome has been investigated as an additional source of biological information that might be useful for individual identification in some circumstances [7, 23].

The scientific basis for microbiome-based personal identification lies in the knowledge that every person has a unique microbial community determined by host genetics, physiology, immune status, exposure to the environment, lifestyle, and daily activities [1, 26]. Many microbial taxa are shared among healthy individuals, but differences in microbial composition, relative abundance, and functional characteristics result in individual microbial signatures that can be distinguished by high-throughput sequencing and advanced bioinformatic analyses [26, 27]. Longitudinal studies also suggest that many of these microbial features are stable enough over time to distinguish between individuals in the face of normal ecological variation [23, 25].

The skin microbiome of all body habitats has received the most forensic attention since it is constantly transferred to objects and surfaces during routine human contact. It has been shown that microbial communities recovered from commonly touched personal items can be similar to the microbial communities of their owners, indicating that skin-associated microorganisms could be used as additional evidence to link a person to a particular object or environment [7, 9]. Similar studies on oral and gastrointestinal microbiomes have also shown good potential for discrimination but their forensic applicability is restricted by sample availability, preservation and analytical methodology [13, 15].

The arrival of next-generation sequencing, metagenomics, bioinformatics, and machine learning has recently made great progress in improving the accuracy of classification and individual differentiation based on the microbiome [36, 42]. These technological advances allow the simultaneous analysis of complex microbial communities and the identification of microbial patterns that may not be detectable by conventional analytical approaches. However, predictive performance is still affected by standardized laboratory protocols, representative population datasets and rigorous computational validation.

Although significant progress has been made, microbiome-based personal identification is still an emerging forensic approach, rather than a well-established identification method. Inter-individual variability, environmental influences, temporal changes and methodological differences still affect the reproducibility and evidential interpretation of microbial profiles [28, 30]. Therefore, according to the available evidence, the use of human microbial signatures as alternative forensic biomarkers could be a complementary, but not alternative, approach to existing forensic DNA profiling methods. Routine use of microbiome-based personal identification in forensic casework will require further multicentre validation studies and the development of internationally standardised analytical frameworks.

Linking Individuals to Objects and Crime Scenes

The transmission of microorganisms between people, objects, and the environment is one of the most promising applications of forensic microbiomics. Just as every contact leaves a trace, according to the forensic truism, human interactions constantly leave characteristic microbial communities on frequently touched surfaces and personal belongings. These transferred microbial signatures can be used as further evidence to connect an individual to an object or to a specific location and can aid forensic reconstruction when interpreted with conventional evidence [9, 46].

The skin microbiome of the different microbial communities associated with the human body has been the most extensively studied because it is easily transmitted through routine physical contact. Microbial communities on mobile phones, keyboards, computer mice, clothes, door handles and other high-touch items tend to resemble those of their owners [9, 47]. Comparative analyses have shown that these microbial signatures can be used to evaluate the associations between individuals and the objects they contact, with the strength of association depending on contact frequency, microbial persistence, environmental exposure and substrate characteristics [47, 48].

Microbial transfer is also a valuable source of information during crime scene reconstruction. Microorganisms associated with humans and deposited on surfaces can complement fingerprint, touch

DNA and other trace evidence by providing additional biological context for contact events or handling of objects. Furthermore, comparing microbial communities isolated from forensic exhibits with those of suspects, victims or crime scene environments can help investigators to estimate possible interactions between people, objects and places [46, 49]. However, microbial transfer should be viewed probabilistically as the transfer efficiency, secondary transfer and environmental contamination can have a substantial effect on the recovered microbial profile.

Several factors affect the persistence and the evidence value of transferred microbial communities. Surface type, temperature, humidity, ultraviolet radiation, cleaning practices, time since deposition, and the biological characteristics of individual microorganisms all affect survival of microorganisms after transfer [48, 30]. Standardized sampling procedures, contamination control, and proper interpretation of statistics are therefore necessary to distinguish meaningful forensic signals from background environmental microbiota.

However, the application of microbial transfer evidence in forensic casework still suffers from a lack of standardized methodologies, validation on larger population sizes and interpretative guidelines despite promising findings obtained from laboratory settings. Future studies should aim to increase the reliability and admissibility of microbiome-derived trace evidence in forensic investigations, including multicentre validation studies, quantitative models of microbial transfer and persistence and internationally harmonised analytical protocols.

Geographical Origin and Biogeographic Inference

Geographical origin is a key component of forensic investigations involving unidentified human remains, missing persons, human trafficking, illegal migration, wildlife crime and transnational criminal activities. Traditional geolocation methods mainly rely on physical traces, geological materials, pollen, stable isotope analysis and intelligence information. Recent advances in forensic microbiomics have also seen the emergence of microbial community profiling as a complementary approach, as human-associated and environmental microbiomes show a measurable geographical variation that mirrors the regional ecological conditions and human-environment interactions [15, 21].

Various geographical factors such as climate, altitude, diet, sanitation, urbanization, cultural habits, and environmental exposure to microorganisms influence the composition of the human microbiome. Therefore, microbial community structures can often be distinguished in different geographical regions even though they share many common microbial taxa [15, 21]. Geographical location has been shown to be one of the major determinants of microbial diversity in large population-based microbiome studies, which is consistent with the hypothesis that microbial signatures could be informative of an individual's geographical origin or environmental history.

Environmental microbiomes provide additional support for forensic geolocation as soil, dust, water, vegetation and built environments harbor characteristic microbial communities that vary between geographic locations. These environmental microbes are routinely deposited on clothing, shoes, personal items, and exposed body surfaces during routine activities leaving microbial traces that may link forensic evidence to a particular location [46]. Thus, the comparison of environmental and human-associated microbiomes allows the study of previous environmental exposure and the geographical attribution in forensic casework.

Recent advances in high-throughput sequencing, microbial source-tracking algorithms, and machine learning have allowed significant improvements in the discrimination of geographically distinct microbial communities. Curated databases such as the Forensic Microbiome Database (FMD) have simplified comparative analyses by integrating publicly available microbiome datasets into uniform analytical frameworks for forensic research [47]. Similarly, recent population-based studies have shown the capacity of microbiome profiling to discriminate between geographically different populations under controlled experimental conditions, providing further evidence that the composition of microbial communities can be a promising marker of geographical origin [48]. However, predictive accuracy still depends on representative reference databases, standardized laboratory protocols, population diversity, seasonal variation, and external validation in different environmental conditions.

These encouraging advances notwithstanding, the use of the microbiome for geolocation should be regarded as a complementary forensic tool, rather than a stand-alone method for determining geographical origin at this time. The routine integration of microbial geolocation evidence into forensic investigations is not possible until more multicentre studies, geographically representative reference databases, harmonised analytical methodologies and robust statistical validation are available. Nevertheless, we expect that further advances in sequencing technologies, computational biology and microbial ecology will improve the reliability and forensic utility of microbiome-based biogeographic inference.

Estimation of Postmortem Interval

The reliable estimation of the postmortem interval (PMI) is one of the most difficult tasks in forensic investigation because the reliability of the conventional methods is affected by environmental conditions, individual variation, and decomposition stage. Traditional methods such as algor mortis, rigor mortis, livor mortis, forensic entomology and biochemical markers are informative but less accurate during advanced stages of decomposition. Therefore, microbiome-based approaches have been proposed as promising complementary tools for improving PMI estimation by investigation of predictable microbial succession after death [8, 46].

Following death, the absence of host immune regulation and physiological processes results in a progressive change in the composition of the microbial community. Endogenous microorganisms proliferate and migrate into previously sterile tissues and interact with environmental microbiota leading to characteristic patterns of microbial succession during decomposition. The postmortem microbial ecosystem, termed the thanatomicrobiome, shows temporal changes that have been correlated with the time since death under different experimental conditions [8, 47].

Decomposition not only changes the microbiological composition of the body, but also the environment around the body. The soil beneath the body experiences predictable changes in its microbial community as the body decomposes, creating what's called the epinecrotic microbiome. Comparison of both thanatomicrobiome and epinecrotic microbiome succession has shown better potential in PMI estimation as these microbial communities respond to

decomposition in complementary ways. Hence, combining body-associated and environmental microbial signatures may enhance the robustness of microbiome-based PMI models [47, 48].

Recent advances in high-throughput sequencing, metagenomics, bioinformatics and machine learning have significantly improved the analysis of postmortem microbial succession. Predictive models based on bacterial community composition have shown promising accuracy in controlled experimental environments, which suggests that microbial succession may serve as a reliable biological indicator of the stage of decomposition. However, predictive performance is still affected by temperature, humidity, geographical location, burial conditions, scavenger activity and other environmental variables affecting decomposition dynamics [42, 49].

While progress has been made, microbiome-based estimation of PMI has yet to be validated sufficiently for routine forensic use. However, differences in sampling protocols, sequencing methods, analytical pipelines and the microbial communities of different populations still hamper the reproducibility across studies. Moreover, the absence of internationally standardized reference datasets limits the comparison between laboratories. Microbial succession should thus be considered an auxiliary source of forensic information that may supplement traditional PMI estimation instead of replacing current forensic practices. The routine application of microbiome-based PMI estimation in forensic practice will depend on ongoing validation studies in multiple centers, standardized analytical frameworks and geographically diverse reference databases.

Sexual Assault Investigations

The identification and interpretation of biological evidence are crucial in sexual assault investigations to establish sexual contact and identify the perpetrator. Traditional forensic examinations are primarily directed towards obtaining semen, epithelial cells, saliva and DNA from sexual assault evidence kits. But the successful recovery of human DNA might be hindered by late reporting, post-assault hygiene, condom use, vasectomy or no ejaculation. Thus, the human genital microbiome has emerged as a promising alternative source of forensic evidence that can provide additional information when conventional biological markers are limited or unavailable [51].

The genital microbiome consists of diverse microbial communities that colonize the male and female reproductive tracts and show both body-site specificity and inter-individual variability. Microorganisms are exchanged between partners during sexual intercourse with a transient transfer of microorganisms that may persist beyond the time period when conventional biological evidence is detectable. Recent studies have shown that it is possible to distinguish between the microbial communities of the penis and vagina using high throughput sequencing and that transfer of microbes after sexual contact may help establish recent interpersonal contact under controlled conditions [51, 52]. The results indicate that microbial signatures of the genital area may serve as an additional type of evidence in sexual assault cases, especially when human DNA evidence is lacking.

Recent advances in next-generation sequencing and bioinformatic analysis have enhanced our ability to characterize genital microbial communities and identify microbial patterns associated with recent sexual activity. Moreover, experimental studies have demonstrated that microbial profiling is able to detect transferred bacterial communities even when the conventional forensic markers are degraded or absent, stressing its use as a complementary investigative tool and not as a replacement of the traditional DNA analysis [52]. Interpretation of genital microbiome evidence remains challenging however, as the microbial composition is affected by the menstrual cycle, hormonal status, personal hygiene, antibiotic exposure, sexual behaviour, contraceptive use and underlying health conditions.

Although research results are promising, evidence of microbiomes in sexual assault investigations is still in the early stages of development. Existing studies are characterized by relatively small sample sizes, heterogeneous methodology and lack of standardized analytical protocols applicable to forensic casework. Before genital microbiome profiling can be routinely incorporated into forensic practice, further multicentre validation studies, population-based investigations and internationally harmonized laboratory guidelines are needed. At present, microbiome analysis can be considered a complementary technique that can aid in the interpretation of evidence in sexual assault cases when used in conjunction with conventional forensic genetics and other biological evidence.

Human Activity and Lifestyle Reconstruction

The composition of human-associated microbial communities is largely shaped by lifestyle, daily activities and environmental exposures of an individual. The composition and functional characteristics of the microbiome are continuously affected by factors such as diet, occupation, smoking, alcohol, physical activity, travel, hygiene, medication and contact with animals or the natural environment [15, 21]. Therefore, microbial profiling has been recognized as a useful tool to reconstruct certain aspects of an individual's lifestyle that could provide significant contextual information in forensic investigations.

Many studies have shown that long-term lifestyle factors induce measurable changes in the structure of microbial communities. Dietary patterns impact the diversity and metabolic capacity of the gut microbiome, and smoking, alcohol consumption, and antibiotic exposure are associated with typical changes in microbial composition [21, 53]. Likewise, microbial communities specific to body sites are likewise influenced by occupational and environmental exposures through repeated exposure to unique microbial ecosystems. The results indicate that microbiome analysis may offer indirect information about habitual behaviours and environmental interactions in an appropriate forensic context.

Recent advances in metagenomics, bioinformatics and machine learning have improved the ability to identify microbial patterns associated with lifestyle characteristics. Predictive models have shown promising performance in discriminating behavioural and environmental factors from complex microbiome datasets and have highlighted the potential of microbial signatures to complement conventional forensic intelligence [42, 54]. But the predictive value of such models is contingent on robust training datasets, standardized analytical pipelines, and external validation across diverse populations.

Yet, lifestyle reconstruction based on microbiome analysis is still subject to important limitations. Many of the microbial signatures with lifestyle are overlapping. Microbial composition is influenced by age, genetics, geographical location, disease status and short-term environmental changes simultaneously. Moreover, the interpretation of microbial evidence is complicated by temporal variation and inter-individual diversity, especially when the goal is to infer specific behaviours from a single biological sample. Lifestyle information

Table 3: Major Applications of Forensic Microbiomics in Criminal Investigations.

Forensic Application	Microbial Evidence Used	Potential Contribution	Current Status
Personal Identification	Skin, oral, gut, and individual-specific microbial communities	Supports differentiation of individuals based on unique microbial signatures	Emerging
Linking Individuals to Objects and Crime Scenes	Skin microbiota transferred to objects and environmental surfaces	Associates individuals with touched objects or crime scenes through microbial transfer	Experimental
Geographical Origin and Biogeographic Inference	Human-associated and environmental microbiomes	Assists in determining geographical origin or previous environmental exposure	Emerging
Postmortem Interval (PMI) Estimation	Thanatomicrobiome and epinecrotic microbiome	Estimates time since death through predictable microbial succession	Advanced research
Sexual Assault Investigations	Genital microbiome and transferred microbial communities	Complements conventional biological evidence by identifying microbial transfer following sexual contact	Experimental
Human Activity and Lifestyle Reconstruction	Gut, skin, and oral microbiomes	Provides information regarding lifestyle, environmental exposure, diet, and occupation	Emerging
Mass Disaster and Decomposed Remains Identification	Postmortem and environmental microbial communities	Supports victim identification and interpretation of highly decomposed remains	Developing

Abbreviations: PMI = Postmortem Interval.

from the microbiome should thus be viewed as an aid and not as conclusive forensic evidence.

Future research should aim at large-scale longitudinal studies, population-specific reference databases, and standardized computational models capable of distinguishing persistent lifestyle-associated microbial signatures from fleeting ecological variation. The combination of microbiome profiling with traditional forensic evidence, environmental data and advanced artificial intelligence is expected to improve the reliability of human activity and lifestyle reconstructions, thus increasing the contribution of microbiome analysis to forensic investigations.

Identification in Mass Disasters and Decomposed Remains

One of the most challenging problems in forensic science is the identification of victims in mass disasters and advanced decomposition. Natural disasters, terrorist attacks, transportation accidents, armed conflict and mass fatality incidents often result in fragmented, burned, skeletonized or severely decomposed human remains, limiting the efficacy of conventional methods of identification. While DNA profiling is still the method of choice for disaster victim identification (DVI), microbial analysis has recently become a promising complementary approach, which can offer additional biological information in case the conventional evidence is compromised [8, 49].

Postmortem microbial communities undergo predictable ecological succession in the body and surrounding environment. Such postmortem microbial changes can last long after the extensive decomposition of soft tissues, yielding biological information that may aid in characterizing decomposition stage and improving interpretation of highly degraded remains [8,50]. Moreover, microbiota associated with bones, teeth and burial environments have been promising in assisting forensic investigations of skeletonized or environmentally exposed remains, especially when considered together with anthropological, pathological and genetic evidence.

Microbiome analysis may also contribute to victim identification in mass disaster investigations through characterization of environmental microbial signatures associated with disaster sites or burial environments. The combination of microbial profiling with forensic genetics, forensic anthropology, odontology and radiological examinations can improve multidisciplinary identification strategies by providing complementary biological information during complex disaster victim identification operations [51]. However, microbiome analysis cannot currently replace established identification techniques, but should be seen as an additional source of forensic evidence.

However, there are still several limitations that hinder the routine application of microbiome analysis for disaster victim identification. Environmental variability, contamination, decomposition conditions, geographical differences and methodological inconsistencies may have a significant impact on microbial community composition and reduce reproducibility between investigations. Additionally, without standardized sampling procedures, validated analysis pipelines, and internationally accepted interpretation guidelines, the evidential value of microbiome data in mass fatality investigations is reduced

Future studies should include large multicentre validation studies, standardised laboratory methodologies and the compilation of extensive microbial reference databases that cover different environmental and geographic settings. In the future, the integration of microbiome profiling with other forensic identification methods and advanced computational techniques may improve the interpretation of highly degraded remains and enhance the overall effectiveness of disaster victim identification. At present, microbiome analysis should be regarded as a complementary tool that could contribute to multidisciplinary forensic investigations in mass disasters and advanced decomposition but not as a substitute for established forensic identification methods (Table 3).

Comparison with Conventional DNA Profiling

Principles of DNA Profiling

For more than 30 years, DNA profiling has been at the core of modern forensic identification because of its high discriminatory power, reproducibility and well-established scientific basis. DNA analysis has revolutionized criminal investigations, identification of victims of disasters, paternity testing and identification of missing persons since its introduction into forensic science, allowing reliable individualization based on genetic variation. Standardized laboratory protocols, validated analytical methods, and comprehensive DNA databases have been implemented worldwide, establishing DNA profiling as the gold standard for human identification internationally [55, 56].

Today, forensic DNA profiling is based on the analysis of short tandem repeats (STRs), highly polymorphic regions of the human genome that are distributed throughout the genome. The number of repeat units in several STR loci varies among persons, so that a unique genetic profile can be generated with a very high discrimination power. DNA is extracted, STR loci amplified by polymerase chain

reaction (PCR) and analyzed by capillary electrophoresis to generate a DNA profile that can be compared to reference samples or national DNA databases [57]. Besides autosomal STRs, Y-chromosomal STRs, X-chromosomal STRs and mitochondrial DNA are routinely analyzed in specific forensic scenarios such as kinship analysis, sexual assault investigations and highly degraded biological samples [58].

Internationally, DNA profiling has been validated, quality assurance systems have been standardized and interpretation guidelines have been universally accepted. Accredited forensic laboratories routinely use rigorous analytical protocols that are designed to ensure reproducibility, minimize contamination, and allow for statistical evaluation of evidential significance. Moreover, the creation of national DNA databases has greatly enhanced the efficiency of investigations by allowing for the comparison of evidence gathered from crime scenes with the profiles of known offenders or missing persons [59].

DNA profiling is a remarkable success, but it is not without its limitations. Environmental degradation, low-template samples, DNA mixtures, contamination or prolonged exposure to adverse environmental conditions can affect the quality and quantity of recoverable DNA. In some forensic cases, biological material may not be enough to carry out a complete genetic profiling, and conventional DNA analysis provides limited information about an individual's lifestyle, environmental exposure, geographical history or postmortem microbial changes [60]. These limitations have motivated the search for complementary biological markers, such as the human microbiome, which may provide additional context beyond the human genome.

The continued development of forensic microbiomics should therefore be considered complementary, rather than a replacement, to traditional DNA profiling. To evaluate the potential advantages of microbiome-based evidence, discussed in the following subsection, it is useful to be aware of the strengths and limitations of the current DNA-based identification.

Advantages of Microbiome-Based Identification

Besides conventional genetic evidence, microbiome analysis offers several unique advantages, but forensic DNA profiling remains the gold standard for human identification. Microbial communities are dynamic biological information reflecting the interaction between an individual and their surrounding environment, whereas human DNA primarily establishes identity or biological relationship. Therefore, microbiome profiling can provide contextual information that can't be obtained from traditional DNA analysis alone [7, 26].

One of the key advantages of microbiome-based identification is the information it can provide when traditional DNA evidence is limited. Microbes associated with humans can persist on the skin, clothing, personal objects, environmental surfaces and decaying bodies, even when host DNA is degraded or present in quantities too low for complete genetic profiling. In these cases, microbial signatures may provide additional biological evidence that can be used together with conventional DNA results to aid in forensic interpretation [49, 50].

Microbiome analyses also provide opportunities for reconstructing biological and environmental interactions. The composition of human microbial communities is determined by body site, geographical location, environmental exposure, diet, lifestyle, occupation and health status, and allows investigators to infer information about the recent activity or environmental history of an individual if the conditions are appropriate [15, 21, 46]. In addition, microorganisms transferred during contact between individuals and objects may offer complementary information on contact events and crime scene reconstruction when analyzed in conjunction with fingerprints, touch DNA and other trace evidence [47].

Another major advantage is the possible use of microbiome analysis for postmortem investigations. The predictable microbial succession during decomposition has proven to be highly promising in estimating the postmortem interval and in assessing decomposition processes, which are applications beyond the reach of conventional DNA profiling [8, 49]. In addition, advances in metagenomics, bioinformatics and machine learning have improved the description of complex microbial communities, increasing the analytical potential of microbiome-derived forensic evidence [42].

Microbiome-based identification, however, should not be considered a substitute for forensic DNA profiling. Its true power lies in its capacity to add biological information to inform forensic interpretation when used in conjunction with existing genetic, anthropological, entomological, and trace evidence. Continued methodological standardization, large-scale validation studies and internationally harmonized analytical protocols will further define the role of microbiome analysis in future multidisciplinary forensic investigations.

Limitations Compared with DNA Profiling

Microbiome-based identification has still many limitations that prevent its application to the same extent of reliability and acceptance as conventional DNA profiling. Forensic microbiomics is getting more and more attention, however. The DNA of humans is stable in the life of an individual (except for very rare somatic mutations) and provides an extremely discriminatory genetic profile that is largely independent of lifestyle or environmental exposure. Unlike forensics, microbial communities are dynamic biological systems that respond to intrinsic and extrinsic factors in real time, leading to temporal and spatial variability that could confound forensic interpretation [19, 21, 23].

One of the main advantages of DNA profiling lies in the international standardization of analytical protocols and the validation of quality assurance systems. STR-based DNA analysis relies on well-established laboratory procedures, supported by accredited forensic laboratories, extensive proficiency testing and internationally accepted guidelines for interpretation. In contrast, microbiome analysis is not yet standardized at the levels of sample collection, DNA extraction, sequencing platforms, bioinformatic pipelines, and statistical interpretation, which leads to variability among studies and limited reproducibility between laboratories [28, 29, 30].

Another key difference between the two approaches is the population scale validation. The availability of large population databases and decades of forensic casework supports robust statistical evaluation of evidential significance Whilst the number of microbiome studies is rapidly increasing, they are often restricted to small cohorts, geographically restricted populations and controlled experimental settings. Consequently, there is a lack of extensive microbial reference databases and validation studies representative of the international population [15, 46].

Microbiome-derived evidence is also more complicated to

Table 4: Comparison Between Conventional DNA Profiling and Microbiome-Based Identification.

Parameter	Conventional DNA Profiling	Microbiome-Based Identification
Primary Biological Target	Human genomic DNA	Human-associated microbial communities
Basis of Identification	Individual genetic variation (e.g., STR markers)	Individual-specific microbial composition and diversity
Stability Over Time	Highly stable throughout life	Dynamic; influenced by age, diet, health, lifestyle, and environmental exposure
Discriminatory Power	Very high	Promising but currently lower than DNA profiling
Sample Requirements	Human biological material containing sufficient DNA	Human or environmental samples containing microbial DNA
Information Provided	Identity, kinship, and biological relationships	Identity support, microbial transfer, postmortem changes, geographical origin, lifestyle, and environmental exposure
Performance with Degraded Samples	May be reduced when DNA is highly degraded	Microbial communities may persist in some circumstances, providing complementary information
Standardization	Internationally standardized protocols and quality assurance systems	Standardization is still under development
Reference Databases	Extensive national and international DNA databases	Limited forensic microbiome databases; global repositories are under development
Judicial Acceptance	Widely accepted as forensic evidence	Emerging; requires additional scientific validation and legal acceptance
Major Limitations	DNA degradation, mixed samples, low-template DNA	Temporal variability, contamination, methodological inconsistency, limited validation
Role in Forensic Science	Primary method for human identification	Complementary tool supporting conventional forensic analyses

interpret from an evidential standpoint than traditional DNA evidence. Age, diet, medication, disease, environmental exposure, hygiene practices and geographical location are factors which may influence the composition of the microbial community, making it more difficult to distinguish between natural biological variation and forensically relevant differences [21, 22]. Moreover, contamination during sample collection or laboratory processing can dramatically affect microbiome profiles, especially in low-biomass forensic samples, necessitating rigorous quality-control measures across the analytical workflow [30, 31].

Legal admissibility is also different for microbiome analysis compared to conventional DNA profiling. Judicial systems widely accept DNA evidence due to its proven scientific validity, standardized methodologies, and well-established statistical framework. By contrast, evidence derived from the microbiome has not yet been subjected to the rigorous validation required for routine courtroom use. Similar to forensic microbiomics, multicentre validation studies, standardised laboratory protocols, transparent reporting standards and internationally accepted interpretation guidelines will be necessary to attain similar evidential status. Therefore, at this stage microbiome analysis should be considered as an additional forensic method that complements but does not replace traditional DNA profiling.

Complementary Use of DNA and Microbiome Evidence

The future of forensic identification will probably see the combination of several types of biological evidence instead of one analytical method. While conventional DNA profiling remains the most reliable method of individual identification, microbiome analysis can provide additional contextual information which may enhance forensic interpretation in cases where human DNA alone cannot fully answer the investigative question. Hence, the complementary use of host genetic and microbial evidence is a multidisciplinary approach combining the benefits of both methodologies and overcoming their respective limitations [7, 36].

DNA profiling provides highly discriminative and genetically stable information for identity, kinship and biological relationship determination. On the other hand, the analysis of the microbiome can reveal microbial transfer, environmental exposure, geographic origin, decomposition processes, and lifestyle-associated characteristics that are not directly available from the human genome [8, 15, 21]. Forensic biologists can integrate these independent biological datasets to achieve a more holistic interpretation of forensic evidence, particularly in complex investigations involving degraded biological samples, mixed evidence or advanced decomposition.

Recent advances in high-throughput sequencing, metagenomics, bioinformatics and artificial intelligence, have made possible the simultaneous analysis of host and microbial DNA from the same forensic specimen. Such integrated analytical workflows may enhance the interpretation of complex biological evidence by combining the high discriminatory power of STR-based DNA profiling with the ecological and environmental information provided by microbial communities [42, 54]. With the continued evolution of sequencing technology, combined host-microbiome analysis is likely to become more feasible within forensic laboratories.

Although integrated approaches have potential advantages, their successful implementation will require standardized laboratory protocols, validated analytical pipelines, comprehensive quality assurance procedures and harmonized reporting guidelines. Furthermore, the establishment of large reference databases, multicentre validation studies and internationally accepted statistical frameworks will be crucial to ensure the reliability and reproducibility of combined forensic analyses. Furthermore, close collaboration between forensic geneticists, microbiologists, bioinformaticians, anthropologists and legal professionals will be required to promote responsible implementation of microbiome-based evidence in routine forensic practice.

The current evidence suggests that microbiome analysis should be used as an auxiliary forensic tool and not as a substitute for standard DNA profiling. Further technological advances and interdisciplinary research are likely to improve the integration of microbial and genetic evidence and hence the accuracy, reliability and evidential value of forensic investigations. This integrated perspective provides the basis to tackle the remaining scientific, technical and legal challenges that continue to influence the routine implementation of forensic microbiomics (Table 4).

Challenges and Limitations

Lack of Standardized Methodologies

A major hurdle to routine application of forensic microbiomics is the lack of universally standardized methodologies. Unlike traditional forensic DNA profiling, which is based on internationally accepted laboratory procedures and quality assurance standards, microbiome analysis exhibits substantial methodological variability throughout the analytical workflow. Sample collection, preservation, DNA extraction, sequencing strategies, bioinformatic processing and statistical analysis all differ and can significantly impact microbial community profiles, making direct comparison among studies and laboratories difficult [28, 29].

Variability starts at the sampling stage in which the variation in sampling devices, collection techniques, storage conditions, transport procedures and time between sample collection and processing may change the microbial composition. These pre-analytical factors are especially important in low-biomass forensic samples, where small methodological differences may influence downstream analysis and introduce analytical bias [30, 31].

Other sources of variability are introduced by subsequent laboratory procedures. Different DNA extraction procedures lyse microorganisms with different cell wall structures with varying efficiency and sequencing platforms differ in terms of read length, sequencing depth, accuracy and analytical performance. Moreover, the choice of target genomic regions, sequencing chemistry, reference databases and taxonomic classification algorithms may result in different microbial profiles from the same biological samples [28, 36].

Another major source of methodological inconsistency is bioinformatic analysis. Variation in quality control procedures, sequence filtering, taxonomic assignment, diversity analysis and statistical modelling can significantly affect the interpretation of microbiome datasets. The absence of harmonized computational pipelines reduces the reproducibility of studies and complicates the comparison of findings reported in the literature, thus impeding the development of universally accepted forensic protocols [39, 40].

Thus, the future development of forensic microbiomics requires the establishment of standardized methodologies. Internationally harmonized sampling procedures, validated laboratory protocols, quality assurance systems, reference materials and standardized bioinformatic workflows will improve reproducibility, allow for inter-laboratory comparisons and increase the scientific credibility of microbiome-derived evidence. The development of internationally accepted and routine forensic practice methodological guidelines will be fundamentally dependent on continued collaboration among forensic scientists, microbiologists, bioinformaticians, standards organizations and regulatory bodies.

Temporal and Spatial Variability

The dynamic nature of the human microbiome is one of the greatest challenges to its routine use in forensics. Unlike the human genome, which is relatively static over the life of an individual, microbial communities are in constant flux responding to biological, environmental and behavioural influences. While many microbial signatures are sufficiently robust to support forensic investigation, temporal and spatial variability can complicate the interpretation of microbiome-derived evidence and its reproducibility across different forensic scenarios [23, 25].

Temporal variability is a result of microbial communities undergoing temporal changes in response to factors such as aging, dietary modification, medication use, antibiotic exposure, disease, hormonal fluctuations, travel, seasonal changes, and environmental interactions. Longitudinal studies have shown that many people have relatively stable core microbial communities, but changes in less abundant taxa can alter microbial profiles and affect comparative analyses, particularly when samples are collected over longer time periods [19, 22, 23].

Spatial variability also complicates forensic interpretation, as microbial populations differ substantially across anatomical regions. Each of these sites—skin, oral cavity, gastrointestinal tract, respiratory tract, and genitourinary tract—has a distinct microbial ecosystem that is adapted to the local physiological conditions. Even within the same body site, microbial composition can vary depending on moisture, temperature, sebum production, oxygen availability and host immunological activity, highlighting the importance of consistent sample procedures in forensic investigations [3, 11, 13].

Ongoing modification of human-associated microbial ecosystems also drives regional variation by environmental factors. The microbial composition is affected to varying degrees by climate, geographic location, occupation, housing conditions, animal interactions, sanitation practices and environmental microbiota [15, 21]. Thus, microbial profiles obtained from individuals residing in different contexts can reveal substantial changes despite the same genetic backgrounds, highlighting a need to consider environmental context during forensic interpretation.

Large longitudinal studies, geographically representative population studies, and standardized sampling protocols are needed to address temporal and spatial variability and to distinguish normal ecological variation from forensically relevant microbial differences. Knowledge of the microbial stability at different body sites and under different environmental conditions will be important to improve the reliability, reproducibility and evidentiary value of microbiome-based forensic analyses.

Environmental Contamination

Environmental contamination poses a significant challenge in forensic microbiomics, as microbial populations are ubiquitous and can be introduced at every step of the forensic process. Unlike traditional DNA profiling, which looks only at mostly human DNA, microbiome analysis includes bacteria naturally found on human skin, laboratory surfaces, collecting tools, chemicals and the environment. Therefore, distinguishing the true forensic microbial signatures from contaminating microorganisms is essential for the accuracy and reliability of the microbiome-based evidence [30, 31].

Contamination can happen at any stage of the process from crime scene investigation, evidence collection, transit, laboratory processing, DNA extraction, sequencing and bioinformatic analysis. Improper handling procedures, inadequate storage conditions, repeated sample manipulation, and the use of contaminated reagents may alter microbial community composition, especially in low-biomass forensic samples where even small quantities of exogenous microbial DNA can substantially influence analytical results [30]. Such contamination may result in erroneous taxonomic assignment, biased diversity estimation and wrong forensic interpretations.

Environmental exposure after evidence deposition further complicates microbiome analysis. Microbial communities recovered

from forensic evidence may be influenced by temperature, humidity, UV radiation, precipitation, soil contact, air circulation and interactions with ambient microbes over time. Furthermore, subsequent microbial transfer between humans, objects, and ambient surfaces may obscure the original microbial signature and complicate the reconstruction of forensic events [9, 46]. These variables highlight the importance of the environmental context for interpreting microbiome-derived data.

Strong quality assurance procedures should be used throughout the analytical workflow to reduce contamination. Potential sources of exogenous microbial DNA can only be detected by sterile collection techniques, DNA-free consumables, extraction blanks, reagent controls, negative controls and contamination-monitoring systems [28, 30, 31]. Standardized laboratory techniques, specialized clean-room facilities, and proven contamination-control protocols all improve analytical reliability and reduce the possibility of false-positive results.

Even with improvements in sequencing technology and computational contamination-detection methods that have increased the reliability of microbiome analysis, environmental contamination remains a major hurdle to routine forensic application. Continued development of standardized contamination-control guidelines, validated laboratory workflows, and robust bioinformatic filtering approaches will be critical to increase confidence in microbiome-derived forensic evidence and to facilitate its future application in forensic investigations.

Reproducibility and Validation Issues

Reproducibility and scientific validation are essential requirements for the acceptance of any forensic method. Although microbiome-based analyses have shown much promise in experimental research, reproducibility between laboratories, populations, and analytical platforms remains a major challenge. Inconsistencies in laboratory procedures, sequencing technologies, computational workflows and statistical analyses can lead to inconsistent microbial profiles, thereby reducing confidence in the interpretation of microbiome-derived forensic evidence [28, 29].

One of the main barriers to reproducibility is the absence of validated, standardized frameworks for forensic microbiomics. Microbiome based methods are still being evaluated in various experimental settings, whereas conventional DNA profiling has been extensively developed, internally validated and inter-laboratory proficiency tested. As a result, differences in study design, sample preparation, sequencing depth, taxonomic classification and data processing often complicate comparisons between published investigations [61].

Another important limitation is the relatively small sample size of many published studies. Studies have been conducted on small groups of people or in highly controlled lab settings, making it difficult to translate the findings to real-life forensic investigations. Moreover, microbial community composition is affected by geographical location, ethnicity, environmental exposure and lifestyle differences, highlighting the need for large multicentre studies with different populations to generate reliable forensic reference standards [62].

Development of robust validation strategies should also include a comprehensive evaluation of analytical sensitivity, specificity, precision, accuracy, repeatability, reproducibility and error rates. Standardized quality assurance programs, certified reference materials, external proficiency testing and clear reporting guidelines are needed to evaluate analytical performance and to guarantee consistency between forensic laboratories. These are already well established in forensic DNA analysis and should similarly form the basis of future forensic microbiome investigations [61].

Recent technological advances in sequencing platforms, computational biology, and machine learning have improved analytical consistency, but these technological improvements cannot substitute for rigorous scientific validation. Reliability and evidential value of microbiome-based forensic analyses will be essential, requiring ongoing international collaboration, multicentre validation studies, standardized reporting practices and independent replication of published findings. Ultimately, the improvement of reproducibility will determine whether forensic microbiomics will be able to move from an emerging research field to a routinely accepted forensic discipline.

Insufficient Reference Databases

The development of comprehensive, representative reference databases is fundamental to successful implementation of any forensic identification system. Traditional forensic DNA profiling relies on large population databases to provide robust statistical comparisons and to improve the interpretation of genetic evidence. Forensic microbiomics, however, currently lacks large-scale standardized reference databases that sufficiently represent microbial diversity across different populations, geographical regions, body sites and environmental conditions. This limitation greatly impedes the routine use of microbiome-derived evidence in forensic investigations [46, 61].

Most microbiome studies published to date have been done on relatively small cohorts, specific geographic locations, or highly controlled experimental settings. Consequently, the available datasets often lack the considerable diversity associated with ethnicity, age, diet, lifestyle, climate, environmental exposure and socioeconomic factors. Since these variables affect microbial community composition, reference databases constructed from small populations may not be representative of larger forensic populations and thus decrease the accuracy of comparative analyses [15, 21, 62]. Thus, increasing the size of microbiome datasets with geographically and demographically diverse populations continues to be a major research priority.

Another major challenge is the inconsistency among publicly available microbiome repositories. Differences in sampling methodologies, DNA extraction methods, sequencing platforms, taxonomic annotation, metadata collection, and bioinformatic processing limit interoperability between datasets and impede cross-study comparisons. In addition, the forensic value of the available microbiome resources is further limited by the lack of consistent information about the characteristics of the participants, environmental conditions, and analytical techniques [63].

Recent efforts have centered on constructing dedicated forensic microbiome libraries that can integrate microbial profiles with standardized metadata and analysis workflows. Resources such as the Forensic Microbiome Database [47] provide advances in the development of unified platforms for microbial comparison, data exchange, and the development of robust forensic classification models. However, the development of validated reference data sets that accurately reflect the global microbial diversity and are suitable for routine forensic casework will require broad international

collaboration.

Standardized sampling methods, thorough metadata collection, stringent quality control, and continuous data curation should be central to future database initiatives. Internationally coordinated efforts between forensic laboratories, research institutions and public health organizations will be necessary to develop reliable microbial reference databases that improve the scientific validity, reproducibility and evidential value of forensic microbiomics.

Interpretation of Complex Microbial Data

The interpretation of forensic evidence from microbiome-derived data is one of the most technically demanding aspects of forensic microbiomics. In contrast to conventional forensic DNA profiling, which targets relatively stable genetic markers, microbiome analysis produces high dimensional datasets of thousands of microbial taxa with complex ecological interactions. Microbial communities are diverse, compositionally complex and dynamic, and their accurate analysis and interpretation calls for sophisticated computational approaches [36, 40].

A major challenge is the complexity of bioinformatic workflows for processing sequencing data. Different analytical pipelines use different quality-control procedures, taxonomic classification algorithms, reference databases, sequence filtering criteria, and statistical models. Thus, the same raw sequencing data can yield different microbial profiles based on the computational methods employed, causing inconsistency between studies and complicating the forensic interpretation [39, 63].

Machine learning and artificial intelligence have greatly improved the analysis of complex microbiome datasets, enabling the detection of patterns that are hard to see with traditional statistical methods. Algorithms such as random forests, support vector machines and deep learning models have shown promising performance in personal identification, postmortem interval estimation and geolocation inference. However, many of these models are “black-box” systems that have limited biological interpretability and it is difficult to explain how specific conclusions are reached. This opaqueness creates major problems for scientific validation and testimony in court [64, 65].

Further limitations include the lack of a universally accepted statistical framework for evaluating evidence from microbiomes. In contrast to DNA profiling, where population frequencies and likelihood ratios are well established, there are currently no standardized probabilistic models to assess the strength of evidence in microbiome analysis. An important research priority is the development of robust statistical methodologies that can quantify uncertainty, measure confidence and support objective interpretation [61, 63].

Addressing these challenges will necessitate further progress in bioinformatics, explainable AI, standardized computational pipelines and forensic statistical modelling. International collaboration between microbiologists, bioinformaticians, statisticians, computer scientists and forensic practitioners will be necessary to establish transparent and reproducible analytical frameworks that improve the reliability and evidential value of microbiome-based forensic investigations.

Admissibility in Court

The value of any forensic technique is not only determined by its scientific validity, but also by its acceptance in the legal system. Forensic microbiomics has shown great promise in the research setting, but the judicial acceptance of microbiome-based evidence has not yet reached the level of traditional forensic DNA profiling. Analytical reliability, reproducibility, limitations and error rates must be rigorously scientifically validated and independently verified before such evidence can routinely be presented in court [61, 67].

In general, the admissibility of scientific evidence requires a showing of the reliability, reproducibility, and acceptance of the underlying methodology in the scientific community. Currently, several factors limit the acceptance of forensic microbiomics in the courtroom, including methodological variability, lack of standardized laboratory protocols, insufficient population-scale validation and limited consensus on the interpretation of complex microbial datasets. These challenges make it difficult to establish consistent evidential standards appropriate for judicial proceedings [28, 29, 30].

Another important consideration is how to statistically interpret evidence derived from the microbiome. Unlike standard DNA profiling, where well-developed population databases and likelihood-based statistical frameworks exist, there is no universally accepted way of expressing the strength of the evidentiary associations for microbiome analysis. Developing transparent probabilistic models, validated reporting standards, and objective measures of uncertainty will be essential to support expert testimony and allow for judicial evaluation of microbiome evidence [61, 66].

Internationally harmonised quality assurance programmes will also be critical for increasing legal confidence in forensic microbiomics. Each forensic laboratory should implement standardized protocols for evidence collection, laboratory processing, sequencing, bioinformatic analysis, contamination control, and reporting to ensure consistency and reproducibility. External proficiency testing, laboratory accreditation and independent validation studies will further contribute to the scientific credibility of microbiome-based analyses and will foster confidence among investigators, legal practitioners and courts [67, 68].

While it is unlikely that evidence from the microbiome will replace traditional forensic DNA profiling in the near future, it has great potential as a complementary line of evidence when supported by robust scientific validation and interpreted in conjunction with traditional forensic techniques. Developing internationally accepted standards that allow the responsible and legally defensible use of forensic microbiomics in future judicial proceedings will rely on continued collaboration between forensic scientists, microbiologists, statisticians, legal experts and regulatory bodies.

Ethical, Legal, and Regulatory Considerations

Privacy and Ownership of Microbiome Data

The expanding use of microbiome analysis in forensic science has raised important questions about the privacy and ownership of microbiome-derived data. Unlike traditional forensic DNA profiling that is primarily used to establish biological identity, the human microbiome can provide information about an individual’s health status, diet, lifestyle, environmental exposures, and geographic history. As microbiome research advances, the potential to infer personal information from microbial communities is likely to increase, underscoring the need for strong privacy protections and responsible data governance [66, 69].

A key issue is who owns the microbiome data generated from

forensic samples. In criminal investigations, biological samples can be taken, but it is unclear who owns the resulting microbiome data, how long the data should be stored, and when it can be reused. Questions also arise when datasets of microbiomes generated for clinical or research purposes are later considered for forensic investigations. Clear policies defining ownership, permissible use, and long-term stewardship of microbiome-derived information are therefore essential to prevent misuse and maintain public confidence in forensic research and practice [70].

Microbiome profiles can reveal sensitive personal information, and therefore the need for effective privacy protection. Unlike traditional forensic genetic markers, microbial communities may reveal medical status, recent drug use, diet, travel history, occupational exposures, and other personal traits that are not relevant to the goals of a forensic investigation. Access to such information without adequate safeguards could risk discrimination, unauthorized surveillance or inadvertent disclosure of private health-related information. Therefore, the collection and analysis of microbiome evidence should be restricted to information that is clearly relevant to the forensic question at hand [66, 71].

Comprehensive governance frameworks that foster transparency, accountability and responsible data stewardship are necessary to protect individual privacy, while also enabling scientific and forensic advancement. Where applicable, policies on informed consent, controlled access to microbiome databases, data minimization, anonymization and well-defined retention and sharing procedures will be essential to strike a balance between investigative needs and fundamental privacy rights. As forensic microbiomics moves toward operational deployment, internationally harmonized standards for privacy and data ownership will be important for maintaining public trust and the ethical use of microbiome-derived evidence.

Ethical Issues in Forensic Investigations

Including microbiome analysis in forensic investigations raises a number of ethical issues that go beyond those raised by traditional forensic methods. Although microbial evidence could enhance criminal investigations and bolster forensic interpretation, its use must be weighed against basic ethical principles of respect for individual autonomy, proportionality, transparency, and justice. Because microbiome profiles may reveal information not relevant to the purpose of an investigation, forensic practitioners must ensure that analyses are restricted to data that are directly relevant to the legal questions at hand [66, 69].

Concerns about the secondary use of biological samples arise with the collection and use of microbiome evidence as well. Samples initially collected for medical diagnosis, biomedical research or public health surveillance may later have forensic value, creating uncertainty whether they can ethically be re-purposed for law enforcement investigations. Such practices may be at odds with the expectations of individuals who have supplied biological samples for non-forensic uses. Hence, clear governance policies and ethical oversight are required to define the circumstances under which secondary use of information derived from the microbiome may be warranted while respecting individual rights and preserving public trust [70, 71].

Another key ethical issue concerns the potential for bias and discrimination in microbiome-based forensic investigations. Microbial communities are affected by geography, socioeconomic status, occupation, diet, health status and environmental exposure. If not carefully considered in the interpretation of data or development of algorithms, these influences can produce systematic bias or differential outcomes in different populations in microbiome-based models. Ethical implementation thus requires representative datasets, transparent analytical methodologies, ongoing evaluation of algorithmic performance, and periodic evaluation of potential sources of bias [72].

Interdisciplinary collaboration between forensic scientists, microbiologists, bioethicists, legal professionals, policymakers and regulatory authorities will be essential for comprehensive ethical governance to ensure the responsible integration of microbiome analysis into forensic practice. Ethical review mechanisms, transparent reporting practices, public engagement, and international harmonization of professional guidelines will be key to ensure that microbiome-based evidence is generated and applied in ways that protect individual rights while supporting the legitimate goals of forensic investigations.

Data Protection and Security

The increasing use of high-throughput sequencing and the existence of large microbiome databases has emphasized the importance of strict data protection and security for forensic microbiomics. Microbiome datasets are typically accompanied by biological and other metadata that may reveal sensitive information about an individual's health, lifestyle, environmental exposures, and geographical background. As the amount of microbiome data grows, it is increasingly important to protect these datasets from unauthorized access, misuse, or accidental disclosure as a key requirement for responsible forensic practice [71, 73].

Effective data protection needs strong governance across the entire data lifecycle – from sample collection to lab analysis, data storage, sharing and long-term archival. To mitigate the risk of unauthorized access and ensure data integrity, secure digital infrastructure, controlled-access repositories, encryption technologies, user authentication systems, and detailed audit trails are critical. In addition, transparent policies for data retention, sharing and disposal are required to satisfy legal and institutional requirements and to preserve the scientific value of forensic microbiome datasets [74].

Another important aspect is the anonymization and de-identification of microbiome-derived information. While anonymization may reduce the risk of direct identification, advances in computational biology and data integration may increase the risk of re-identification when microbiome data are merged with other biological or demographic datasets. Therefore, forensic laboratories and research institutions should use strong de-identification strategies, minimize the collection of extraneous personal information, and restrict data access to authorized personnel with clearly defined responsibilities [71, 73].

To enhance data protection and cybersecurity, ongoing investments in secure information technology systems, standardized governance frameworks, and periodic assessments of security will be necessary. International collaboration among forensic laboratories, regulatory bodies, data protection specialists and cybersecurity experts will be key to establish harmonized standards that protect sensitive microbiome data, while allowing legitimate forensic research and investigations. The future application of forensic microbiomics will depend on creating trust of the public for the safety and ethical management of microbiome derived data.

Legal Frameworks and Courtroom Acceptance

However, the successful incorporation of forensic microbiomics into routine forensic practice is not only a matter of scientific progress, but also the establishment of appropriate legal and regulatory frameworks. Although microbiome analysis is promising in forensic investigations, its use in judicial proceedings remains limited since the field has not achieved the same level of validation, standardization, and legal acceptance as forensic DNA profiling. Thus, regulatory oversight will be critical to ensure the consistent and transparent use of microbiome-derived evidence in a manner consistent with accepted forensic standards [61, 67].

Validation studies must prove, without any doubt, that the basic analytical methods used to obtain microbiome evidence are scientifically sound, reliable, reproducible and have known error rates in order to be admissible in court. Confidence in analyses based on the microbiome requires standardized laboratory protocols, accredited testing facilities, quality assurance programmes and independent proficiency testing. Moreover, clear reporting guidelines and transparent statistical interpretation will allow judges, legal practitioners and expert witnesses to objectively assess the evidential value and limitations of microbiome-derived findings [67, 68].

Regulatory frameworks must also consider the roles of forensic laboratories, investigators, researchers and expert witnesses involved in microbiome analysis. Standardized international protocols for sample collection, laboratory procedures, bioinformatic analysis, data reporting, quality control and evidence preservation will ensure consistency across jurisdictions and reduce variability in forensic practice. Thus, it will be important that scientific organizations, accreditation bodies, regulatory agencies and legal institutions work together in developing uniform operational standards suitable for routine forensic application [68, 69].

As forensic microbiomics grows up, legal frameworks need to be flexible enough to accommodate new scientific knowledge and consistent with the principles of fairness, transparency and due process. Ongoing interdisciplinary collaboration between forensic scientists, microbiologists, legal scholars, policy makers and regulators will contribute to the development of evidence-based regulations that permit the responsible use of microbiome-derived evidence. In the long term, the development of legally recognized international standards will improve public confidence and judicial acceptance, and facilitate the gradual integration of forensic microbiomics into modern criminal justice systems.

Future Directions and Emerging Perspectives

Development of Global Forensic Microbiome Databases

It is widely accepted that the creation of extensive global forensic microbiome databases is one of the most important prerequisites for the routine application of forensic microbiomics. Standardized microbiome repositories would, like traditional forensic DNA databases, provide reference data sets allowing for robust comparisons of microbial profiles between individuals, populations, body sites and geographic locations. Such databases would assist in interpreting evidence obtained from the microbiome, by providing typical baseline data to compare forensic samples against [47, 63].

Current microbiome databases are fragmented and mostly designed for biological or ecological research, not for forensic use. Current datasets often differ greatly in sampling strategy, DNA extraction method, sequencing platform, collection of metadata, and bioinformatic analysis. These methodological weaknesses hinder interoperability between datasets and reduce their forensic value. Thus, future forensic microbiome repositories should implement unified methods for sample collection, sequencing, quality control, metadata annotation, and taxonomic classification to facilitate meaningful cross-study comparisons and inter-laboratory reproducibility [61, 64].

The establishment of global reference databases should also prioritize population diversity and extensive metadata collection. Microbial communities differ by geography, ethnicity, age, diet, environmental exposure, climate and lifestyle and representative coverage of the population is required to enable robust forensic interpretation. International collaborative efforts between forensic laboratories, research institutions, public health agencies and academic organizations will be necessary to establish databases that are representative of the true global microbial diversity and also adhere to high standards of data quality and scientific integrity [46, 62].

The future success of forensic microbiomics will thus depend on the availability of large datasets, but also on their accessibility, sustainability and governance. Standardized database management, secure data-sharing mechanisms, regular updates, and internationally accepted quality assurance frameworks will underpin the long-term value of forensic microbiome repositories. The establishment of globally coordinated databases will enable scientific validation, comparative analyses, and the building of predictive models and will accelerate the transition of forensic microbiomics from experimental research to routine forensic practice.

Standardization of Protocols

The successful translation of forensic microbiomics from research laboratories to routine forensic practice will require the establishment of standardized protocols. Currently, a high degree of methodological variability exists across the entire analytical workflow from sample collection, preservation, DNA extraction, sequencing, bioinformatic analysis, and statistical interpretation. These inconsistencies cause differences in microbial profiles between studies and laboratories, thus limiting reproducibility and lowering confidence in microbiome-derived forensic evidence [28, 61].

Future standardization efforts should concentrate on the development of internationally accepted guidelines for each step of microbiome analysis. Standardized procedures for evidence collection, storage conditions, contamination control, sequencing strategies, quality assurance and data reporting will enhance consistency across laboratories and allow comparison of results generated using different analytical platforms. Standard operating procedures should also define minimum quality criteria for forensic microbiome analyses, so that evidence based on microbiomes meets the scientific standards expected in forensic investigations [67, 76].

Along with laboratory methodologies, bioinformatic standardization is equally important. Different ways of filtering sequence quality, algorithms for taxonomic classification, reference databases and statistical analyses may lead to different interpretations of the same sequencing datasets. This will improve reproducibility and increase confidence in microbiome-based forensic investigations through the implementation of validated computational pipelines,

harmonized reporting formats and transparent analytical workflows. International proficiency testing and external quality assessment schemes should provide additional support for harmonisation between forensic laboratories [39, 40].

Consistent global standardization will require ongoing collaboration between forensic scientists, microbiologists, bioinformaticians, standards organizations, accreditation bodies, and regulatory agencies. International efforts to build consensus guidance, certified reference materials and harmonized validation frameworks will speed up the use of forensic microbiomics in operational laboratories. Standardized protocols will improve analytical reliability, but also strengthen scientific credibility, facilitate judicial acceptance and promote the responsible incorporation of microbiome analysis into modern forensic science.

Portable Sequencing Technologies

Portable sequencing technologies are developing rapidly and are expected to lead the future of forensic microbiomics by enabling more rapid and flexible microbial analysis outside traditional laboratory settings. Recent advances in miniaturized sequencing platforms have allowed the potential to characterize microbial communities in real-time at crime scenes, disaster sites, border crossings, and remote locations where immediate access to a laboratory may not be feasible. These technological advances can reduce sample transportation times, minimize degradation, and speed up forensic decision making while maintaining the integrity of microbiome derived evidence [79, 80].

One of the most important advances is nanopore sequencing, which results in direct analysis of DNA molecules, by small, portable devices that can generate sequencing data in real time. Conventional sequencing platforms require significant laboratory infrastructure, while portable nanopore devices facilitate rapid microbial profiling with streamlined workflows and real-time data generation. Recently, they have been shown to be useful for pathogen surveillance, environmental microbiology, and microbial community analysis, indicating a great potential for future forensic microbiome investigations, especially in time-sensitive cases [79].

Notwithstanding these benefits, several technical issues need to be addressed before portable sequencing technologies can be routinely incorporated into forensic practice. Sequencing accuracy, DNA quality requirements, environmental factors, contamination control, computational resources, and standardized analytical workflows continue to impact the fidelity of microbiome analyses in the field. Validated protocols, quality assurance, and rigorous performance evaluation will be required for forensic use, to ensure that portable sequencing platforms generate data comparable in quality to that generated by conventional laboratory-based systems [80, 81].

Further improvements in sequencing chemistry, instrument accuracy, bioinformatics and cloud-based computational analysis are expected to improve the performance of portable sequencing technologies for forensic applications. Integration with artificial intelligence, automated sample processing and standardized quality control procedures may further increase analytical efficiency and reliability. As these technologies mature, portable sequencing may become an important part of routine forensic microbiomics, allowing for rapid, on-site microbial characterization that complements conventional laboratory investigations.

Artificial Intelligence-Assisted Forensic Microbiomics

The explosion of microbiome sequencing has generated data sets that are often extremely large and complex, at the limits of what can be handled by traditional statistical approaches. Hence, artificial intelligence (AI) and machine learning have emerged as transformative technologies in forensic microbiomics by allowing for automated analysis of high-dimensional microbial data, identification of subtle biological patterns, and improved predictive modelling. With the ongoing development of computational methods, AI-assisted analyses are anticipated to increasingly contribute to improved accuracy, efficiency, and scalability of microbiome-based forensic investigations [42, 65].

Machine learning algorithms, such as random forests, support vector machines, artificial neural networks and deep learning models, have shown great promise in a variety of applications such as personal identification, postmortem interval estimation, geolocation inference and microbial source attribution. These computational methods can analyse thousands of microbial variables at the same time and find complex relationships that might not be apparent using standard analytical techniques. Recent studies have shown promising predictive performance, emphasizing the growing contribution of AI to forensic microbiome research [65, 66].

However, there are still some challenges ahead before the AI assisted microbiome analysis can be widely applied in forensic practice. Many machine learning models are data hungry, requiring substantial, diverse, and well-annotated datasets for reliable performance; insufficient training data can also limit model generalizability and raise the risk of overfitting. Also, the opacity of some "black-box" algorithms makes it hard to interpret results scientifically, to testify as an expert and to get the judicial acceptance. Future work should thus be directed at explainable AI, transparent model validation and standardized performance evaluation to increase trust in AI-based forensic evidence [82].

The future integration of artificial intelligence with high-throughput sequencing, cloud computing, automated bioinformatics and worldwide microbiome databases, has the potential to revolutionize forensic microbiomics into a more efficient, data-driven discipline. The development of robust AI frameworks that are scientifically reliable, legally defensible, and amenable to routine forensic implementation will rely on ongoing collaboration among forensic scientists, microbiologists, computer scientists, statisticians, and regulatory organizations. With the continuous improvement of computational methods, artificial intelligence is expected to be a key element of forensic microbiome analysis in the future.

Integration into Routine Forensic Practice

Coordinated advances in technology, infrastructure, policy, and professional training will be needed to successfully translate forensic microbiomics from experimental research to routine forensic casework. Although many studies have demonstrated the potential of microbiome analysis for forensic investigations, the operational implementation will depend on the development of standardized laboratory workflows, validated analytical methods, comprehensive quality assurance programmes, and internationally accepted reporting standards. These developments are critical to ensuring that microbiome-derived evidence is reliable, reproducible, and fit for use in forensic laboratories and judicial proceedings [61, 67].

Integrated analytical workflows linking microbiome profiling

Table 5: Major Challenges, Current Limitations, and Future Perspectives in Forensic Microbiomics.

Challenge	Current Limitation	Potential Future Direction
Lack of standardized methodologies	Variability in sample collection, DNA extraction, sequencing platforms, and bioinformatic workflows	Development of internationally standardized protocols and quality assurance guidelines
Temporal and spatial variability	Microbial communities change with time, body site, lifestyle, and environmental exposure	Longitudinal studies and standardized sampling strategies to improve interpretation
Environmental contamination	Contamination during sample collection, laboratory processing, and environmental exposure may influence microbial profiles	Improved contamination-control procedures, validated laboratory workflows, and quality assurance measures
Limited reproducibility and validation	Small study populations, methodological differences, and insufficient multicentre validation	Large-scale validation studies, inter-laboratory collaborations, and standardized validation frameworks
Insufficient reference databases	Limited geographically and demographically representative microbiome datasets	Development of comprehensive global forensic microbiome databases with standardized metadata
Complex data interpretation	High-dimensional microbial datasets require advanced computational analysis and lack standardized statistical frameworks	Integration of explainable artificial intelligence, machine learning, and standardized bioinformatic pipelines
Legal and regulatory challenges	Limited judicial acceptance, absence of internationally harmonized evidential standards	Development of evidence-based regulatory frameworks, laboratory accreditation, and internationally accepted reporting guidelines
Routine forensic implementation	Limited operational experience and infrastructure for microbiome analysis	Integration with conventional forensic methods, professional training, and continued technological innovation

Abbreviations: DNA = Deoxyribonucleic Acid; AI = Artificial Intelligence.

with traditional forensic techniques such as DNA profiling, forensic anthropology, forensic pathology, toxicology and digital forensics will likely be implemented in future forensic laboratories. Microbiome analysis will likely not replace traditional methods, but rather be a complementary source of biological information with added information on microbial transfer, postmortem changes, geographical origin and environmental interactions. Such multidisciplinary approaches may provide for better evidence interpretation and enhance the overall accuracy of forensic investigations [54, 66].

Routine forensic microbiomics will also require significant investment in laboratory infrastructure, training of personnel, and interdisciplinary collaboration. Forensic practitioners need to acquire expertise in microbiology, molecular biology, bioinformatics, biostatistics and computational data analysis for the correct interpretation of complex microbiome datasets. Moreover, the partnership between academic institutions, forensic laboratories, health care organizations, regulatory agencies and technology developers will foster knowledge transfer, method validation and the development of internationally harmonized best-practice guidelines [76, 83].

With the decreasing costs of sequencing technologies, the development of computational tools and the increasing size of global microbiome reference databases we expect the practical application of forensic microbiomics to be increasingly feasible. Validation in multicentre studies, accreditation of analytical procedures, and the development of evidence-based regulatory frameworks will help to progressively integrate microbiome analysis into routine forensic practice. Ultimately, successful integration will require practitioners to maintain the same scientific rigor, quality assurance and legal reliability that are characteristic of established forensic disciplines, while embracing emerging technological innovations.

Research Gaps and Recommendations

Although great advances have been made in forensic microbiomics over the past decade, there are still several important research gaps that prevent its incorporation into regular forensic practice. Most of the published studies have been carried out on relatively small sample sizes, geographically restricted populations or controlled laboratory conditions that may not represent the complexity of real forensic casework. Future research should be focused on large-scale multicentre studies with varied populations and environmental conditions to enhance the generalizability and reliability of microbiome-based forensic analyses [62, 63].

Development of standardized validation frameworks to assess the analytical performance throughout the complete forensic workflow is another research priority. In future studies, systematic assessment of sensitivity, specificity, accuracy, precision, reproducibility, robustness and error rates should be performed using harmonized experimental designs. Moreover, internationally agreed standards for sample collection, sequencing, bioinformatic processing, statistical analysis and reporting should be established to enable inter-laboratory comparisons and to ensure consistent interpretation of microbiome-derived evidence [61, 67].

Continued technological innovation will further influence the future of forensic microbiomics. We expect that new approaches such as long-read sequencing, multi-omics integration, explainable artificial intelligence, automated bioinformatic pipelines, and cloud-based computational platforms will improve the resolution, efficiency, and interpretability of microbial analyses. Equally important will be the development of comprehensive global microbiome reference databases, with standardized metadata that accurately reflect different populations, body sites, environmental conditions and geographical areas. These resources will provide the scientific foundation necessary for reliable forensic comparisons and predictive modeling [65, 77, 78].

The future success of these efforts will ultimately be dependent on the continued interdisciplinary collaboration of forensic scientists, microbiologists, molecular biologists, bioinformaticians, statisticians, computer scientists, legal experts, policymakers, and international regulatory bodies. Investment in collaborative research programmes, standardised methodologies, professional training and evidence-based regulatory frameworks will accelerate the responsible adoption of forensic microbiomics. Future research may help to overcome the current scientific and operational challenges and to make the analysis of the microbiome a valuable additional tool for traditional forensic investigations and to advance forensic science (Table 5).

Conclusion

Forensic microbiomics is a rapidly evolving field that has the potential to supplement traditional forensic methods by providing novel biological information that goes beyond human DNA analysis. Recent advances in high-throughput sequencing, metagenomics, bioinformatics and computational analytics have

substantially improved the characterization of human-associated and environmental microbial communities, allowing their investigation in several forensic contexts. Current evidence suggests that microbial signatures have a large potential for applications such as individual identification, linking individuals to objects and crime scenes, inferring geographic origin, estimating postmortem interval, investigating sexual assault, reconstructing lifestyle and identifying disaster victims. Many of these applications are still being investigated, but they show the growing role of microbiome research in modern forensic science.

However, despite these promising developments, several scientific and practical challenges still prevent routine implementation of forensic microbiomics. Major hurdles to reproducibility and judicial acceptance include the dynamic nature of microbial communities, variability in methods, environmental contamination, limited reference databases, computational complexity, and lack of internationally standardized analytical frameworks. Rigorous validation studies, harmonised laboratory protocols, transparent statistical methodologies, comprehensive quality assurance programmes and internationally coordinated efforts will be required to overcome these challenges and establish robust microbiome reference databases. These advances are vital to guarantee that microbiome-based evidence meets the scientific and legal standards required for forensic application.

Microbiome analysis should be viewed as a complementary approach rather than a replacement for conventional forensic DNA profiling, providing additional strength to forensic investigations by integrating multiple sources of biological evidence. The integration of microbial profiling with well-established forensic disciplines such as genetics, anthropology, pathology, toxicology and digital forensics provides new opportunities to improve evidence interpretation and to reconstruct complex forensic scenarios. Further progress in artificial intelligence, portable sequencing technologies, multi-omics approaches, and computational biology is likely to enhance the analytical capability and practical utility of forensic microbiomics.

Future success will require continued interdisciplinary collaboration between forensic scientists, microbiologists, molecular biologists, bioinformaticians, statisticians, legal professionals, policy makers, and international regulatory organizations. To elevate forensic microbiomics from an emerging research discipline to a reliable part of routine forensic practice, investment in collaborative research, technological innovation, standardized methodologies and ethical governance will be essential. With continued scientific validation and responsible application, the human microbiome could become a valuable complementary source of forensic evidence and contribute to more complete, accurate and scientifically sound forensic inquiries in the years to come.

Declarations

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Not applicable. This review article is based exclusively on previously published literature and did not involve human participants, animals, or identifiable personal data.

Consent for Publication

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Competing Interests

The author declares that there are no competing interests.

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