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Investigating the Role of Environmental and Human Microbiome in Health and Disease: Focus on Pathogen's Monitoring and Control

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Abstract

The main lines of the research conducted during my PhD aimed to shed light on the role and influence of the microbiome, both in the environment and in the human body, trying to find correlations between environmental and human microbiomes, and between the human microbiome and the human health or disease condition.

To this purpose, different approaches were adopted: on one side the hospital environment was characterized and monitored, in order to optimize surveillance strategies of hospital contamination and counteract the risk of acquisition of the so-called Healthcare-Associated Infections (HAIs); on the other side, the human microbiome and its role were investigated in defined anatomical niches, in particular in the oral and vaginal sites, with the aim to clarify whether specific microbiome compositions may be related to increased risk to develop specific virus-induced diseases (namely those associated with HPV and SARS-CoV-2 infection).

In particular, we investigated the microbiome of the hospital environment, of the nasal cavities of hospitalized babies, of the oral cavity and of the vaginal tract.

The environmental microbiome: the hospital as a paradigm of built confined environments The microbiome of built confined environments, such as the hospital one, is constantly influenced by the human presence and *vice versa*. In particular, in the healthcare settings, the environment represents a *reservoir* for microbial pathogens, which contribute to the onset and transmission of HAIs, representing one of the most frequent and severe complications occurring in nosocomial settings worldwide.

Based on the need to implement the monitoring and control of microbial contamination in the hospital environment, we aimed to evaluate the use of molecular methods potentially more sensitive compared to conventional microbiological methods used so far. To this purpose, Next Generation Sequencing (NGS) technologies, customized qPCR arrays and qPCR microarrays were tested for hospital microbiome characterization, in comparison with microbiological culture-based methods (CFU/m^3 count). The study was performed at the Institute for Maternal and Child Health "IRCCS Burlo Garofolo", Trieste, Italy. Different wards and surfaces were included in the study, including pediatric clinic' rooms, surgical rooms, intensive care units and delivery rooms, and floor, bed foot-board and sink surfaces. The results showed a persistent contamination in most wards and surfaces, and, overall, NGS was proven to be the most sensitive method, able to characterize the whole bacterial population residing on hospital surfaces and to detect unsearched bacteria. On the other hand, microbiological methods gave quantification of alive microorganisms,

and molecular PCR methods allowed the quantification of mycetes and the characterization of the antibiotic resistance genes (resistome) of the whole contaminating population, providing important data for antimicrobial resistance (AMR) monitoring. In conclusion, NGS could be considered a useful tool for microbial environmental surveillance, especially if flanked by PCR methods for species identification and resistome characterization, to improve monitoring strategies and counteract HAIs transmission in hospital settings (Comar *et al.*, 2019).

Next, since the risk for contracting HAIs is particularly high in preterm infants, the potential of pathogens colonizing the Neonatal Intensive Care Unit (NICU) to colonize preterm newborns was explored. To this aim, NGS was used to characterize simultaneously the bacterial composition of nasal swabs of preterm infants and environmental surfaces microbiome, at the time of delivery and during hospitalization in NICU. The resistome profile was also evaluated, by qPCR microarray. The results showed that HAIs-associated environmental pathogens were colonizing the newborn nose increasing with stay time in NICU, and the analysis of nasal resistome as well evidenced the acquisition of resistant pathogens absent at the time of birth. Thus, these data evidenced the role of the hospital microbiome in pathogen transmission and evidenced the importance of environmental microbial surveillance (Cason *et al.*, 2021).

Last, since we previously reported that an innovative probiotic-based cleaning system (PCHS, Probiotic Cleaning Hygiene System; Copma Scrl, Ferrara, Italy) was able to stably decrease pathogens and their AMR in adult Internal Medicine wards, and to inactivate SARS-CoV-2 virus in controlled laboratory conditions (Caselli *et al.*, 2016b, 2018; D'Accolti *et al.*, 2021), we tested its impact in the Emergency ward of the Institute for Maternal and Child Health "IRCCS Burlo Garofolo" (Trieste, Italy) during the COVID-19 pandemics, in comparison with chemical disinfection. The monitoring of microbial contamination, including the resistome profile, was evaluated by microbiological analysis, NGS, and qPCR microarray. In parallel, the presence of SARS-CoV-2 was assessed by qPCR after retrotranscription (RT-qPCR). The results showed that the use of PCHS induced a stable decrease of pathogens (>80% compared to chemical disinfection), confirming previous results. In parallel, resistance genes were decreased up to 2 logarithms. Notably, SARS-CoV-2 RNA was never detected during the PCHS usage, despite the presence of infected patients, thus suggesting the effectiveness of PCHS in preventing SARS-CoV-2 contamination also in real-life conditions. In conclusion, the collected data suggest that PCHS may allow the control of HAI-associated pathogens and SARS-CoV-2 spread, without worsening the AMR threat (Soffritti *et al.*, 2022).

The human microbiome: the oral and vaginal niches The second focus of my PhD thesis regarded the study of the human microbiome, in health and disease condition, with particular interest in the oral and vaginal microbiomes.

Since a detailed map of oral microorganisms (including also eukaryotes and viruses) and their relative abundance was missing, we first aimed to obtain a site-specific and comprehensive view of the oral microbiome of young adult healthy individuals (healthy oral microbiome, HOM). To this aim, the Whole Genome Sequencing (WGS) technology was used, allowing to evidence simultaneously prokaryotes, eukaryotes, and viruses. The results evidenced that each investigated oral microhabitat (tongue dorsum, hard palate, buccal mucosa, keratinized gingiva, supragingival and subgingival plaque, saliva with and without rinsing) is characterized by a different microbial community, including bacteria, fungi, and viruses. Interestingly, alpha-diversity values highlighted significant differences among the different sampled niches, but not among patients, supporting the existence of a recognizable core microbiome of a healthy oral cavity. Oral rinse microbiome resulted most representative of the whole microbiomes, compared to that of saliva, suggesting that this specimen can be used to represent the whole oral microbiome. In addition, the HOM AMR was also characterized, to define HOM resistome profile in healthy conditions. The results showed that HOM harbored several R genes, conferring resistance to macrolide, lincosamides, streptogramin and tetracycline (Caselli *et al.*, 2020b). In conclusion, our data provided for the first time a comprehensive view of HOM microbiome, useful as a reference for future studies aiming at correlating microbiome dysbiosis with specific diseases.

Next, since SARS-CoV-2 virus enters the body through the oropharynx, and the virus replication at the site of entry might be influenced by local microbiome, we investigated the oral microbiome in COVID-19 patients, to evidence the eventual association between virus-induced disease and the microbial environment of the oral cavity. As applied in the HOM study, the oral microbiome was characterized by WGS. In parallel, the presence of inflammation and/or local secretory immune response (IgA) was assessed, by specific ELISA assays. The results showed that COVID-19 patients harbored dysbiotic oral microbiomes, with lower alpha-diversity and species richness, compared to controls. Moreover, the oral dysbiosis correlated with symptoms severity and increased inflammation, with augmented concentration of inflammatory cytokine belonging to the so-called COVID-19 cytokine storm. In parallel, oral dysbiosis inversely correlated with the development of mucosal sIgA response, which in turn inversely correlated with symptoms severity, suggesting the important role of local immune response in the early control of virus infection. In conclusion, our data support a role of the oral microbiome in defining individual susceptibility to SARS-CoV-2 infection and could be useful to better understand

the features of the oral environment that could potentially promote viral infection, and to identify potential markers for the risk of developing a severe disease (Soffritti *et al.*, 2021).

Another aspect of the microbiome role in virus-induced disease was investigated related to the vaginal microbiome in human papillomavirus (HPV) infection and oncogenesis. The dysbiosis of vaginal microbiome was in fact reported to be associated with the persistence of high-risk human papillomaviruses (hrHPVs) infection and the development of precancerous (cervical intraepithelial neoplasia, CIN) and cancerous lesions (cervical cancer, CC). However, no data were available on the microbiome role in determining the persistence or clearance of HPV infection in CIN patients undergoing surgical excision of the lesion by LEEP (Loop Electrosurgical Excision Procedure). To clarify this aspect, we characterized the vaginal microbiome, together with the inflammatory cytokine microenvironment, in a cohort of women with CIN2/CIN3 lesions undergoing LEEP. Before LEEP, the results showed a high prevalence of dysbiotic microbiomes and pro-inflammatory environment in CIN patients, that correlated with disease severity. At 6-months follow-up after excision, specific microbiome and cytokine profiles were associated with hrHPV clearance or persistence. In particular, hrHPV-cleared patients showed a decrease of microbiome dysbiosis and pro-inflammatory cytokines levels, compared to women with hrHPV recurrence. These data thus highlight the crosstalk between HPV and the local vaginal microbiome and open the way to novel approaches to enhance viral clearance and CIN progression, by remodulating vaginal microbiome (Caselli *et al.*, 2020a).

In conclusion, the data collected during my PhD support the existence of a deep interconnection between the environmental and human microbiome, and a significant role of the human microbiome in maintaining the health or favoring disease onset. This open the way to novel approaches, aimed to remodel the microbiome, whatever it is the environmental or human one, in order to maintain and improve the health of humans, animals, and the whole ecosystem.

This approach would be also in line with the "One Health" concept, considering the environmental and human microbiomes strongly interconnected. In this perspective, the data already obtained by us in the hospital environment are strongly suggestive that such an approach, through a comprehensive characterization of microbiomes, may be successful in achieving a deeper understanding of the environment-humans microbial network and a consequent better control of the infectious risk.

Sinossi

Le principali linee di ricerca condotte durante il mio Dottorato hanno avuto lo scopo di far luce sul ruolo e l'influenza del microbioma, sia nell'ambiente che nel corpo umano, cercando di trovare correlazioni tra il microbioma ambientale e quello umano, e tra il microbioma umano e lo stato di salute o malattia.

A questo scopo, sono stati adottati diversi approcci: da un lato abbiamo cercato di caratterizzare microbiologicamente l'ambiente ospedaliero, al fine di ottimizzare le strategie di sorveglianza della contaminazione ospedaliera e contrastare il rischio di acquisizione delle cosiddette Infezioni Correlate all'Assistenza (ICA); dall'altro lato, è stato studiato il microbioma umano in definite nicchie anatomiche, in particolare nel sito orale e vaginale, con l'obiettivo di chiarire se specifiche composizioni del microbioma possano essere correlate ad un aumento del rischio di sviluppare specifiche malattie virus-indotte (in particolare quelle associate all'infezione da HPV e SARS-CoV-2). In particolare, abbiamo investigato il microbioma dell'ambiente ospedaliero, delle cavità nasali di neonati ospedalizzati, della cavità orale e del tratto vaginale.

Il microbioma ambientale: l'ospedale come paradigma degli ambienti confinati costruiti Il microbioma degli ambienti confinati costruiti, come quello ospedaliero, è costantemente influenzato dalla presenza umana e viceversa. In particolare, nelle strutture sanitarie, l'ambiente rappresenta un serbatoio per gli agenti patogeni microbici, che contribuiscono all'insorgenza e alla trasmissione delle ICA, le quali rappresentano una delle complicanze più frequenti e gravi che si verificano negli ambienti ospedalieri su scala mondiale.

Data la necessità di implementare il monitoraggio e controllo della contaminazione microbica nell'ambiente ospedaliero, abbiamo voluto valutare l'uso di metodi molecolari potenzialmente più sensibili rispetto a quelli microbiologici convenzionali finora utilizzati. A questo scopo, abbiamo testato e confrontato tecniche di Next Generation Sequencing (NGS), microarray in real time PCR quantitativa (qPCR), e metodi microbiologici colturali classici (conta CFU/m^3 per la caratterizzazione del microbioma ospedaliero. Lo studio è stato svolto all' Istituto IRCCS Materno Infantile Burlo Garofolo di Trieste, Italia. Diversi reparti e superfici sono stati considerati nello studio, incluse le stanze della clinica pediatrica, chirurgia, terapia intensiva e sale parto, e superfici quali pavimento, pediera del letto e lavandino. I risultati hanno mostrato una contaminazione persistente nella maggior parte dei reparti e delle superfici e, nel complesso, l'NGS si è dimostrato il metodo più sensibile nella rilevazione dei batteri, in grado di caratterizzare l'intera popolazione batterica residente

sulle superfici ospedaliere e di rilevare anche i batteri non ricercati. D'altro lato, i metodi microbiologici hanno fornito la quantificazione dei microrganismi vitali, e i metodi molecolari di PCR hanno permesso la quantificazione dei miceti e la caratterizzazione dei geni di resistenza (resistoma) dell'intera popolazione contaminante, fornendo dati importanti per il monitoraggio dell'antibiotico resistenza (AMR). In conclusione, l'NGS può essere considerato un utile strumento di sorveglianza della contaminazione ambientale, soprattutto se affiancato a metodiche molecolari di PCR per l'identificazione di specie e la caratterizzazione del resistoma, andando a migliorare le strategie di monitoraggio e controllo della diffusione delle ICA nell'ambiente ospedaliero (Comar *et al.*, 2019).

In seguito, dato che il rischio di contrarre ICA è particolarmente elevato nei neonati prematuri, è stata valutata la potenziale capacità dei patogeni contaminanti il reparto di terapia intensiva neonatale (TIN) di colonizzare i neonati pretermine ospedalizzati in questo reparto. A questo scopo, l'NGS è stato utilizzato per caratterizzare simultaneamente la composizione batterica dai tamponi nasali dei neonati e il microbioma delle superfici ospedaliere, al momento del parto e durante l'ospedalizzazione nel reparto TIN. Il profilo del resistoma è stato valutato tramite qPCR microarray. I risultati hanno mostrato che i patogeni ambientali ICA-associati hanno colonizzato progressivamente le cavità nasali dei neonati, con l'aumentare del tempo di permanenza nel reparto, e l'analisi del resistoma nasale ha evidenziato simultaneamente l'acquisizione di patogeni resistenti, assenti al momento della nascita. Questi dati supportano quindi il ruolo del microbioma ospedaliero nella trasmissione dei patogeni ed evidenziano l'importanza della sorveglianza microbiologica ambientale (Cason *et al.*, 2021).

Infine, poiché abbiamo precedentemente riportato la capacità di un innovativo sistema di sanificazione a base di probiotici (PCHS, Probiotic Cleaning Hygiene System; Copma Scrl, Ferrara, Italy) di ridurre stabilmente i patogeni e la loro AMR nei reparti di Medicina Interna, e di inattivare SARS-CoV-2 in condizioni controllate di laboratorio (Caselli *et al.*, 2016b, 2018; D'Accolti *et al.*, 2021), abbiamo testato il suo impatto nel reparto di Pronto Soccorso dell'IRCCS Burlo Garofolo di Trieste, durante la pandemia COVID-19, in comparazione diretta con la disinfezione chimica. Il monitoraggio della contaminazione microbiologica ambientale ed il profilo del resistoma sono stati valutati mediante analisi microbiologiche, NGS e qPCR microarrays. In parallelo, la presenza ambientale di SARS-CoV-2 è stata valutata mediante qPCR dopo retrotrascrizione (RT-qPCR). I risultati hanno mostrato che l'uso del PCHS, ha indotto una diminuzione stabile dei patogeni (>80% in comparazione alla disinfezione chimica), confermando i risultati ottenuti precedentemente. In parallelo, i geni di resistenza sono calati fino a 2 logaritmi. In aggiunta, non è stata rilevata la presenza dell'RNA di SARS-CoV-2 durante l'utilizzo del PCHS,

anche in presenza di pazienti infetti, suggerendo l'efficacia del PCHS nel prevenire la contaminazione da SARS-CoV-2 anche in condizioni real-life e non solo in condizioni controllate in vitro. In conclusione, i dati raccolti suggeriscono che il PCHS possa consentire di controllare i patogeni ICA-associati e la diffusione di SARS-CoV-2, senza aggravare il problema dell'AMR (Soffritti *et al.*, 2022).

Il microbioma umano: le nicchie orali e vaginali Il secondo obiettivo della mia tesi di Dottorato ha riguardato lo studio del microbioma umano, in condizioni di salute e di malattia, con particolare focus sul microbioma orale e vaginale.

Poiché non era ancora disponibile una mappa dettagliata dei microorganismi orali (compresi anche microrganismi eucarioti e virus) e della loro abbondanza relativa, abbiamo avuto come primo obiettivo quello di ottenere una mappa complessiva e sito-specifica del microbioma orale sano in soggetti giovani (healthy oral microbiome, HOM). A questo scopo, abbiamo utilizzato la tecnica di Whole-Genome Sequencing (WGS), in grado di evidenziare simultaneamente procarioti, eucarioti e virus. I risultati hanno evidenziato che ogni micro-habitat investigato (dorso della lingua, palato duro, mucosa buccale, gengiva cheratinizzata, placca sopragengivale e sottogengivale, saliva con e senza risciacquo) è caratterizzato da una diversa comunità microbica, comprendente batteri, funghi e virus. Da notare, i valori di alfa-diversità hanno evidenziato significative differenze tra le diverse nicchie campionate, ma non tra soggetti diversi, suggerendo l'esistenza di un HOM indicativo dello stato di "salute" della cavità orale. Il microbioma del risciacquo orale è risultato il più rappresentativo di tutti i microbiomi sito-specifici, rispetto a quello della saliva, suggerendo che questo tipo di campione possa essere utilizzato per rappresentare l'intero microbioma orale. In parallelo, è stato anche caratterizzato il resistoma dell'HOM, per definirne il profilo in condizioni di salute. I risultati hanno mostrato che l'HOM presenta diversi geni R, che conferiscono resistenza a macrolidi, lincosamidi, streptogramine e tetracicline (Caselli *et al.*, 2020b). In conclusione, i nostri dati hanno fornito per la prima volta una visione comprensiva del microbioma HOM e potranno essere utili come riferimento per studi futuri, volti a definire la disbiosi del microbioma orale in associazione con specifiche malattie.

Successivamente, dato che il virus SARS-CoV-2 penetra nell'organismo umano attraverso l'orofaringe, e la replicazione virale nel sito di ingresso potrebbe quindi essere influenzata dal microbioma locale, abbiamo investigato il microbioma orale in pazienti COVID-19, allo scopo di evidenziare l'eventuale associazione tra la malattia indotta dal virus e l'ambiente microbico del cavo orale. Come nello studio sull'HOM, il microbioma orale è stato caratterizzato mediante WGS. In parallelo, è stata valutata la presenza di infiammazione e/o risposta immunitaria secretoria locale (IgA), mediante specifici saggi ELISA. I risultati hanno evidenziato che i pazienti

COVID-19 presentavano microbiomi orali disbiotici, con diminuita alfa-diversità e ricchezza di specie, rispetto ai controlli. La disbiosi orale correlava con la severità della sintomatologia e con una maggiore infiammazione, con un aumento della concentrazione di citochine infiammatorie, appartenenti alla cosiddetta "tempesta citochinica" caratteristica della patologia COVID-19. La disbiosi orale correlava invece inversamente con lo sviluppo di risposta mucosale sIgA, che a sua volta era inversamente correlata alla severità dei sintomi, suggerendo il ruolo importante della risposta immunitaria locale nel controllo iniziale dell'infezione virale. Nel complesso, i nostri dati supportano un ruolo del microbioma orale nel definire la suscettibilità individuale all'infezione da SARS-CoV-2 e potrebbero essere utili per comprendere meglio le caratteristiche del microambiente orale che potrebbero potenzialmente promuovere l'infezione virale, e per identificare potenziali marcatori del rischio di sviluppare una malattia grave (Soffritti *et al.*, 2021).

Un altro aspetto del ruolo del microbioma nelle malattie virus-indotte preso in considerazione è stato quello relativo al microbioma vaginale nell'infezione e oncogenesi da papillomavirus umani (HPV). La disbiosi del microbioma vaginale è stata infatti riportata essere associata alla persistenza dell'infezione da HPV ad alto rischio (hrHPV), e allo sviluppo di lesioni precancerose (neoplasia intraepiteliale cervicale, CIN) e cancerose (cancro cervicale, CC). Tuttavia, non erano disponibili dati sul ruolo del microbioma nel determinare la persistenza o la clearance dell'infezione da HPV nelle pazienti CIN sottoposte a rimozione della lesione tramite escissione elettrochirurgica ad ansa (Loop Electrosurgical Excision Procedure, LEEP). Per chiarire questo aspetto, abbiamo caratterizzato il microbioma vaginale, insieme al microambiente infiammatorio citochinico, in una coorte di donne con lesioni CIN2/CIN3 sottoposte a LEEP. Prima della LEEP, i risultati hanno mostrato un'elevata prevalenza di microbiomi disbiotici e di un ambiente pro-infiammatorio nelle pazienti CIN, che correlavano con la severità della lesione. Al follow-up a 6 mesi dall'escissione chirurgica, specifici microbiomi e profili citochinici sono risultati associati alla persistenza o alla risoluzione dell'infezione da hrHPV. In particolare, le pazienti negativizzate hanno mostrato un calo della disbiosi del microbioma e dei livelli di citochine pro-infiammatorie, rispetto alle donne con persistenza di hrHPV e lesioni ricorrenti. Questi dati sottolineano l'interazione tra HPV e il microbioma vaginale locale e aprono la strada a nuovi approcci per migliorare la clearance virale e la progressione CIN, rimodulando il microbioma vaginale (Caselli *et al.*, 2020a).

In conclusione, i dati raccolti durante il mio Dottorato supportano l'esistenza di una profonda interconnessione tra il microbioma ambientale e umano, e un ruolo significativo del microbioma umano nel mantenere lo stato di salute o favorire l'insorgenza di malattie. Questo apre la strada a nuovi approcci, volti a rimodulare il microbioma, sia esso ambientale o umano, al fine di mantenere e migliorare

la salute degli esseri umani, degli animali e dell'intero ecosistema.

Questo approccio è in linea con il concetto di "One Health", che considera i microbiomi ambientali e umani fortemente interconnessi. In questa prospettiva, i dati già da noi ottenuti nell'ambiente ospedaliero suggeriscono fortemente che un tale approccio, attraverso una caratterizzazione completa dei microbiomi, possa essere efficace per ottenere una comprensione sempre più profonda della relazione microbica ambiente-uomo e un conseguente miglior controllo del rischio infettivo.

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Chapter 1

Introduction

Microbiota and Microbiome: historical overview and current definitions The field of microbiome research has become a topic of constantly growing interest over the past few decades. Nevertheless, a clear definition of the terms "Microbiota" and "Microbiome" is still debated among experts (Berg *et al.*, 2020). The term "Microbiota" represents a basic microbiology expression that has been in common use for at least 60 years (Prescott, 2017), but its most common definition is attributed to Prof. Joshua Lederberg in 2001, who described it as "*the ecological community of commensal, symbiotic, and pathogenic microorganisms that literally share our body space and can be determinants of health and disease*" (Lederberg and McCray, 2001). The term "Microbiome", as originally postulated by Whipps et al. in 1988, more broadly denotes the community of the microbes that define the microbiota and their "*theatre of activity*", i.e., all types of molecules produced by those microorganisms (structural elements, metabolites/signal molecules, and the surrounding environmental conditions) (Whipps *et al.*, 1988). Recently, the definition of Whipps et al. has been updated in order to provide a comprehensive and integrated description of the term (Berg *et al.*, 2020), as shown in Figure 1.1. The microbiota includes bacteria, archaea, fungi, algae, and small protists, in other words all living members of the "Microbiome" (Marchesi and Ravel, 2015). A controversial point concerns the inclusion in the definition of "relic DNA" (extracellular DNA released after cell death), plasmids, viruses and phages: as all listed elements are not considered as living organisms, they are not considered members of the Microbiota, although they are known to have an impact on microbial interactions and are included in the term Microbiome (Berg *et al.*, 2020).

Originally, according to Robert Koch's postulates, microbes were considered unidirectionally associated with infection diseases, and this negative connotation, considering microorganisms as agents that must be eradicated in order to preserve health, was valid until the end of the nineteenth century (Segre, 2013; Berg *et al.*, 2020). Similarly, the studies on environmental microbiology showed that the mi-

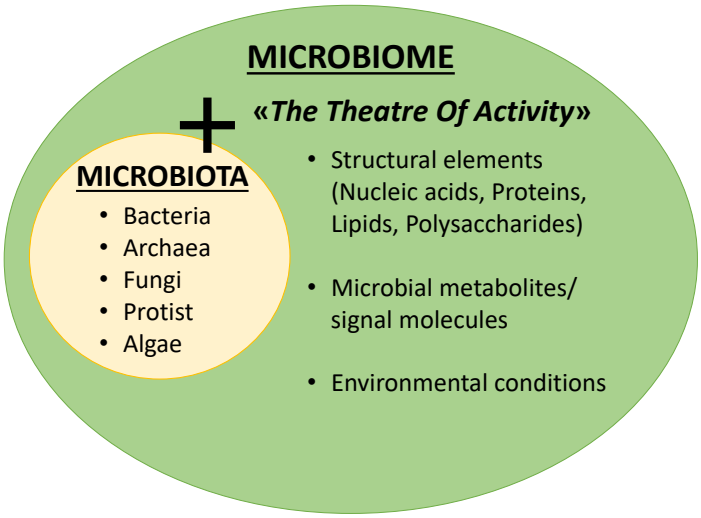


Figure 1.1: Graphical representation of the terms Microbiome and Microbiota: Microbiome includes both the community of microorganisms (Microbiota) and the "Theatre of activity". Modified from Berg *et al.* (2020).

croorganisms colonizing natural environments are essential for the ecosystem maintenance, and a first mention of "beneficial" microorganisms was reported in 1910 (Metchnikoff and Mitchell, 1910). Now it is recognized that only a small portion of living microbes are pathogenic and can cause disease (Berg *et al.*, 2020), and that microbes interact each other forming complex and dynamic communities through cell-to cell interactions and under the constant influence of environmental factors (Bassler, 2002; Banerjee *et al.*, 2018). Essentially, microbial interactions can exist within one species or even between different genera and domains, and can be classified as positive, negative, or neutral, based on the effect on the population dynamics. Several fitness strategies foresee competitive mechanisms, including predation, parasitism, antagonism, or competition, whereas positive pathways include mutualism, synergism, or commensalism. Microorganisms interact each other through direct interaction, horizontal gene transfer and via secondary metabolites, the latter representing important modulators in Quorum Sensing mechanisms and biofilm formation (Papenfort and Bassler, 2016). The investigation of microbial communities has grown rapidly over the past decades, as a result of increasing scientific interest and continuous improvement of molecular technologies. The launch of The Human Microbiome Project (HMP) paved the way for a more in-depth understanding of microbiota composition, and its influence both in human health and disease conditions, taking advantage of the developed high-throughput sequencing technologies (Turnbaugh *et al.*, 2007). Since HMP completion, genome sequencing technologies have continued to evolve (Goodwin *et al.*, 2016), providing efficient, relatively fast, and accessible methods to study the microbiome. However, the development of

High-throughput Next Generation Sequencing (NGS) methods has made it possible to study microbiota at unprecedented scales, providing for the first time a complete picture of the whole bacterial community colonizing a specific environment or niche. The use of phylogenetic markers for NGS sequencing is now broadly utilized, including the 16S rRNA gene most commonly used for archaea and bacteria, the 18S rRNA gene and the 28S rRNA gene specifically used for eukaryotic microorganisms, and the internal transcribed spacer (ITS) specifically used for fungi.

A further essential step in the microbiome investigation was obtained with the development of the Whole-Genome Shotgun (WGS) metagenomic approach, which focuses on the sequencing of the complete genomic sequences present in specimens, allowing a simultaneous evaluation of bacterial, archaeal, fungal and viral components of the microbiome.

The Holobiont Theory and One Health Approach Microorganisms can not only interact each other via different mechanisms (Bassler, 2002), but also can establish complex interactions with their host (human, animals and plants), with whom they have coevolved (Moran, 2007). Microbial-host coevolution has been initially considered using the "separation approach", according to which microorganisms can be subdivided into pathogens, symbionts and neutral, based on their interaction with the host (Lederberg and McCray, 2001). New observations instead support the so called "holistic approach", that rather consider the host and its associated microbiome as one unit (the holobiont) (Zilber-Rosenberg and Rosenberg, 2008; Berg *et al.*, 2020) (Figure 1.2).

This important concept is the basis of the paradigm shift we are currently dealing with. Based on this approach, the healthy state of the holobiont is obtained with the presence of a healthy microbiome (defined as "eubiosis", generally characterized by elevated biodiversity and stability), which in turn affects the health of human and environment components (Trinh *et al.*, 2018; van Bruggen *et al.*, 2019). On the other hand, the presence of a dysbiotic microbiome is associated with disease, both in animals, humans, and environment. Importantly, dysbiotic microbiome can diffuse from animals and environment to humans, contributing to the spread in infections, food safety problems and antimicrobial resistance (AMR). These deep interconnections between the environment, animals and humans have led to the development of the "One Health" concept, which refers to "the collaborative effort of multiple health science professions, together with their related disciplines and institutions – working locally, nationally, and globally – to attain optimal health for people, domestic animals, wildlife, plants, and our environment" (World Health Organization, 2017a). In this integrated approach, schematically represented in Figure 1.3, it is recognized that the health of animals, humans, plants and environment is tightly

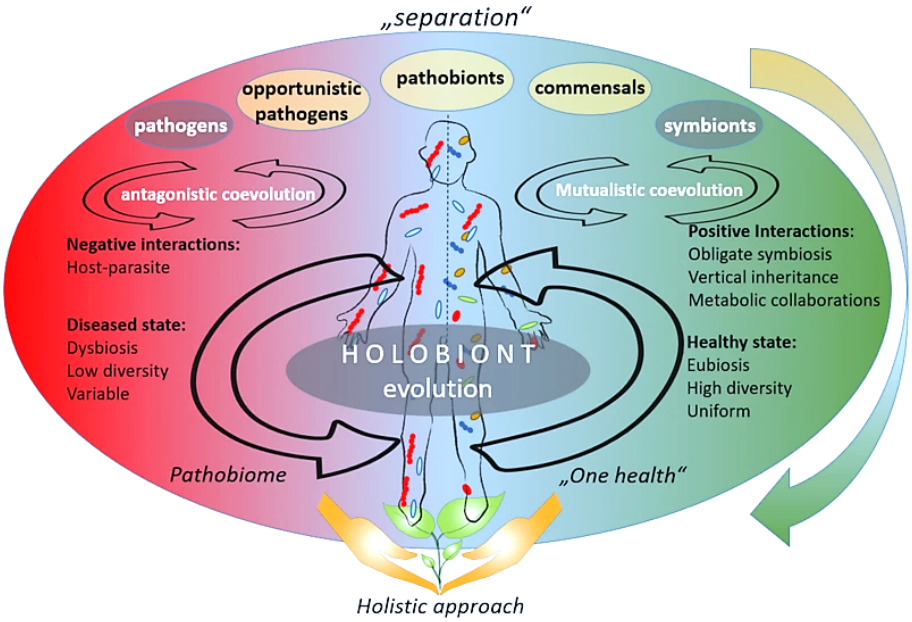


Figure 1.2: The Holobiont Theory: the beneficial interplay of the host and its microbiome is essential for maintaining the health of the holobiont, in which the dynamic interactions between humans, animals, and environment are described by the One Health concept. On the other hand, microbial dysbiosis is often associated with diseases (Berg *et al.*, 2020).

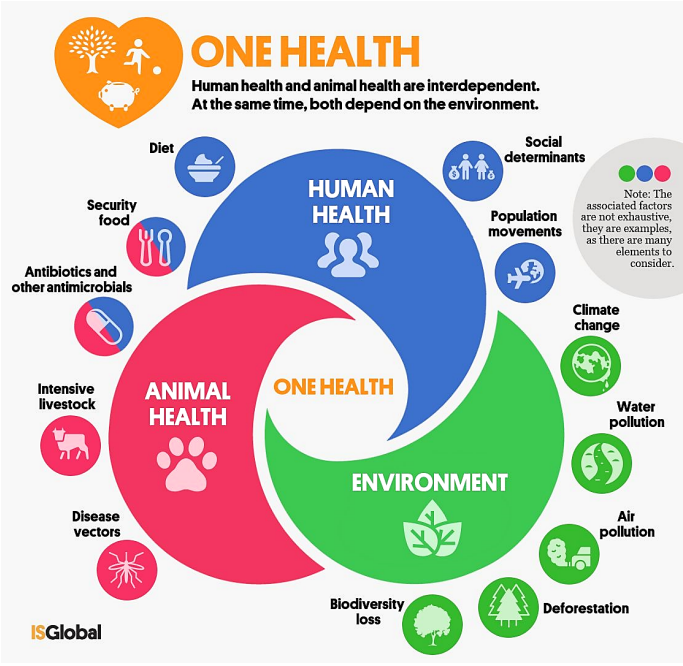


Figure 1.3: The One Health approach: the health of humans, animals and our shared environment is strongly interconnected. A collaborative and transdisciplinary approach is needed in order to counteract global issues (e.g. antibiotic resistance, emerging zoonotic diseases, environmental pollution) and achieve global health (One Health Breakthrough Partnership, 2021).

linked and this must be considered when developing strategies directed toward the prevention and control of infection and AMR spread, as expressed the World Health Organization document of reference (World Health Organization, 2017a).

The growing availability of microbiome data has led to an increasingly comprehension of microbiome features and role, and the main current challenges for microbiome research are achieving a deeper understanding of the mechanisms associated with microbiome eubiosis and dysbiosis, investigating strategies to remodel the microbiomes in order to improve the health of humans, animals, plants, and the whole ecosystem.

1.1 The Environmental Microbiome in Health and Disease

Microorganisms can be found across all types of environments on Earth, and are even adapted to live in extreme environments, thus the term "Environmental microbiome" widely describes the ubiquitous distribution of microorganism among different habitats, including air, soil, aquatic environment, and indoor spaces inhabited by humans. Notably, in recent times the human beings spend most of their life in indoor spaces and built environments (BEs), including homes, workplaces, schools, public transports, and hospital settings. Consequently, in the urban settings, the continuous exposure to the BE indoor microbiomes, rather than those from the external environment of the rural life, affects the health conditions of the human occupants with potential risk to develop human diseases (Dai *et al.*, 2017). According to the holistic approach, also BEs can be considered as super-organisms, similarly to what accepted for living organisms, with their own microbiome made of bacteria, viruses, and fungi, which can persist long and potentially be transmitted to individuals via contact with contaminated surfaces (Smith *et al.*, 1996; Kramer *et al.*, 2006; Otter and French, 2009). A "healthy" BE microbiome is predominantly composed of commensal and beneficial microbes spread by healthy humans and pets, but it can also contain pathogenic microorganisms, potentially responsible for human diseases, disseminated by infected individuals and/or selected by the massive use of disinfectants and antibiotics. Not only surfaces but also the air can be a *reservoir* for pathogens, especially in indoor BEs equipped with air ventilation systems, and breathing unhealthy BE air can lead to the so-called "Sick Building Syndrome" (SBS) (Prussin and Marr, 2015), including symptoms such as headache, eye, nose or throat irritation, nausea, fatigue, difficulty in concentrating and other acute distress, in absence of a specific cause diagnosable, and therefore linked to the occupation of localized rooms or wide areas of a building (EPA, 1991). Among chemical and biological con-

taminants contributing to SBS onset, there are SBS-associated pathogens including bacteria, fungal spores, and molds, which can proliferate under specific humidity and temperature conditions and can be transported by air ventilation (Joshi, 2008).

The BE microbiome is part of a highly dynamic and complex ecosystem characterized by several organisms that interact each other and with the surrounding environment (Dai *et al.*, 2017), with continuous interactions between the building itself and BE occupants (Mahnert *et al.*, 2019). Recent studies focused on the characterization of the BE microbiome and showed that it can include several taxonomic groups (Marimuthu *et al.*, 2014), with different types of indoor spaces exhibiting different microbiome structure, abundance, and diversity (Adams *et al.*, 2015; Shin *et al.*, 2015; Bragoszewska and Biedroń, 2018). The BE microbiome is influenced by geographical and seasonal variations, as well as by abiotic, biotic, and anthropogenic factors (e.g. human activities and occupancy) (Rai *et al.*, 2021). Humans principally contribute to the BE microbiome by spreading their own microbes in the occupied indoor spaces, and higher levels of occupancy correspond to higher abundance of microbes. In addition, human movements contribute to displacement and resuspension of the settled particles (Adams *et al.*, 2016). Of note, more confined BEs have lower microbiome biodiversity and higher presence of multi-drug resistant (MDR) species (Kang *et al.*, 2018; Mahnert *et al.*, 2019; Nowrotek *et al.*, 2019), consistent with the strong selective pressure exerted by the massive use of disinfectants and antimicrobials in confined BEs, such as hospitals. Besides hospitals, also animal husbandry and agriculture display similar conditions (Chokshi *et al.*, 2019), and MDR bacteria can also be detected in other indoor environments, including domestic ones (Jovel *et al.*, 2016; Li *et al.*, 2018; Xu *et al.*, 2018).

In parallel, the significant loss of microbial diversity observed in urbanization has been associated with increased risks of allergies, asthma, and other chronic diseases (Flandroy *et al.*, 2018). A reduced biodiversity of the environmental microbiome from soil and air has been implicated in adverse health outcomes and despite the importance of the environmental microbiome on human health, especially in shaping the microbiome in the early childhood, there are sparse studies investigating this aspect (Hanski *et al.*, 2012). However, an increased microbial diversity has been linked to prevention of allergy and asthma, and children growing up on a farm showed reduced risk of inflammatory respiratory diseases compared to kids raised in more urban environments (Havstad *et al.*, 2011; Dominguez-Bello *et al.*, 2016). Interestingly, up to 25% of the human microbiome variability seems related to the environmental input, rather than ethnicity (Rothschild *et al.*, 2018). Drugs and disinfectants can heavily affect the microbiome, in parallel an increase in allergic and chronic inflammatory diseases was observed in Western countries, whereas low-income countries are reported to be less affected by these illnesses (Hugg *et al.*, 2008;

Anandan *et al.*, 2010; James *et al.*, 2010), likely consistent with the reduced environmental microbial stimulation correlated to increased sanitation, which is consistent with the "hygiene hypothesis" (Strachan, 1989; Ege *et al.*, 2011; Rook, 2013).

Characterization of the Built Environment (BE) microbiome The BE microbiome composition is largely affected by human presence and commonly include high amounts of human skin colonizers such as Gram-positive Staphylococci, but also Gram-negative bacteria including Enterobacteriaceae family, fungi, and viruses. In particular, data from the Home Microbiome Project highlighted a strict relation between microbes, people and their homes, suggesting rapid colonization of the home environment by the family's microbiota (Lax *et al.*, 2014; Li *et al.*, 2021). Various bacterial species can reside in the toilet, kitchen, and refrigerators, potentially being a direct source of food borne diseases (Jeon *et al.*, 2013). Proteobacteria, Firmicutes, Actinobacteria, and Bacteroidetes has been detected in domestic environment, including *Propionibacterium, Bacteroides*, and *Staphylococcus* genera (Jeon *et al.*, 2013). Moreover, *Staphylococcus* and *Micrococcus* genera were also detected in washing machines, half of which potential opportunistic pathogens, emphasizing the need for effective cleaning and control strategies (Jacksch *et al.*, 2021). *Staphylococcus* species, usual colonizers of the human skin and upper respiratory tract are commonly spread from humans to BE surfaces and air (Xu *et al.*, 2015b; Madsen *et al.*, 2018; Cave *et al.*, 2021b), and have been associated with the development of chronic conditions and allergic diseases, in the context of domestic environments and urbanized areas (Shan *et al.*, 2020), due to their ability to persist long in domestic dry environments and surfaces (Oie and Kamiya, 1996; Smith *et al.*, 2001). Among *Staphylococcus* species, *Staphylococcus aureus* can cause community and hospital-associated infections (Aires De Sousa and De Lencastre, 2004; Gupta *et al.*, 2015), and its increasing AMR makes it one of the major pathogens in both nosocomial and community-associated infections (Tong *et al.*, 2015). Methicillin resistant *S. aureus* (MRSA) was originally detected only in the healthcare settings but has now spread also to domestic environments (Cave *et al.*, 2021a). Similarly, coagulase-negative staphylococci (including *S. epidermidis*, *S. haemolyticus*, *S. hominis* and *S. saprophyticus*, initially associated with severe infections in the hospital environment, now are frequently detected in non-healthcare settings (Becker *et al.*, 2014), playing an important role in horizontal transfer of AMR genes (Davis *et al.*, 2012).

Enterobacteriaceae, Gram-negative bacteria commonly present in the human gut microbiota, but also found in food, water, and home surfaces (Manshadi *et al.*, 2013; Ashbolt, 2015), show widespread beta-lactam resistance and carbapenem resistance (Denisuik *et al.*, 2013) and have also often been reported in non-healthcare environments, highlighting the potential threat for public health (Meletis, 2016; Kelly *et al.*,

2017). Of note, the recent spread of mobile colistin resistance (mcr) genes among MDR Enterobacteriaceae rendered inefficient even the last-resort colistin drug (Morrill *et al.*, 2015), with further important consequences for human health (Wang *et al.*, 2017).

Fungi are also detectable, and the indoor mycobiome is largely composed by saprotrophs, including *Cladosporium*, *Aspergillus*, and *Penicillium* (Nevalainen *et al.*, 2015; Flannigan *et al.*, 2016). Its diversity, abundance, and composition are influenced by factors including climate and local environmental variations (Adams *et al.*, 2016; Stephens, 2016). Like other contaminant microorganisms, fungi can survive for days to months on BE surfaces (Kramer *et al.*, 2006), where the release of spores, hyphal fragments, and mycotoxins represent a source of indoor pollution.

The viral community harboring BE microbiome is less explored, although it is known that the main sources of viruses are represented by humans, pets, plants, indoor ventilation and air-conditioning systems, and dust (Prussin and Marr, 2015). The new pandemic caused by the Severe Acute Respiratory Syndrome Coronavirus type 2 (SARS-CoV-2) highlights the important role of indoor spaces as primary sites of viral transmission, both by direct human-to-human interaction and through the air (Cai *et al.*, 2020; Dietz *et al.*, 2020; Liu *et al.*, 2020). In addition, recent data also highlight the potential role of contaminated surfaces and fomites as a possible transmission route for SARS-CoV-2 (Chen *et al.*, 2020; Contini *et al.*, 2020; D'Accolti *et al.*, 2020), based on its ability to survive up to days on inanimate surfaces (Kampf *et al.*, 2020), similarly to other enveloped viruses including influenza viruses and herpesviruses (Kramer *et al.*, 2006; Dublineau *et al.*, 2011).

The Threat of Antimicrobial Resistance The term antimicrobial resistance (AMR) refers to the ability of microorganisms to be resistant to the action of one or more antimicrobial agents, and today represents one of the most urgent threats to the public health. According to the European Centre Disease and Control (ECDC), each year in Europe at least 4 million people acquire an antibiotic resistance infection, leading to over 40,000 deaths (European Centre for Disease Prevention and Control, 2020). The World Health Organization (WHO) estimated at least 10 million deaths within 2050, if no concrete and rapid global action is taken against AMR (O'Neill, 2016). AMR primarily development resulted from the selective pressure exerted by the widespread use of disinfectants and antibiotics in the hospital settings, as well as in agriculture, livestock, and in the general community (Wand *et al.*, 2016). Microorganisms persistently contaminating those environments develop drug, multi-drug, or pan-drug resistant phenotypes (MDR, panDR), then were spread rapidly through the environment (including settings with low antibiotic usage such as private homes), the food chain, the frequently touched surfaces, or via direct

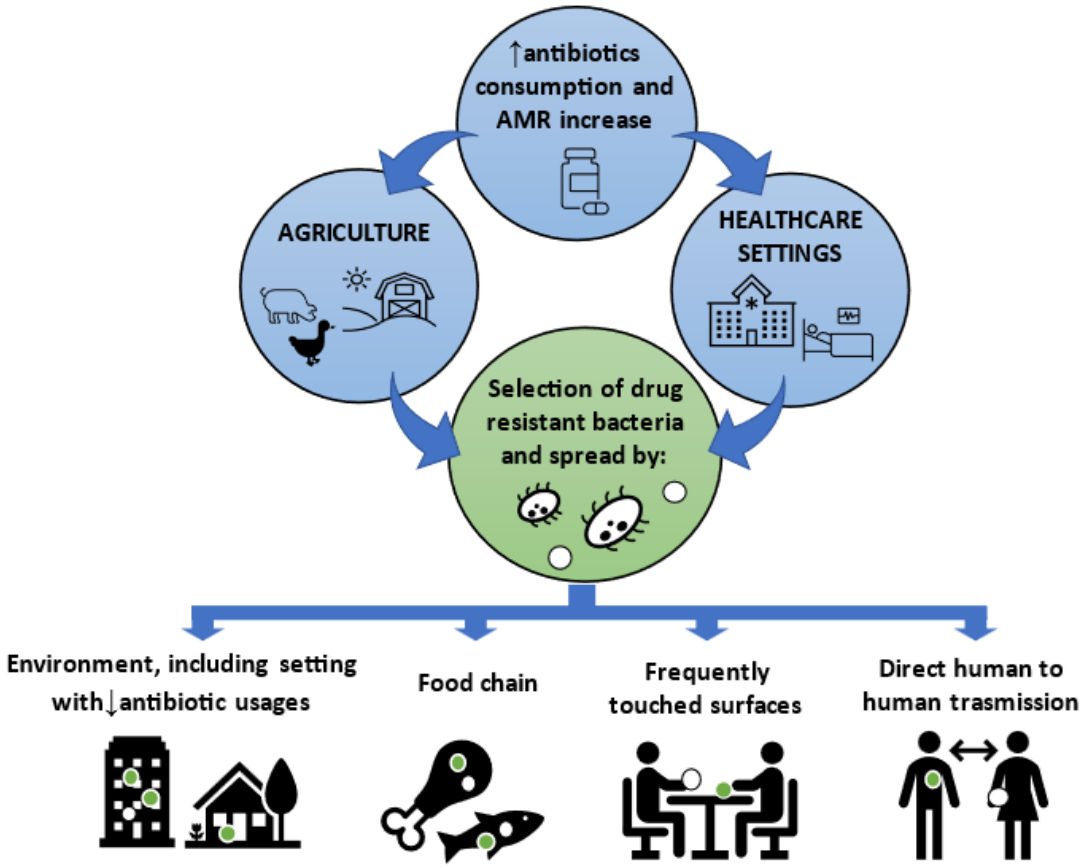


Figure 1.4: Antimicrobial resistance selection and spread in different environments (D'Accolti *et al.*, 2022).

contact between humans (Figure 1.4) (D'Accolti *et al.*, 2022). The AMR onset and spread in the general population is influenced by microbial, host, and environmental factors, including exposure to antibiotics in clinical medicine, agriculture, livestock, food production, and environmental decontamination (Singer and Williams-Nguyen, 2014; Woolhouse *et al.*, 2015; Holmes *et al.*, 2016). Non-healthcare highly frequented settings, including public transports, schools, universities, can become reservoirs for AMR pathogen spread, which can also finally take place in domestic environments. However, in this "chain" of AMR transmission, healthcare settings play a major role in the selection and spread of resistant pathogens.

1.1.1 The Hospital Microbiome

Healthcare settings represent a focal environment for AMR selection and spread, due to the high selective pressure exerted by the continuous and massive use of disinfectants and antimicrobials. Infections caused by AMR pathogens, also called "superbugs", are particularly dangerous for hospitalized patients, as they are very difficult, or even impossible, to treat. Consistently, AMR spread in healthcare settings

is tightly associated with the severity of the so-called healthcare-associated infections (HAI), defined as a "*localized or systemic condition occurring as an adverse reaction to the presence of an infectious agent(s) or its toxin(s) that was neither present nor incubating upon the patient's admission to the acute care facility*" (Horan *et al.*, 2008). HAIs represent one of the most frequent complications occurring in healthcare facilities worldwide, affecting up to 15% of all hospitalized patients (Allegranzi *et al.*, 2011). According to the ECDC, over 4 million patients contract a HAI in EU each year, and >37,000 patients die as a direct HAI consequence (Brusaferro *et al.*, 2015; European Centre for Disease Prevention and Control, 2020). In Italy, the HAI incidence varies from 5-15% for hospitalized patients to 1% for home care patients, with an up to 30% mortality rate (Messineo and Marsella, 2015). In addition, HAI occurrence is responsible of lengthening of the duration of stay, long-term disability, and additional economic burden, with extra costs of about 1.1 billion Euros each year (World Health Organization, 2011).

The hospital microbiome in HAI transmission

The hospital microbiome represents an important *reservoir* of the pathogens listed by WHO in the so-called "ESKAPE" and "Dirty Dozen" groups. In particular, the acronym ESKAPE includes six nosocomial pathogens exhibiting MDR phenotype and high virulence: *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp. (Rice, 2008). The "Dirty Dozen" group is a global priority pathogens list (PPL) made by WHO in 2017, including 12 bacteria against which new antibiotics are urgently needed (Tacconelli *et al.*, 2018). Methicillin- and vancomycin-resistant *S. aureus* (MRSA, VRSA) and vancomycin resistant *E. faecium* (VRE) are included in the high priority group of the list, while carbapenem-resistant Enterobacteriaceae, *A. baumannii* and *P. aeruginosa* are included in the "critical" priority group (World Health Organization, 2017b).

The hospital microbiome has a crucial role in HAI onset (Green *et al.*, 1998; Martinez *et al.*, 2003; Dancer, 2008), as most of HAI-associated pathogens can survive long on inanimate surfaces, which represent the pathogen *reservoir* (Kramer *et al.*, 2006; Otter *et al.*, 2011). Consistent with this, the risk to acquire a specific infection increases when a patient is admitted in a room that has been formerly occupied by an infected or colonized patients by that specific infectious agent (Huang *et al.*, 2006). However, several pathogenic microbes have been detected on hospital surfaces even in the absence of infected/colonized patients, including MRSA and VRE, thus suggesting a general persistent contamination by these pathogens (Trick *et al.*, 2002; French *et al.*, 2004). Microbes that are able to survive long on surfaces can be transmitted to patients by direct or indirect contact, including horizontal

transmission from asymptomatic subjects, healthcare workers or patient's visitors (Kramer *et al.*, 2006; Riggs *et al.*, 2007; Otter *et al.*, 2013). In particular, frequently hand-touched surfaces are considered critical for pathogens spreading (Bhalla *et al.*, 2004). The persistence of microorganisms is also favored by their ability to form biofilm, as its production allows microorganisms to be more resistant to disinfectants and cleaning procedures. For example, *S. aureus* dry-surface biofilms have been shown to resist even to sodium hypochlorite's action (Almatroudi *et al.*, 2018).

According to the ECDC, the most frequently reported HAIs include respiratory tract (pneumonia 19.4% and lower respiratory tract 4.1%), surgical site (19.6%), urinary tract (19.0%), bloodstream (10.7%), and gastro-intestinal infections (7.7%) (European Centre for Disease Prevention and Control, 2013). The risk of acquiring a HAI is influenced by the host susceptibility, being higher in fragile patients such as extremely preterm infants receiving care in Neonatal Intensive Care Unit (NICU) (Plano, 2010; Resende *et al.*, 2015). HAIs cause in fact important morbidity and mortality in NICUs (Borghesi and Stronati, 2008), with a 6%-50% prevalence and 20%-80% mortality (Bolat *et al.*, 2012; Tawfik *et al.*, 2017). Among preterm infant HAIs, late onset sepsis (LOS), frequently caused by coagulase-negative staphylococci (CoNS) (39%) and *E. coli* (9%) (Bizzarro *et al.*, 2005), is considered the main cause of morbidity and mortality (Greenberg *et al.*, 2017), also leading to serious clinical sequelae in surviving infants, including necrotizing enterocolitis, bronchopulmonary dysplasia, and neurodevelopmental impairment (Tsai *et al.*, 2014). Other frequent HAIs in newborns include ventilator-associated pneumonia, mainly caused by Gram-negative bacteria, and ventricular shunt infections, mainly sustained by Gram-positive pathogens (McGirt *et al.*, 2003; Lee *et al.*, 2017). The prolonged use of antibiotics has been reported to increase the incidence of neonatal resistant bacterial HAIs (Silva *et al.*, 2018). The role of NICU surfaces in the transmission of HAIs has been suggested, based on different studies investigating the risk of HAI onset in premature infants related to pathogens resident on NICUs surfaces (Bokulich *et al.*, 2013; Brooks *et al.*, 2014).

Based on these considerations, it is clear that a correct cleanliness of hospital environments is essential to control pathogens' spread, including MDR ones, and consequent HAI onset (Rampling *et al.*, 2001; Tacconelli *et al.*, 2014).

Hospital sanitation methods

The decontamination of hospital surfaces has been so far addressed by the use of chemicals-based detergents and disinfectants (National Patient Safety Agency, 2007), which however show some important limitations:

1. First, chemical disinfectants have a high environmental impact and can in-

crease earth and water pollution (Nabi *et al.*, 2020; Zhang *et al.*, 2020).

2. Second, they have a temporary effect, as more than 50% of surfaces cleaned with chemical products did not result adequately decontaminated (Carling *et al.*, 2008), and several microbes have been found to persist on treated surfaces (Kramer *et al.*, 2006; Goodman *et al.*, 2008; Lawley *et al.*, 2010; Boyce, 2016). Also, chemical sanitation is ineffective in preventing recontamination phenomena, which occurs rapidly, within 30 minutes after disinfection (Rutala and Weber, 2014; D'Accolti *et al.*, 2021).

3. Last, but very importantly, chemical sanitation can enhance the selection of microbes resistant to disinfectant itself (Mcdonnell and Russell, 1999; Caini *et al.*, 2013; Cornejo-Juárez *et al.*, 2015), but also exhibiting cross-resistance towards antimicrobial drugs. For example, the disinfectant chlorhexidine has been observed to be able to induce resistance against the antibiotic colistin (Wand *et al.*, 2016) and can additionally favor resistance to several antibiotics including ceftazidime, sulfamethoxazole, imipenem, cefotaxime, and tetracycline (Kampf, 2018). Gram-negative species adapted to benzalkonium chloride can become resistant to ampicillin, cefotaxime, and sulfamethoxazole; cross-resistance to antibiotics was also found with triclosan, octenidine, sodium hypochlorite, and didecyldimethylammonium chloride (Kampf, 2018). Thus, in the light of the increasing AMR diffusion, these "side effects" of chemical sanitation appear particularly undesirable and unsafe, both for human and environmental health.

In recent years, several alternative methods for hospital surface sanitation have been developed, and included automated no-touch technologies, the use of hydrogen peroxide or ultraviolet (UV) light (Weber and Rutala, 2013; Boyce, 2016), and the use of self-disinfecting surfaces, based on the action of antimicrobial agents such as iron, copper and silver (Lansdown, 2006; Noyce *et al.*, 2006; Casey *et al.*, 2010). However, despite the effectiveness of such technologies, they can often be applied only in the absence of patients, they are not suitable for all types of surfaces and are scarcely sustainable in terms of costs (Dancer, 2014).

Research techniques for hospital environmental microbiome investigation

Despite the importance of contamination monitoring in the management of infectious risk in the hospital environment, so far it has mostly been performed by using fluorescent markers or chemical methods (Dancer, 2009, 2014), allowing the assessment of cleaning performance, but not the actual microbial contamination (Arvanitakis *et al.*, 2018). Similarly, methods based on adenosine triphosphate (ATP) by biolu-

minescence assays show low accuracy, as they can identify not only alive microbes but also organic materials, with risk of false positive results (Nante *et al.*, 2017).

Microbiological methods, consisting of culture isolation and CFU (Colony Forming Unit) count, allow instead the detection and quantification of the searched total or specific microbial contaminants per surface unit, and are used basically for high-risk areas monitoring. Microbiological sampling is performed by RODAC plates (Replicate Organism Detection and Counting plates, containing general or specific selective media), dip slides and nitrocellulose membranes, or swabs, sponges, and wipes able to collect microbes from surfaces. However, although effective, these conventional methods are very time-consuming and complex, especially if applied for the analysis of whole microbial population resident on hospital surfaces (D'Accolti *et al.*, 2019).

An efficient environmental monitoring system should instead provide precision, rapidity, and the most comprehensive characterization of the investigated microbiome. To address this need, molecular methods based on DNA technologies represent a useful tool for accurate and informative analysis of high number of different parameters, via both qualitative and quantitative approaches (D'Accolti *et al.*, 2019). Among quantitative approaches, the use of specific qPCR microarrays represents an efficient method for monitoring the hospital microbiome, providing also information on AMR genes present in the contaminant population (Caselli *et al.*, 2016b, 2018, 2019a). The advent of technologies based on DNA sequencing has significantly improved the microbiome investigation, allowing definition of complex populations (Jovel *et al.*, 2016), thus becoming a potentially useful strategy for environmental surveillance (D'Accolti *et al.*, 2019) and representing one of the aims of our research on environmental microbiome monitoring.

New paradigm about environmental health

The concept of a "super-sanitation" was applied for many years in hospital environments, but more recent evidences highlighted that a sanitation approach directed toward a complete elimination of microbial contamination in the environment is not effective, but rather harmful (Vangay *et al.*, 2015).

A habitat free of natural contamination, with the absence of "good" competitors bacteria, can facilitate the colonization of pathogenic species, leading to the spread of resistant pathogens and more virulent strains. An alternative approach has been proposed and positively considered: rather than "over-sterilizing" the environment, could be useful to try to manage the balance of microbial populations by restoring beneficial microbial species. This method has been defined by Al-Ghalith and Knights as "Bidirectional Hygiene" or "Bygiene" (Al-Ghalith and Knights, 2015) (Figure 1.5).

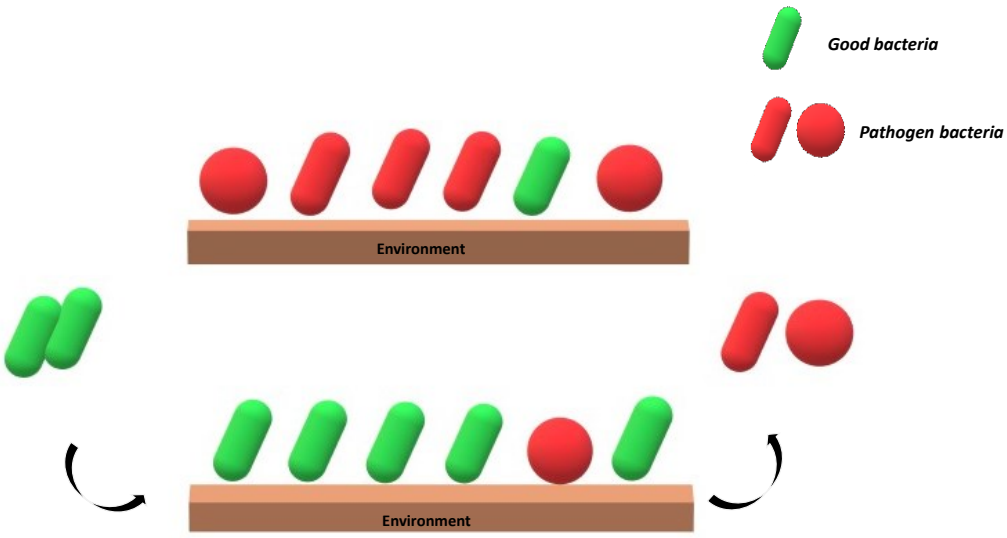


Figure 1.5: Schematic representation of the "Bygiene" principle. The introduction of good bacteria (green) in the environment counteracts the colonization of potential pathogens (red) (D'Accolti *et al.*, 2019).

The reported new approach originates from the recent acquisition regarding the human microbiome, on which many examples highlight how microbiota depletions can lead to pathogenic microorganisms taking over and to the establishment of a dysbiotic condition.

Probiotic cleaning for environmental sanitation: the PCHS system

Probiotics are defined as "*live microorganisms that confer a health benefit to the host, when administered in adequate amounts*", accordingly with FAO/WHO guidelines (Food and Agriculture Organization and World Health Organization, 2002).

Probiotics have been reported effective in counteract the occurrence of different nosocomial infections including diarrhea (Squellati, 2018), necrotizing enterocolitis (Patel and Underwood, 2018), upper respiratory infections (Banupriya *et al.*, 2015), and infections in surgical patients (Rayes *et al.*, 2002, 2012; Sommacal *et al.*, 2015).

In an effort to find an effective method for environmental sanitation in healthcare settings, numerous investigations of our group of research focused on the application of an innovative eco-sustainable cleaning system, defined "Probiotic Cleaning Hygiene System" (PCHS), based on non-pathogenic probiotic bacteria of *Bacillus* genus, namely *B. subtilis*, *B. pumilus*, and *B. megaterium*. Bacteria belonging to *Bacillus* genus are gram positive rods, spore-forming, and ubiquitously found in environment (for example in soil and water), and also in human and animal gut. Except for *Bacillus anthracis* and *Bacillus cereus* species, they are considered safe

for application in humans (EFSA, 2020), and have been broadly applicated in food production, aquaculture and agriculture, human and animal therapy.

The ability of *Bacillus* to persist in sporogenic form made them suitable for addition to concentrated detergents, maintaining their viability and the ability to germinate when diluted and applied on surfaces. *In vitro* studies showed that PCHS-*Bacillus* could almost completely inhibit the growth of Gram-positive and Gram-negative bacteria, as well as of mycetes (Caselli *et al.*, 2019b). The main mechanism of action is based on competitive antagonism (Gause's law), moreover the production of antibacterial compounds, known as bacteriocins, has been observed.

Several studies on field proved that PCHS application is able to induce a stable remodulation of hospital microbiome, and even a more relevant modulation of AMR among hospital microbiome. Investigation on field involved different public and private healthcare settings, in which PCHS results were directly compared to conventional chlorine-based cleaning (Vandini *et al.*, 2014), or analyzed in the same wards in pre-post intervention studies (Caselli *et al.*, 2016b, 2018). PCHS was observed to stably abate pathogen contamination up to 90% more efficiently than chemical disinfectants, without selecting antimicrobial resistant pathogens, evaluated by Real Time qPCR targeting 84 Resistance genes.

An implemented microbiological surveillance in all treated hospitals assured both the genetic stability of PCHS-derived *Bacillus* species, and the absence of pathogenic potential in hospitalized patients (Caselli *et al.*, 2016b,a).

Another positive outcome of PCHS application resulted in a 52% reduction of HAI incidence in hospitals with probiotic cleaning compared to those with conventional chemical sanitation (Caselli *et al.*, 2018). In addition, HAI reduction significantly impacted antimicrobial consumption and related costs (Caselli *et al.*, 2019a).

The major limitation of PCHS system in surface disinfection resides on its gradualness and non-specific action, based on this consideration a potential improvement with the addition of bacteriophages has been considered (D'Accolti *et al.*, 2021). Bacteriophages are ubiquitous viruses infecting exclusively bacteria, with high specificity and rapid action. Due to their antimicrobial activity, they have been broadly utilized prior to the discovery of antibiotics, especially in eastern countries. With the alarming spread of microbial resistance to antibiotics, they have been currently revalued as alternative antimicrobial strategy. Several data demonstrate that they are safe for human health, and can be a successful option in several fields, including farms, food industry, agriculture, aquaculture and wastewater plants (D'Accolti *et al.*, 2021).

Our research therefore focused on their potential application in the hospital environment. Initial *in vitro* tests assessed bacteriophages activity on different type of

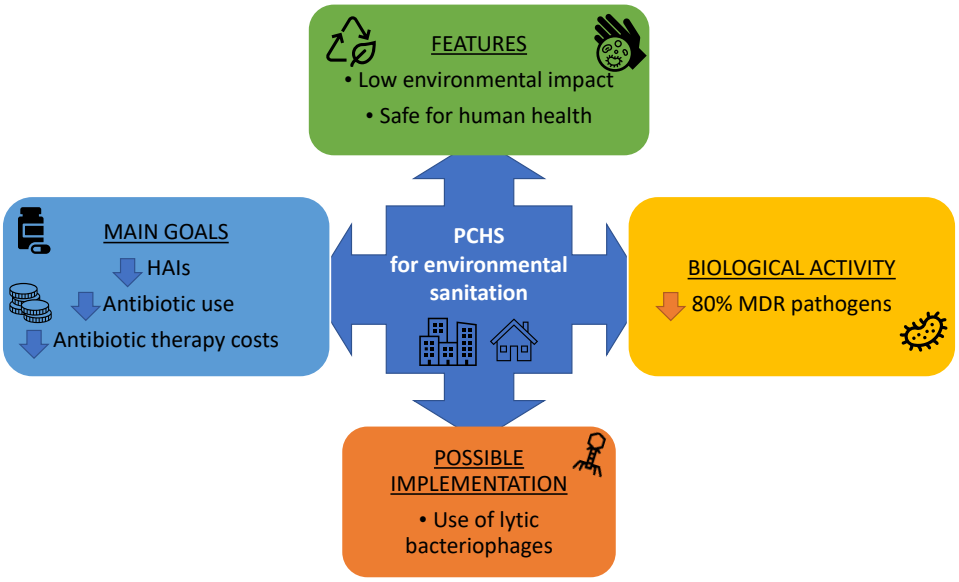


Figure 1.6: Features of PCHS system for environmental sanitation, main goals, biological activity, and possible implementation of the system (D'Accolti *et al.*, 2022).

hard nonporous surfaces, similar to those present in nosocomial environments. In a further study on field, routinary PCHS sanitation in healthcare setting has been implemented with the addition of lytic and target-specific bacteriophages. Staphylococcal contamination in the bathrooms of General Medicine wards of an Italian hospital was monitored, being those areas the most contaminated and Staphylococci the most prevalent bacteria in such settings. Results highlighted that phage application induced a rapid decrease in *Staphylococcus* spp. load on treated surfaces, up to 97% more than PCHS alone (D'Accolti *et al.*, 2019). Taking together, the results obtained with the application of probiotic bacteria and bacteriophages can be a clear example of how the environmental microbiome can be remodeled, counteracting the AMR spread and promote a healthier environment. The main features of PCHS system are reported in Figure 1.6.

The PCHS system during COVID-19 pandemics

The new SARS-CoV-2 human coronavirus has spread worldwide from end of 2019 causing a global health problem. Corona Virus Disease 2019 (COVID-19) has globally caused nearly 300 million cases, with over 5 million deaths (World Health Organization, 2021). The ongoing COVID-19 pandemic has profoundly influenced the habits relative to hygiene and sanitization measures, in all aspect of human life and above all, in the hospital environment. SARS-CoV-2 is mainly spread by respiratory droplets and has been reported to stably persist on inanimate surfaces (Chin *et al.*, 2020; Kampf *et al.*, 2020; Marquès and Domingo, 2021). Although the transmis-

sion via contaminated fomites is difficult to assess definitively (Centers for Disease Control and Prevention, 2021), a few cases have been reported (Kampf *et al.*, 2020; Meyerowitz *et al.*, 2021) and the risk of hand-to-fomite transmission has been addressed (Liu *et al.*, 2021; Pitol and Julian, 2021; Wilson *et al.*, 2021). The current condition of health emergency has imposed the massive use of high-level virucidal chemical disinfectants, including 0.1-0.5% sodium hypochlorite (NaClO) and 1% hydrogen peroxide (H_2O_2), in both healthcare and non-healthcare settings (CDC, 2020; ISS, 2020; World Health Organization, 2020). As already mentioned, chemical disinfectants present several disadvantages, foremost the risk of selection of AMR pathogens. The problem of AMR spread is considered so crucial that the WHO organization feared a risk for a next AMR pandemic, caused by the anti-COVID-19 sanitation measures worldwide applied (World Health Organization, 2020). In addition, AMR microbes may worsen the care of COVID-19 patients, further exacerbating the impact of COVID-19 pandemic (Knight *et al.*, 2021).

The new approach based on PCHS system could be a valid sanitation alternative, in order to overcome the negative effects of chemical disinfectants yet preserving the effectiveness of sanitation against enveloped viruses. In addition, the monitoring of the hospital environment during COVID-19 pandemics could be a useful tool for AMR surveillance, and also test the potential effectiveness of PCHS sanitation compared to chemical one. These aspects of PCHS application represented part of the aim of our research, that have been included in this thesis.

1.2 The Human Microbiome in Health and Disease

The human microbiome comprises more than 100 trillion of microorganisms, including bacteria, archea, viruses and eukaryotic microbes (O'Hara and Shanahan, 2006), with more than 1000 different bacterial species and 150 times more microbial genes than the entire human genome (Ursell *et al.*, 2014). Commensal bacteria colonize the human body shortly after birth, then these primary communities gradually evolve into complex and dynamical balanced microbial ecosystems, during the host growth (Rogier *et al.*, 2014). During human life, the microbiome exerts a wide range of beneficial function for the human body, including food metabolization and nutrient acquisition (Gill *et al.*, 2006), the establishment of a protective barrier from foreign pathogens colonization through competitive exclusion and production of antimicrobial substances (Wang *et al.*, 2017), and the development and maturation of the host immune system (Macpherson and Harris, 2004).

Several recent evidences have highlighted a crucial role of the microbiome in human health and disease, where the beneficial microbiome-host interplay generally associated with high microbial diversity is responsible for maintaining a healthy state

of the host, whereas the microbiome dysbiosis frequently associated with biodiversity loss has been linked to human diseases.

Among the human body sites, our research works focused in particular on oral and vaginal microbiomes.

1.2.1 The Oral Microbiome

The oral cavity hosts the second most complex, highly dynamic and populated microbial environment of the human body, after the gut, harboring over 700 species of bacteria, fungi, viruses and protozoa (Bäckhed *et al.*, 2012). Over 250 bacterial species have been culturally isolated and investigated, including several pathogens associated to dental caries and periodontitis, such as *Streptococcus mutans*, *Porphyromonas gingivalis*, and *Tannerella forsythia* (Holt and Ebersole, 2005; Mineoka *et al.*, 2008). The subsequent development of metagenomic approaches has led to an increasingly in-depth characterization of oral microbiota composition and function.

Within the oral cavity, distinct microenvironments are present (including soft tissues of tongue dorsum and mucosal membranes and hard tissue of teeth plaques), each one is colonized by niche-specific microorganisms (Rosan and Lamont, 2000). In addition, oral surfaces are continuously exposed to foreign substances, saliva and gingival crevicular fluid (GCF) (Lamont *et al.*, 2018; Baker and Edlund, 2019). Tissue-specific tropisms are largely influenced by the adhesion capacity, useful to provide resistance to mastication and to mechanical forces of salivary flow inside the mouth (Lamont *et al.*, 2018). Last, diet and lifestyle play an important role on oral microbiome, influencing its composition.

The oral microbiome development and variations

The oral cavity has been long considered sterile until birth, and then rapidly colonized after delivery, on contact to the external environment (Perez-Muñoz *et al.*, 2017), but recent findings show the presence of bacteria within the uterus (Aagaard *et al.*, 2014; Perez-Muñoz *et al.*, 2017), and data showed that oral neonatal microbiota appeared significantly associated mainly with that of maternal placenta, suggesting a plausible prenatal origin (Tuominen *et al.*, 2019).

Several studies investigate the composition of placental microbiome, finding bacterial species belonging to *Streptococcus*, *Fusobacterium*, *Neisseria*, *Prevotella* and *Porphyromonas* genera, that have been frequently observed in oral diseases (Han, 2011; Aagaard *et al.*, 2014; Cobb *et al.*, 2017). On the other hand, the presence of oral pathogens in the uterus has been associated with adverse pregnancy outcomes, such as preeclampsia and preterm delivery (Han, 2011; Cobb *et al.*, 2017; Vander Haar *et al.*, 2018). For example, *Fusobacterium nucleatum*, a typical oral

pathogen, has been isolated in amniotic fluid and cord blood from women with adverse pregnancy outcome, suggesting an hematogenous translocation from inflamed gingival tissue to decidua tissue (Vander Haar *et al.*, 2018). Zaura et al. hypothesized that during pregnancy, an increased gingival inflammation promotes the passage of maternal oral microbes to placenta, that become an antigen-collecting site for the development of fetal tolerance mechanism (Zaura *et al.*, 2009; Kaan *et al.*, 2021).

Following delivery, the colonization of the neonatal oral cavity begins early, within 8-16 hours after birth, driven by vertical transmission from the mother (in particular through exposure to maternal vaginal and skin microbiomes), transmission from the mother's diet prior to delivery, and horizontal transmission (from other human-infant interactions) (Nelson-Filho *et al.*, 2013). Vaginal or cesarian section delivery lead to a wide range of hormonal, metabolic, immunologic differences, in both mother and infant (Hyde *et al.*, 2012), and influences microbial colonization of the newborn's oral cavity. Vaginal microbiota, such as *Lactobacillus* species, *Prevotella* species, and *Sneathia* species, are dominant in babies delivered vaginally, while skin-associated microbes *(Staphylococcus* spp., *Corynebacterium* spp., *Propionibacterum* spp.) result more abundant in babies delivered by cesarian section (Dominguez-Bello *et al.*, 2010). Infant oral microbiome is shaped also by maternal exposure to antibiotics during pregnancy, in fact exposed infants showed higher proportion of Protobacteria phylum, while Firmicutes phylum and *Lactobacillus* species were found predominant in unexposed neonates (Gomez-Arango *et al.*, 2017). *Streptococcus, Staphylococcus* and *Fusobacterium* species are considered the major early colonizers of the oral cavity (Dzidic *et al.*, 2018). Among them, *Streptococci* represent a dominant bacterial group in breast milk, and their adhesion ability makes them the principal constituent of the infant oral microbiota (Boix-Amorós *et al.*, 2016). Additional early colonizers are represented by *Gemella, Rothia, Granulicatella* and *Haemophilus* species, found at 3-6 months of age and increased with ages (Dzidic *et al.*, 2018). A recent study by Mason et al. found that predentate children shares over 85% of oral bacteria with their mother, suggesting an early maternal imprinting (Mason *et al.*, 2018).

Oral microbiome composition changes throughout life, shaped by host genetics and environmental factors (Sedghi *et al.*, 2021). Oral microbial diversity increases in early life, in particular the eruption of primary teeth introduces new substrata for microbial colonization, allowing an ecological shift of microbial niches (Costello *et al.*, 2009, 2012). The acquisition of permanent dentition, together with the acquisition of independent oral-hygiene practices, cause the substantial stabilization of microbiotas during children stage. In adult life, oral microbiome is profoundly shaped by environmental factors, including dietary habits, oral hygiene practices, smoking, alcohol consumption, stress conditions and systemic factors. In addition,

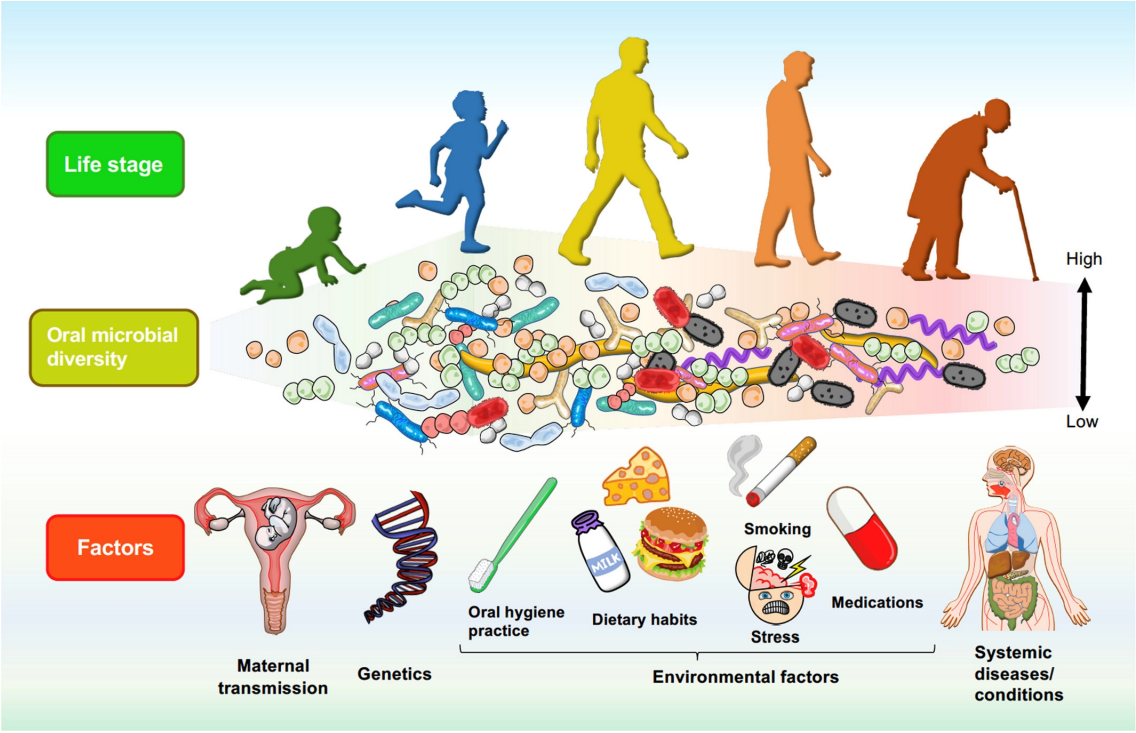


Figure 1.7: Development and factors influencing the human oral microbiota composition throughout life (Sedghi *et al.*, 2021).

the role of hereditary factors has been widely discussed, and current data highlights the influence of genetic predisposition to the development of dental caries and periodontal diseases (Demmitt *et al.*, 2017; Esberg *et al.*, 2020). Oral microbiome diversity has been shown to decline with age (Burcham *et al.*, 2020). Figure 1.7 shows the trend of oral microbial diversity and the factors influencing the onset/alteration of oral microbiota composition during the different life stages.

Research techniques for oral microbiome investigation

The microbiome of the oral cavity represents a complex environment, of which only 54% of species are cultivable and identifiable, the 14% represents the percentage of species that are cultivable but not identifiable, and the remaining 32% not even cultivable (Wade, 2013). Thus, it is clear that conventional approaches, involving culture-dependent techniques, have several limitations. In recent decades, the use of germ-free mice was considered the gold standard for studies of the microbiota, allowing individual colonization with defined microbes to investigate their effects (Kennedy *et al.*, 2018). Such studies have been employed in the investigation of the oral microbiota in diabetes pathogenesis (Xiao *et al.*, 2017) and inflammatory periodontal disease (Hajishengallis *et al.*, 2011; Dutzan *et al.*, 2018). However, this procedure requires specialized facilities and training, together with high costs and expertise, making it unaffordable to many researchers. Based on this, the use of

antibiotics-treated mouse model has emerged, representing a less demanding and expensive method. Besides, *in vitro* biofilm culture systems from saliva inocula have been extensively used to investigate mechanisms involved in microbial adherence, inter or intra-species interactions and pathways existing into the microbial communities (Bunetel *et al.*, 2000; Samarian *et al.*, 2014; Li *et al.*, 2017). These systems present however limitations related to different growing conditions on specialized culture media, including anaerobic conditions, and the difficulty of maintaining the complexity of oral microbiome and to recreating the conditions of salivary flow and oral microenvironment (Edlund *et al.*, 2013; Samarian *et al.*, 2014).

The development of metagenomics have instead provided a comprehensive characterization of the microbial communities that colonize the oral cavity, rendering possible to identify specific microbial taxa found within the human oral cavity that are difficult or impossible to cultivate (Ursell *et al.*, 2012). However, 16S ribosomal RNA sequencing by NGS presents some limitations, as sequencing of hypervariable genes allows identification of bacteria at the level of species, but do not provide information of sub-species characteristics and limit the resolution of phylogenetically related species or strains. Moreover, this technique considers neither the viral and mycotic component of the microbiome, nor provide information of functional genes content. Based on this, shotgun metagenomics sequencing (WGS) should be preferred, allowing a parallel assessment of all microbial kingdoms (bacteria, fungi, viruses) in the same sample (Quince *et al.*, 2017b,a; Han *et al.*, 2020).

Oral microbiota in fact includes also non-bacterial species, including eukaryotic microbes (fungi, protozoa) and viruses. Furthermore, the oral cavity hosts several unique ecological niches, characterized by specific nutrient, environmental conditions, and related unique microbial communities (Aas *et al.*, 2005; Avila *et al.*, 2009), thus each microhabitat should be sampled following appropriate procedures, as described in the Human Microbiome Project (HMP), which provides the protocols described below and already reported in published papers (Mcinnes, 2010; Segata *et al.*, 2012; Eren *et al.*, 2014; Yu *et al.*, 2017):

1. Saliva sampling: collection of saliva non-stimulated or stimulated by administration of an unflavored gum, into a labeled 50 ml collection tube; it is also indicated the sampling of oral rinse specimens (saliva after rinsing), to enhance the collection of the adherent bacteria fraction,

2. Soft tissues sampling: to be performed by using sterile brushes or nylon sterile microbrushes (Mager *et al.*, 2003; Zaura *et al.*, 2009),

3. Hard tissues sampling: to be performed by using sterile Gracey curettes for plaque sampling on teeth (Segata *et al.*, 2012; Eren *et al.*, 2014; Yu *et al.*, 2017).

Besides HMP protocols, others sample collection procedures, such as endodontics paper cones, sterile toothpicks and floss, are extensively reported in literature (Xie *et al.*, 2010; Welch *et al.*, 2016; Hall *et al.*, 2017).

Oral microbiome characterization: one oral cavity, different microbial niches

The mouth is characterized by different oral niches, where the microbial colonization is influenced by the diverse ability of bacteria to adhere and by the availability of oxygen and nutrients, as depicted in Figure 1.8.

For example, the dorsum of the tongue, as well as supra- and subgingival dental plaques, provide a suitable habitat for strict anaerobes colonization (Xu *et al.*, 2015a). The hard, non-shedding dental surfaces represent a site for multispecies biofilm formation. The enamel surfaces acquire a salivary pellicle, made up of proteins, enzymes, glycoproteins, lipids and mucins, that enable the first adherence by primary colonizers, essentially Streptococci (Cavalcanti *et al.*, 2017; Chawhuaveang *et al.*, 2021), which then allow the subsequent adhesion of other microorganisms, such as *Fusobacteria*, *Veillonella*, and *Rothia* (Whittaker *et al.*, 1996). Once established, the conglomerate of bacteria begins to replicate, leading to the formation and maturation of a multispecies biofilm (Rosan and Lamont, 2000). By contrast, the desquamating mucosal surfaces do not allow the formation of mature biofilm (Lamont *et al.*, 2018), while having an important role in maintaining tissue integrity and prevent microbial colonization via secretory IgA (Hannig *et al.*, 2017). Saliva is

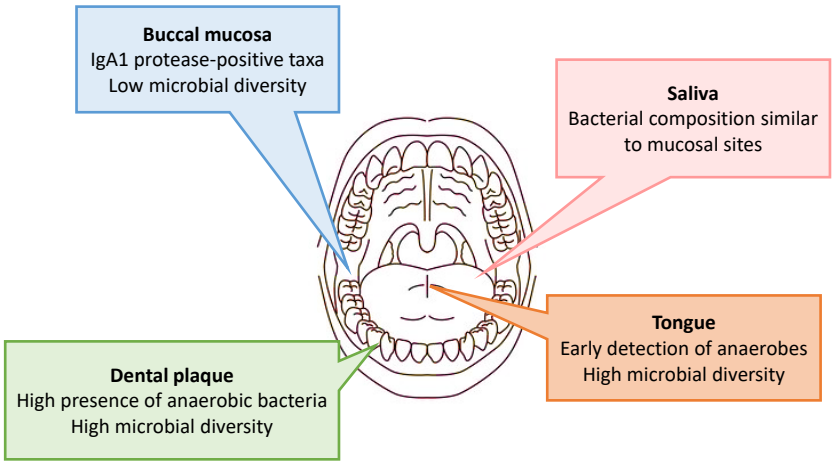


Figure 1.8: Different microbial niches inside the oral cavity. Modified from Kaan *et al.* (2021).

not considered a defined oral niche, but rather a pool of microorganisms dislodged from various oral niches, and bacterial profile of saliva is closer to those of mucosal sites, than to those of dental surfaces (Faust *et al.*, 2012). Velocity of salivary flows, nutrient and oxygen availability, eating and mastication forces, and oral hygiene practices have a strong effect on microbial dynamics (Proctor *et al.*, 2018).

Oral microbiome role in health and disease

A healthy oral condition is characterized by a homeostatic balance between the host and the commensal microbial communities, which have been recognized as being able to contribute to the development of healthy tissues and immune system, and to maintain a healthy status of oral microhabitats by competing with pathogens through competitive exclusion (Vollaard and Clasener, 1994). Data from literature reported different mechanisms of competition against periodontopathogens: the passive occupation of the same niche, the interference with adhesion capability on surfaces, the competition for essential nutrients, and the ability to modify/regulate the production of virulence factors (Socransky *et al.*, 1998).

Oral commensal, such as *S. sanguinis*, *S. cristatus*, *S. salivarius*, *S. mitis*, *Actinomyces naeslundii*, and *Haemophilus parainfluenzae*, have been reported to exert direct antagonism against oral pathogens, by reducing the adhesion of periodontopathogens such as *P. gingivalis*, in *in vitro* models (Van Hoogmoed *et al.*, 2008; Wu and Xie, 2010). In particular, *S. mitis* has been suggested as possible candidate for "replacement therapy", consisting of the deliberate colonization of pockets with antagonistic bacteria in order to restore healthy periodontal microflora promoting the healing of periodontitis (Allaker and Stephen, 2017). Clinical isolates from subgingival plaque of healthy patients were reported to inhibit pathogens including *P. intermedia*, *P. gingivalis*, *A. actinomycetemcomitans*, and *F. nucleatum* more efficiently compared to isolates collected from periodontitis patients (van Essche *et al.*, 2013). Of note, recent studies highlighted that nitrate reduction induced by the use of mouthwash containing chlorhexidine may negatively impact on commensal oral bacteria activity (Govoni *et al.*, 2008). Oral commensals also contribute to general health through the production of bacteriocins; for example, *Lactobacillus lactis* produce nisin, a bacteriocin that has been found to inhibits head and neck cancer tumorigenesis and prolongs survival (Kamarajan *et al.*, 2015). Nisin has been recently suggested as potential antimicrobial and anti-tumorigenic agent, thanks to its ability to modulate cancerogenic pathways promoted by periodontal pathogens (Kamarajan *et al.*, 2020). Several other commensal species, including *S. gordonii*, *S. sanguinis*, *Lactobacillus casei*, have been involved in maintain an healthy oral microenvironment re-establishing pH homeostasis of saliva, by counteracting the lowering of the pH induced by caries-associated pathogens (Burne and Marquis,

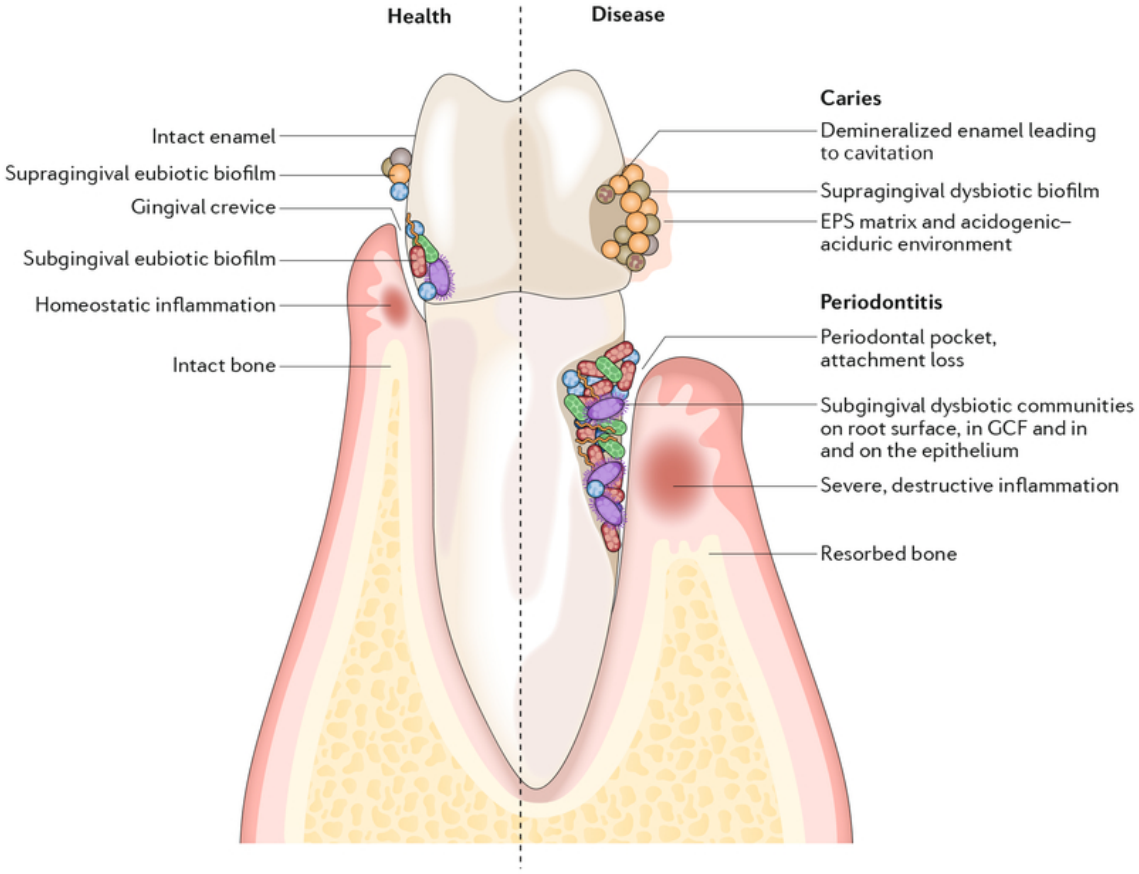


Figure 1.9: Eubiotic or dysbiotic state of the oral cavity (Lamont *et al.*, 2018).

2000).

Various conditions, such as diet, inflammatory and immunological response, tobacco or alcohol assumption, can lead to oral dysbiosis (Pitiphat *et al.*, 2003; Cekici *et al.*, 2014; Wu *et al.*, 2016; Wang *et al.*, 2019), potentially leading to increased risk of oral and systemic diseases onset, including cardiovascular diseases, rheumatoid arthritis and the recently emerged COVID-19 pathology.

Periodontal diseases: caries and periodontitis Site-specific oral diseases associated with altered oral microbiome includes caries, that involve the supragingival dental surface, and periodontitis, affecting subgingival niche (Figure 1.9).

Dental caries is defined as an ecological collision of microbial, host, diet and time factors. It affects around 23% of children and over 60% of adolescents (Dye *et al.*, 2015), possibly leading to complications such as abscesses or pulpal implication (Monse *et al.*, 2010). Notably, recent data also highlighted that untreated caries are the most frequent cause of preventable hospitalization among children (Acharya *et al.*, 2018). The microbial biofilm that is associated with caries is characterized by the prevalence of *S. mutans*, which is considered the most cariogenic bacterium, often accompanied by *Lactobacillus*, *Bifidobacterium* spp., *Scardovia* spp. and *Acti-*

nomyces spp., and fungi (*Candida albicans* (Hajishengallis *et al.*, 2017; Mira *et al.*, 2017; Eriksson *et al.*, 2018). The cariogenic biofilm changes dynamically as caries develops from the initial stage of enamel demineralization to a more extensive and severe lesion reaching the dentin layer of the tooth (Sedghi *et al.*, 2021).

Periodontal diseases affect subgingival tissues, possibly leading to the loss of tooth attachment. This pathology is characterized by a dysbiotic subgingival microbiome that determines uncontrolled inflammatory response at the gingiva level, inducing tissue destruction and formation of anaerobic subgingival pockets (Lamont *et al.*, 2018). Periodontal diseases involve multiple pathways, including both microbial and immunological responses, reciprocally reinforced. However, some key pathogens have been recognized thank to the metagenomics approach, including bacteria of the so called "red-complex", able to stimulate both epithelial and bone damage (tissue destruction, bone loss, destructive inflammation, deepening of gingival crevice) (Kinane *et al.*, 2017). In particular, *P. gingivalis*, *T. forsythia* and *T. denticola* are associated with high risk of progressive periodontitis (Holt and Ebersole, 2005; Socransky and Haffajee, 2005), and several other microorganism have been recently suggested as possible periodontal pathogens, including Gram-positive *Filifactor alocis* and *Peptoanaerobacter stomatis*, Gram-negative *Dialister* spp., *Megasphaera* spp. and *Selenomonas* spp., and species of the genera *Prevotella, Desulfobulbus* and *Synergistes* (Dewhirst *et al.*, 2010; Griffen *et al.*, 2012; Abusleme *et al.*, 2021).

Role of oral microbiome in systemic diseases The dysbiosis of the oral microbiome has been also associated with oral cancer (in particular oral squamous cell cancer), and systemic diseases, including Alzheimer's disease, Rheumatoid arthritis, cardiovascular diseases, and cancer affecting other body sites (for example, colorectal and pancreatic cancers).

Oral squamous cell cancer (OSCC) is highly prevalent worldwide, and several evidences have associated its onset with the high levels of *Porphyromonas*, *Fusobacterium* and *P. gingivalis* (Nagy *et al.*, 1998; Katz *et al.*, 2011). Oral pathogens are hypothesized to contribute to cancer development due to the ability to induce a chronic and dysregulated inflammation, and to interfere with cells pathways controlling cell viability, proliferation and differentiation (Han and Wang, 2013; Atanasova and Yilmaz, 2014; Whitmore and Lamont, 2014). *P. gingivalis*, *T. denticola*, and *F. nucleatum* have been demonstrated to enhance OSCC cell migration, invasion, tumor formation, and tumorigenesis *in vivo*, contributing to a highly aggressive cancer progression (Kamarajan *et al.*, 2020).

P. gingivalis and periodontal disease have been also suggested as risk factors for Alzheimer's disease pathology. Recent evidence identified the pathogen in the brain

of Alzheimer's patients and pointed out the role of a toxic protease produced by *P. gingivalis*, shown to be neurotoxic *in vitro* and *in vivo*, affecting the functionality of tau protein in neurons (Dominy *et al.*, 2019).

Rheumatoid arthritis (RA), an autoimmune disease characterized by chronic inflammation of the joint, has also been correlated with tooth loss and periodontitis (de Pablo *et al.*, 2008), and correlation between periodontal index (periodontal inflamed surface area, PISA) and tooth loss with disease-related parameters was observed in patients with RA and ankylosing spondylitis (AS) (Schmalz *et al.*, 2021). Periodontitis and rheumatoid arthritis share many common genetic and immunological risk factors (Volkov *et al.*, 2020), thus can be considered bidirectional linked, as one enhance the risk of the other and *vice versa* (Thomas *et al.*, 2021). The presence of bacterial DNA and peptidoglycans in the joints of patients may support the hypothesis that bloodstream dissemination of bacteria and pro-inflammatory mediators from oral cavity may trigger or enhance joint inflammation (Thomas *et al.*, 2021). Second, the role of *P. gingivalis* has been proposed in particular because of its ability to synthetize the peptidyl-arginine-deiminase (PAD) enzymes, determining the citrullination of bacterial and human proteins, and stimulating the production of antibodies against the host's citrullinated peptides, which is recognized as typical signs of RA (Wegner *et al.*, 2010; Johansson *et al.*, 2016).

In recent years, there has been increasing evidence on the links between oral dysbiosis and systemic diseases. The principal oral pathogens implicated in systemic diseases are reported in Figure 1.10. The increasingly thorough investigation of the molecular pathways involved in oral dysbiosis and its general impact on human health are allowing the oral microbiota to be considered as a biomarker, diagnostic tool and target for systemic disease assessment and treatment (Thomas *et al.*, 2021).

Role of oral microbiota on viral infection: SARS-CoV-2 As already mentioned, the dysbiosis of the oral cavity has been associated with several diseases, including those of viral origin (Baghbani *et al.*, 2020; Cagna *et al.*, 2020). Since the microbiota colonizes the sites where the virus enter the host, the interaction between these two components has been widely hypothesized in the past (Wilks and Golovkina, 2012), suggesting that the microbiota can prevent, suppress or exacerbate the viral infection, by direct or indirect mechanisms (Baghbani *et al.*, 2020) (Figure 1.11).

Concerning the oral cavity, the role of oral microbiome in the establishment of infection of viruses entering the body via oropharynx route has been reported (Baghbani *et al.*, 2020). An healthy microbiota could inhibit pathogen colonization by competitive exclusion mechanisms or the enhancement of the host immune response, and a mutual interaction between microbiome and viruses has been rec-

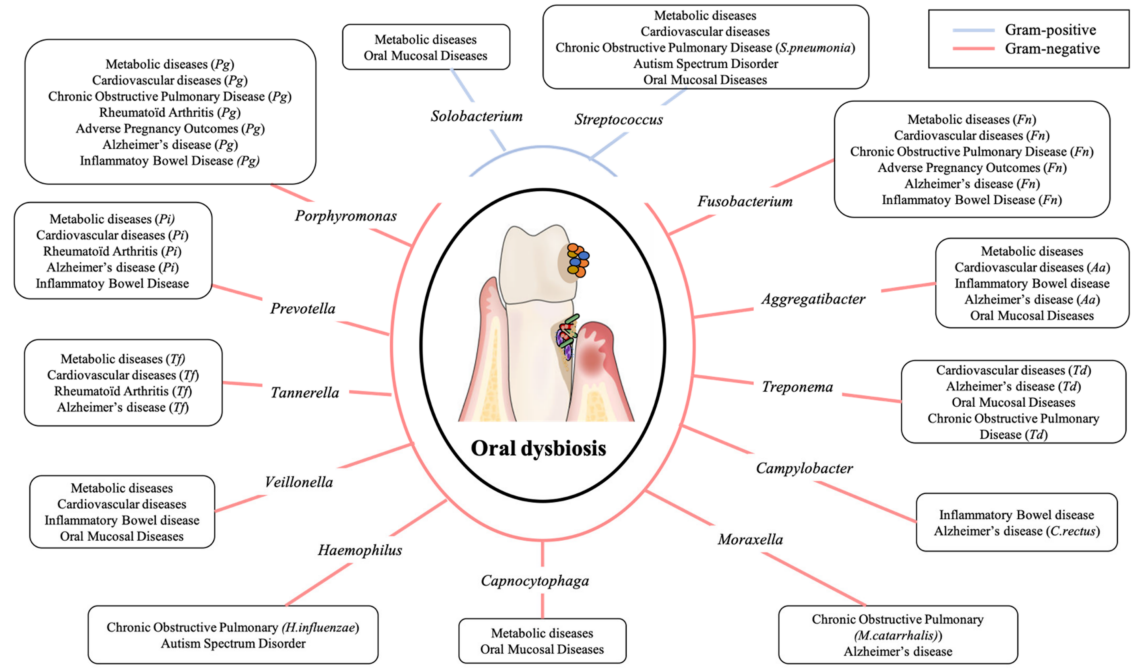


Figure 1.10: The main pathogens involved in oral dysbiosis and linked to systemic diseases (Thomas *et al.*, 2021).
Abbreviations: Fusobacterium nucleatum (Fn), Aggregatibacter actinomycetemcomitans (Aa), Treponema denticola (Td), Tannerella forsythia (Tf), Prevotella intermedia (Pi), Porphyromonas gingivalis (Pg).

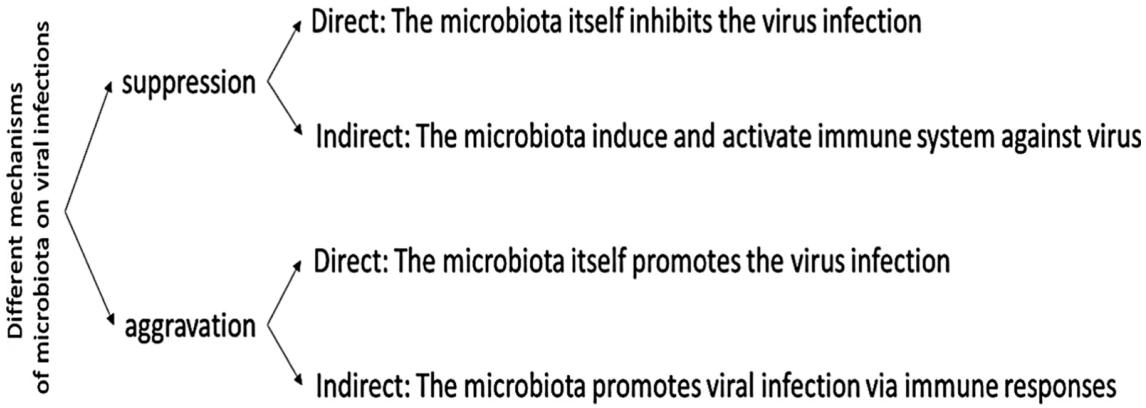


Figure 1.11: Mechanisms of microbiota and viral infection (Baghbani *et al.*, 2020).

ognized (Wilks and Golovkina, 2012).

The new pandemic SARS-CoV-2 enters the body via the oropharynx, thus, similarly to many others respiratory viruses, its replication at the site of entry could be deeply influenced by local microbiome. Interestingly, a recent investigation associated the presence of periodontitis in COVID-19 patients with a 3.5 fold increased risk of admission to intensive care units, a 4.5 fold higher risk of the need of assisted ventilation, and more than 8 fold risk of death (Marouf *et al.*, 2021). Despite the extraordinary high number of studies concerning SARS-CoV-2, little data are available about the oral microbiome profile in COVID-19 subjects. Few papers focused on the bacterial component of the oral microbiome by NGS methods (Iebba *et al.*, 2020; Ward *et al.*, 2021; Wu *et al.*, 2021), but did not consider the non-bacterial component of microbiome (mycobiome and virome), which can have an impact on human susceptibility to infection, including SARS-CoV-2.

Oral microbiome modulation as a therapeutic approach

As already mentioned, different factors (e.g., diet, smoking, drug treatments, stress, and hormonal changes) can influence the balance of the oral microbiota, causing a shift towards a state of dysbiosis and consequently favoring the establishment of periodontal diseases. Prevention methods consist in the non-specific mechanical displacement of oral bacteria and plaque from dentition and gingiva, in order to decrease bacterial accumulation and limit long-term damage from their metabolic products. Devices commonly used include manual or powered toothbrush, interproximal devices (floss or interproximal brush), toothpaste and mouthwash. All these devices has been shown to present several limitations, for example, toothbrushes may become heavily contaminated from the oral cavity, environment, hands and aerosol (Frazelle and Munro, 2012), although there has been little data on how a contaminated toothbrush could influence oral microbiota (Shang *et al.*, 2020); chlorhexidine mouthwash has been recently demonstrated to induce a shift on oral microbial composition, with significant reduction in salivary pH and buffering capacity of saliva, all factors that have been associated with dysbiosis and periodontal diseases (Brookes *et al.*, 2021).

Based on the limitations of traditional methods of oral hygiene, the use of oral probiotics has been recently evaluated, to modulate and restore the eubiosis status.

Consistently, a growing number of studies support probiotic therapy to prevent or treat gingivitis and periodontitis. The study by Krasse et al. shows that consumption of chewing gum containing *L. reuteri* would reduce bleeding, gingivitis pocket probing depth and clinical attachment loss, as it is able to reduce the amount of periodontal pathogens (*A. actinomycetemcomitans*, *P. intermedia* and *P. gingivalis* (Krasse *et al.*, 2006; Vicario *et al.*, 2013). The administration of *L. brevis* has proved

effective in the treatment of periodontal disease, leading to pH remodulation and reducing inflammatory immune response of the host to periodontal pathogens (Riccia *et al.*, 2007). Another strategy that has been taken in account is the selection of probiotic bacteria with inhibitory properties toward oral pathogens, such as *S. mutans*, that has been associated with biofilm development and formation of dental caries (Eriksson *et al.*, 2018). A novel strain of *Streptococcus* A12, isolated from plaque of an healthy subject (without caries) exhibited inhibitory activity against *S. mutans* and has been suggested as possible therapeutic agents able to promote health-associated biofilm communities by maintaining normal pH levels and interfering with the growth and virulence of caries-associated pathogens (Huang *et al.*, 2016).

Besides, also the use of prebiotics (non-digestible carbohydrates that can promote the growth of specific bacteria) may be useful to improve the health of oral microbiome. Prebiotics can be used as supplement of probiotic treatment, stimulating probiotic activity, and inhibiting the growth and activity of potentially harmful bacteria, restoring an ecological balance or regain biodiversity of the oral microbiota (Reddy *et al.*, 2011; Zarco *et al.*, 2012).

Last, several clinical trials also investigated the efficacy of oral bacteriophage application, based on the results obtained in the treatment of wound infection in burn patients, ulcers and chronic otitis (Rhoads *et al.*, 2009; Wright *et al.*, 2009; Rose *et al.*, 2014). Interestingly, phages are reported to eradicate bacterial biofilms, where the antibiotic therapy resulted insufficient, for example in cases of chronic infections sustained by biofilm-producing bacteria (Maciejewska *et al.*, 2018). The use of bacteriophages has been also proposed for modulating the oral microbiota (Norris, 1991), but this application is still quite unexplored.

1.2.2 The Vaginal Microbiome

The vaginal microbiome represents about 9% of the total human microbiome (Sirota *et al.*, 2014) and is considered a complex and dynamically balanced microecosystem that fluctuates throughout a woman's life, influenced by physiological and external factors, including age, menses, sexual behaviors, pregnancy, and hygiene practices. Contrary to the majority of sites of the human body, where a high level of microbial diversity is associated with healthy conditions, the vaginal environment of healthy women appeared dominated by few species of lactic acid-producing bacteria (*Lactobacillus* species), with low microbial diversity (Boskey *et al.*, 2001; Kroon *et al.*, 2018). *Lactobacillus* spp. are found at 10^7–10^9 per gram of vaginal fluid, that correlated with copious amount of lactic acid and vaginal pH < 4.5 (Daniel *et al.*, 2006; Srinivasan *et al.*, 2010). By contrast, a more diverse microbiome, enriched of

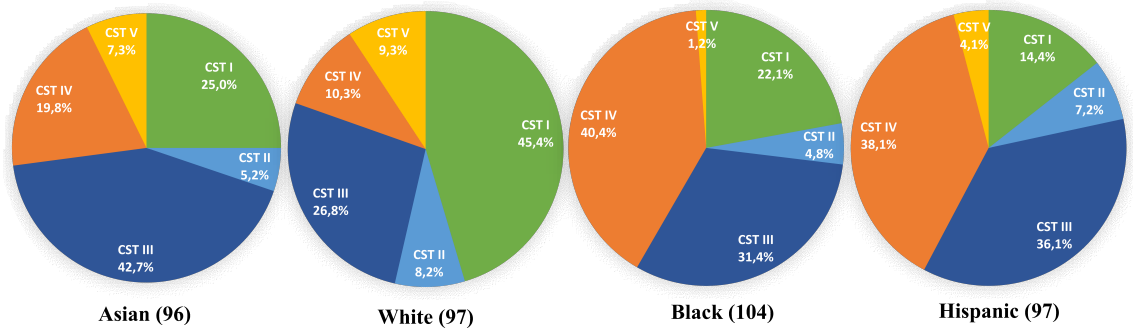


Figure 1.12: Representation of vaginal CSTs distribution within each ethnic group of women. Modified from Ravel *et al.* (2011).

strict or facultative anaerobic bacteria, correlates to vaginal disease, increased risk to develop cervico-vaginal infections and adverse pregnancy outcomes (Kroon *et al.*, 2018). Following the first in-depth and accurate investigation of vaginal microbial ecosystem by 16S rRNA gene sequencing (Ravel *et al.*, 2011), the vaginal microbiome have been classified into five different Community State Types (CSTs), based on the predominant species:

1. CST- I: characterized by the dominance of *Lactobacillus crispatus*
2. CST-II: dominated by *Lactobacillus gasseri*
3. CST- III: with *Lactobacillus iners* dominance
4. CST-IV: the most heterogeneous group, characterized by depletion of *Lactobacillus* spp., significantly higher pH, higher microbial diversity, and higher proportion of anaerobic bacteria including *Prevotella*, *Dialister*, *Atopobium*, *Gardnerella*, *Megasphaera*, *Peptoniphilus*, *Sneathia*, *Eggerthella*, *Aerococcus*, *Finegoldia*, and *Mobiluncus*.
5. CST-V: *Lactobacillus jensenii*-dominated

CST-I, II, III and V are thus *Lactobacillus*-dominated group, whereas CST-IV represents the only non-*Lactobacillus* dominated cluster, that has been associated with Bacterial Vaginosis (BV). The proportions of each community group differed among the four ethnic groups evaluated, as reported in Figure 1.12 (Ravel *et al.*, 2011). Several hypotheses have been formulated to explain the differences between ethnic groups, including genetic factors, that could influence for example differences in innate and adaptive immune systems, the composition and quantity of vaginal secretions, and ligands on epithelial cell surfaces. Other studies have also shown the influence of human habits and practices, including personal hygiene, methods of birth control, and sexual behaviors (Schwebke, 2009).

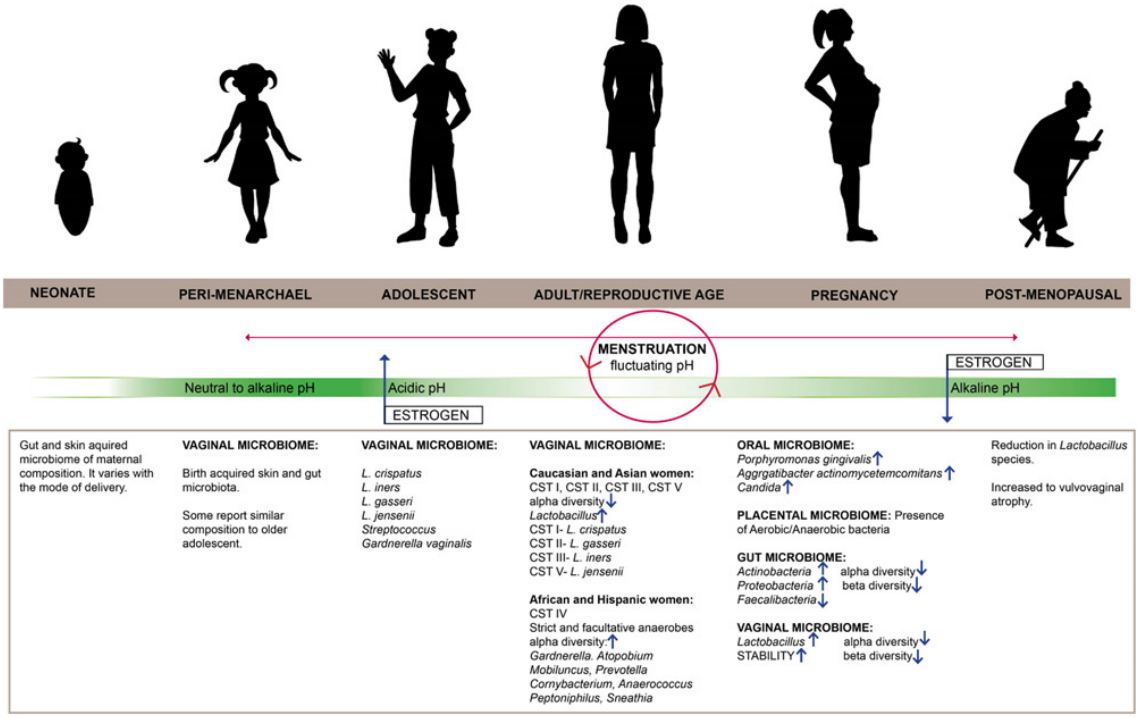


Figure 1.13: The change in pH and the microbiota of the vaginal microbiome with age and hormonal fluctuations (Deka *et al.*, 2021).

Research techniques for vaginal microbiome investigation

Conventional culture-based methods present several limitations, and the advent of cultivation-independent and high throughput molecular techniques has paved the way to a more comprehensive characterization of the vaginal microbiome. Consistently, NGS and WGS methods are currently extensively approved for examination of vaginal microbial communities (Sharma *et al.*, 2021).

The vaginal microbiome development and variations

The vaginal tract, sterile at birth, is rapidly colonized by *Lactobacillus* species, under the influence of estrogens produced by the mother (Figure 1.13). After 3-4 weeks post-birth, the drop of estrogen levels leads to a reduction of *Lactobacillus* species and an increase of anaerobic and enteric species, which is conserved until adolescence (Cruickshank and Sharman, 1934). The vaginal microbiota of peri-menarcheal adolescents is thus characterized by neutral or slightly alkaline vaginal pH, and a relative stable microbial composition, derived from skin and gut microbiota acquired during birth (Deka *et al.*, 2021). First relevant changes occur during puberty, along with hormonal important fluctuations. Preceding menarche, the rise of estrogen and progesterone levels promotes glycogen deposition in the vaginal epithelium, whose enzymatic degradation (by human α-amylase to maltose, maltotriose, and α-dextrins and eventually to lactic acid by lactic acid-producing bacteria) results in the devel-

opment of a highly acidic microenvironment, which inhibits the growth of many bacteria and support colonization of acid-producing bacteria. During reproductive years, menstrual cycle and estrogen cyclic fluctuations strongly influence Lactobacilli abundance, lactic acid production and vaginal microbiota composition (Gajer *et al.*, 2012). During pregnancy, a high microbial stability is observed, with overall decrease of Alpha diversity and a stable composition of *Lactobacillus*, *Clostridiales*, *Bacteriodales*, and *Actinomycetales* species, maintained by the sustained secretion of estrogens (Deka *et al.*, 2021). Any imbalance of vaginal microbiota composition in pregnant women has been associated with complications, among all preterm delivery (Fettweis *et al.*, 2014). The imbalanced microbiota usually correlates also with pro-inflammatory cytokines levels in vaginal fluids (Fettweis *et al.*, 2019). During post-partum period, the drop of estrogen associates with the depletion of the *Lactobacillus* component and a decrease of community stability, subsequently followed by gradual revert to the pre-pregnancy status (Mitra *et al.*, 2015). Finally, during menopause, the decline of estrogen levels results in the decrease of *Lactobacillus* species, with presence of *Streptococcus* and *Prevotella* species, that has been in addition associated to vulvovaginal atrophy (VVA) condition (Brotman *et al.*, 2014a).

Several other influencing factors, including ageing, lifestyle, sexual activity, diet, alcohol consumption, smoking and the use of contraception devices, have been related to alterations in the composition of vaginal microbiome (Gajer *et al.*, 2012; Brotman *et al.*, 2014a; Gupta *et al.*, 2017; Song *et al.*, 2020). Among them, for example, the use of hormonal contraceptives has been associated with a greater stability of the vaginal microbiota and a reduction in the risk of BV. Smoking and sexual intercourse, instead, correlate with a reduction in the relative proportion of *L. crispatus* and increase diversity of bacterial species (Mitra *et al.*, 2016).

Vaginal microbiome role in health and disease

In vaginal environment, *Lactobacillus* species play an important regulatory role, and they are considered a key marker of a healthy vaginal microhabitat. It has been long agreed that the dominance of *Lactobacillus* spp. confers the vagina protection from potentially pathogenic bacteria, that may cause urinary tract infections and sexually transmitted infections (STI) (Saraf *et al.*, 2021). The beneficial action of *Lactobacillus* spp. is associated with several mechanisms:

Maintaining an acidic vaginal pH : *Lactobacillus* species produce enzyme able to metabolize glycogen, highly present in cervical and vaginal epithelium and stimulated by estrogen peaks. The glycogen fermentation led to the production of lactic acid, which assures an acidic vaginal microenvironment. As a results, the growth of several pathogens, such as *C. trachomatis*, *N. gonorrhoeae* and

G. vaginalis, is inhibited (Linhares *et al.*, 2011).

Production of bacteriocins and other antimicrobial molecules : *Lactobacillus* species may also inhibit pathogen growth by expressing bacteriocins (Borges *et al.*, 2014). Among them, *L. gasseri*, *L. crispatus* and *L. reuteri* have been demonstrated to produce Gasserin, a bacteriocin with wide spectrum range of activity towards both Gram negative and Gram positive bacteria (Mitra *et al.*, 2016). Different strains of Lactobacilli have been found to produce biosurfactants, defined collectively with the term Surlactin, reducing liquid surface tension and thus inhibiting the adhesion of pathogens on epithelial surface (Reid *et al.*, 1999). *L. crispatus* has been shown to excrete *Lactobacillus* epithelium adhesin (LEA), a molecule that inhibiting activity towards *G. vaginalis* adhesion to genital mucosa (Ojala *et al.*, 2014). In addition, bacteriocins and biosurfactants produced by lactic acid bacteria have been considered as potential antiviral agents and therapeutic agents in cancer disease (Shaikh *et al.*, 2012; Al Kassaa *et al.*, 2014). Some species of *Lactobacillus* are also known to produce hydrogen peroxide, which possesses antimicrobial activity (Aroutcheva *et al.*, 2001; O'Hanlon *et al.*, 2013).

Competitive exclusion : Lactobacilli can also prevent pathogen colonization as a result of competitive exclusion, through competition for space and nutrients.

Consistently, the dysbiosis of vaginal microbiome is generally associated with a decrease of *Lactobacillus* species, an increase of microbial diversity, with the overgrow of facultative or strict anaerobic bacteria species. Despite vaginal dysbiosis can occur naturally during pre-puberal and post-partum periods, dysbiotic changes of vaginal microbiome in the adult fertile woman are associated with several pathologic conditions, including BV, urinary tract infections and STI. Figure 1.14 represents the wide number of factors that have been documented/suggested to influence and regulate eubiosis and dysbiosis of vaginal microbiome, and to drive transitions between the two states (Kroon *et al.*, 2018).

BV is the most common and studied vaginal dysbiosis, affecting about the 20-30% of reproductive-aged women worldwide (Lev-Sagie *et al.*, 2022). However, despite the high prevalence of BV in the population and the existence of numerous molecular and genomic investigations, there is still no consensus on the group of taxa/bacteria directly involved in disease etiology. Overall, BV is characterized by the lack of a predominant *Lactobacillus* species and proliferation of various gram-negative and/or anaerobic bacteria, including *G. vaginalis*, *A. vaginae*, *Megasphaera* types, *Leptotrichia amnionii*, *S. sanguinegens*, *Porphyromonas* and *Prevotella* genera (Fredricks *et al.*, 2005; Swidsinski *et al.*, 2005). In particular, *G. vaginalis* has

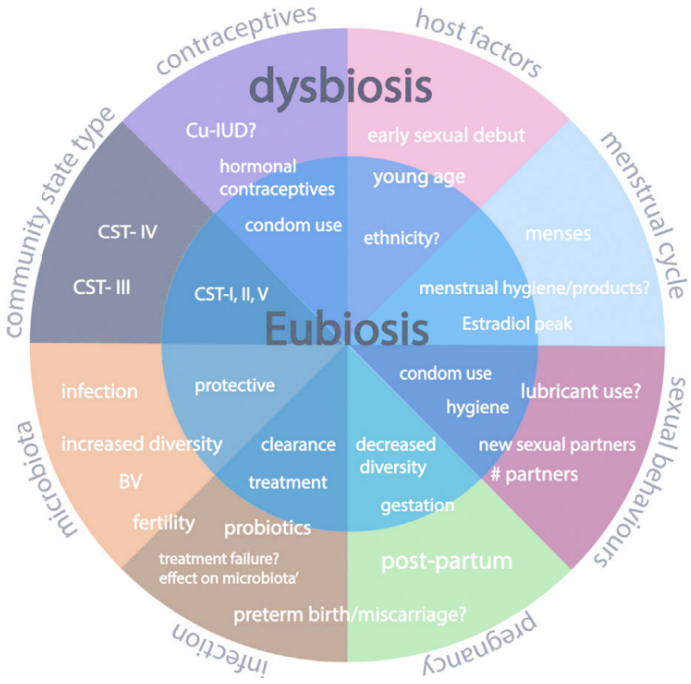


Figure 1.14: Factors driving or associated with dysbiosis or eubiosis of the vaginal microbiota (Kroon *et al.*, 2018).

been suggested to have a predominant role in causing vaginosis condition (Lev-Sagie *et al.*, 2022).

Vaginal microbiota has been also associated with adverse pregnancy outcomes such as pre-term birth. In particular, women with *Lactobacillus*-dominated flora evaluated during the first trimester, resulted in a 75% lower risk of pre-term pregnancy (Petricevic *et al.*, 2014), whereas the exclusive dominance of *L. iners* may be a risk factor for preterm birth (Petricevic *et al.*, 2014). Lower levels of Lactobacilli and higher microbial diversity also characterize the microbiome of women with premature rupture of membranes (Jayaprakash *et al.*, 2016), and several investigations have associated microbiome dysbiosis with early and late miscarriage conditions, chorioamnionitis and intra-amniotic infections (Silver *et al.*, 1989; Gibbs, 1993; Hay *et al.*, 1994; Llahí-Camp *et al.*, 1996; Ralph *et al.*, 1999; Donders *et al.*, 2000).

BV-related microbiota has been further associated with infertility in women of reproductive age and a positive correlation between BV-associated bacteria and higher risk of pregnancy failure was observed (Wilson, 2002; Mania-Pramanik *et al.*, 2009; Sirota *et al.*, 2014; Campisciano *et al.*, 2017; Vander Haar *et al.*, 2018).

Vaginal dysbiosis has been also associated with increased risk to contract sexually transmitted infections (STI), including those by *N. gonorrhoeae*, *C. trachomatis*, *T. vaginalis*, Herpes Simplex Virus (HSV), Human Immunodeficiency Virus (HIV) and Human Papillomavirus (HPV) (Torcia, 2019). In detail, secretion of pro-inflammatory cytokines and chemokines, local recruitment of immune cells, and reduced viscosity of cervical mucus were observed as a consequence of BV-associated

anaerobic gram-negative bacteria presence (Olmsted *et al.*, 2003; Torcia, 2019). The degradation of the protective cervical mucus and vaginal epithelium decreases the efficiency of mucosa as a protective barrier against sexually transmitted pathogens (Plesniarski *et al.*, 2021), and women with BV result more prone to acquire STI, even in absence of clinical symptomatology (Amabebe and Anumba, 2018).

Among STI, particular focus was put on HPV infections, since they represent the most common STI of viral origin, and high-risk genital HPVs (hrHPVs) are associated with the development of precancerous cervical lesions (Cervical Intraepithelial Neoplasia, CIN) and cervical cancer (CC). Cervical cancer is the fourth most common malignancy in women worldwide (Siegel *et al.*, 2020), with over 600.000 new cases (3.1%), and about 340,000 deaths (3.3%) around the world in 2020. A world incidence and mortality rates of 13.3% and 7.3% respectively has been reported by the World Health Organization (World Health Organisation, 2020). Hr-HPV include genotypes 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 66 and 68 (De Villiers *et al.*, 2004), and persistent infection with one of these 13 oncogenic HPV genotypes is responsible for 99.7% of CC cases. However, CIN lesions develop in less than 10% of infected women, suggesting the need for other cofactors (Schiffman *et al.*, 2007). The factors influencing the persistence and clearance of HPV infection are not yet fully understood, and emerging data support the hypothesis that vaginal microbiome is involved in persistence, regression or progression of HPV infection (Mitra *et al.*, 2016). It was in fact reported that HPV positive women harbored a higher microbial variability and a lower predominance of Lactobacilli, compared to their HPV negative twin sisters (Lee *et al.*, 2013). Besides, HPV-positive women were reported to harbor CST-III or CST-IV microbiomes, dominated by *Atopobium*, *Prevotella*, and *Gardnerella* species, whereas the CST-II group, dominated by *L. gasseri*, was associated with a faster clearance of the HPV infection (Brotman *et al.*, 2014a).

A correlation between CIN severity and increased microbial diversity, together with a reduction of *Lactobacillus* quote, was observed, showing that women with high-grade lesions harbored higher levels of *S. sanguinescens*, *Anaerococcus tetradius*, *Fusobacteria* and *Peptostreptococcus anaerobius* and lower levels of *L. jensenii*, compared to patients with low-grade lesions (Mitra *et al.*, 2015). The CST-IV subgroup, including *Gardnerella*, *Prevotella*, *Megasphoera*, *Atopobium*, was observed in 43% women with persistent HPV infection, and in only 7.4% of women with cleared infection (Di Paola *et al.*, 2017). Moreover, several studies have identified *Atopobium*, *Mycoplasma hominis* and *Haemophilus* as markers for the persistence of HPV infection (Brotman *et al.*, 2014b; Adebamowo *et al.*, 2017; Di Paola *et al.*, 2017; Arokiyaraj *et al.*, 2018).

Based on these observations, it has been proposed that specific cervicovaginal microbiota compositions are correlated with the development of invasive CC. In par-

ticular, bacteria including *Sneathia*, *Atopobium*, *Parvimonas*, *Fusobacterium*, *Anaerococcus* and *Peptostreptococcus* seem to be more associated with cervical carcinoma (Mitra *et al.*, 2015; Audirac-Chalifour *et al.*, 2016; Łaniewski *et al.*, 2018). CST-IV bacteria may promote neoplastic development through molecular mechanisms such as the induction of DNA oxidative damage, and the production of high amount of nitrosamines, that have a known carcinogenic effect (Piyathilake *et al.*, 2016). Among *Lactobacillus* subspecies, *L. iners* (characterizing the CST-III group) resulted associated with increased risk for hr-HPV and CC, while *L. crispatus* appears to have a protective role. A recent study confirmed that high-grade dysplasia and invasive cancer strongly correlated with marked depletion of *L. crispatus* and enrichment of anaerobes *A. vaginae*, *Dialister invisus*, *Finegolda magna*, *G. vaginalis*, *Prevotella buccalis* and *timonensis* (So *et al.*, 2020). Some further investigations focused on the non-bacterial components of the vaginal microbiome (mycetes, protozoa), observing an association with increased risk of cervical cancer (Liang *et al.*, 2019).

Despite several evidences of association, the mechanisms by which the vaginal microbiota may influence the persistence of HPV infection and thus its carcinogenic potential have not yet been fully understood. Chronic inflammation has been hypothesized, based on the presence in CIN patients compared to controls, of higher levels of proinflammatory cytokines, including interleukin (IL)-1α, IL-1β, and IL-8, and a decrease of anti-inflammatory mediators and antimicrobial peptides (Mhatre *et al.*, 2012). In CC patients, the presence of *Fusobacterium* species was associated with higher expression levels of IL-4 and TGF-β1, that have been associated to tumor onset and progression (Audirac-Chalifour *et al.*, 2016). A substantial upregulation of pro-inflammatory cytokines (IL-36γ, TNFα, RANTES, MIP-1α, MIP-1β, IP-10), of hematopoietic cytokines (Flt-3L, GM-CSF), of mediators of adaptive immune system mediators (IL-2, IL-4, sCD40L), and of the anti-inflammatory IL-10 were detected in patients with invasive CC, but not in CIN patient, with IL-36γ significantly associated with invasive CC, regardless of the type of microbiota Community State Type (Łaniewski *et al.*, 2018). A significantly increased levels of pro-inflammatory cytokines, including IL-4, IL-5, IL-10, IL-12, IL-13, TFN-α, and IFN-γ was also reported in women with a *G. vaginalis* dominant microbiome (Moscicki *et al.*, 2020). A dysbiotic vaginal microbiome may also impair apoptosis, angiogenesis and lipid metabolism, involved in carcinogenesis process (Ilhan *et al.*, 2019). On the other hand, *Lactobacillus* spp. may inhibit cancer cells viability, down-regulate HPV oncogene expression, and increase the expression of the tumor suppressor p21 (Wang *et al.*, 2018; Łaniewski *et al.*, 2019).

The excisional treatment of CIN lesions by Loop Electrosurgical Excision Procedure (LEEP) has become one of the standard treatments of CIN2/3 globally (Hoffman and J Mann, 2021), as it has been associated with a significantly decreased risk

of disease persistence at 6 months, and risk of recurrence at 12 months follow-up, compared to other surgical treatments (D'Alessandro *et al.*, 2018). However, the effect of LEEP on the cervico-vaginal microbiota has not yet been fully explored. Evidence reported in literature so far showed that surgical treatment of HPV-related cervical lesion can alter the vaginal microbiota, however more evidence is needed in order to support this correlation. With this in mind, a systematic review and meta-analysis study has been recently reported (Janicka-Kośnik *et al.*, 2021), pointing out the importance to provide information on the role of surgical treatments on vaginal microbiome changes, in order to find the more beneficial procedure, also in correlation with the importance of vaginal microbiome eubiosis (Janicka-Kośnik *et al.*, 2021).

Vaginal microbiome modulation as a therapeutic approach

The modulation of the vaginal microbiome has been proposed as a therapeutic approach to restore vaginal eubiosis and promote women's health. Recently, probiotics have been proposed for the treatment of BV, proving to be a potential effective strategy. *Lactobacillus*, *Bifidobacterium*, *Streptococcus*, *Enterococcus*, and *Escherichia* have been reported to have a successful action (Guarner and Schaafsma, 1998). *Lactobacillus* is a universally administered probiotic genus due to its natural abundance in the woman genital tract. Several clinical trials have been performed testing a single or a combination of probiotic species. *L. fermentum*, *L. gasseri*, *L. rhamnosus*, *L. acidophilus*, *L. crispatus*, *L. casei*, and *L. salivarius* has been evaluate for treatment of fertility disorders (Arroyo *et al.*, 2010; Borges *et al.*, 2014). Oral administration of the probiotic *L. rhamnosus* GR-1 and *L. fermentum* RC-14 strains have been reported to revers BV condition and restore a *Lactobacillus*-dominated vaginal microbiome (Reid *et al.*, 2001). Intravaginal administration of exogenous *Lactobacillus* strains, consisting on vaginal tablets containing *L. brevis*, *L. salivarius* subsp. *salicinius*, and *L. plantarum* has also been shown effective in treatment of symptomatic BV (Mastromarino *et al.*, 2009). Petricevic and Witt showed effectiveness of intravaginal treatment with *L. casei* (Petricevic and Witt, 2008). Several clinical trials reported that probiotics *L. rhamnosus* GR-1 and *L. fermentum* RC-14, together with *L. acidophilus*, have been also effective in the treatment of vulvovaginal candidiasis (Falagas *et al.*, 2006).

Probiotics have also been suggested as possible treatment for viral infections, including HPV. In particular, the daily consumption of probiotics was observed to double the chance of clearance of cytological abnormalities related to HPV-precancerous lesions, compared to control group (Verhoeven *et al.*, 2013). Similarly, a 6-month vaginal administration of *L. rhamnosus* BMX resulted in a higher chance to solve HPV-related cytological abnormalities and to clear HPV infection, compared to a

3-months treatment (Palma *et al.*, 2018).

In addition, a great interest has been reserved also to the addition of prebiotics in vaginal treatment by probiotics (Rousseau *et al.*, 2005; Pranckute *et al.*, 2016), and the data collected so far suggest that the use of pre and probiotics could have an important role in the clearance of HPV-related cervical lesions.

1.3 The Microbiome Balance and Modulation

It has become clear that the importance of microbiomes goes beyond the health of individual hosts. Microbiome of different hosts and ecosystems can strongly interact and influence each other. Of particular interest, the environmental microbiome has been observed to strongly influence human health and based on these observations the concept of "One Health" approach is currently applied. Its slogan "*A healthy environment promotes healthy humans*" supports the importance of an integrated approach between different disciplines, in order to achieve the health of animals, humans, plants and environment, that are fundamentally linked.

The increasing interest in microbiome research and the rapidly advancing of molecular technologies have led to a more comprehensive characterization of the whole human microbiome, and its pathways and microbial interactions, characterizing both the state of health and disease of the host. The eubiosis condition has been predominantly associated with a high level of microbial diversity (except in the vaginal microbiome, where the healthy condition foresees the wide predominance of *Lactobacillus* species). On the contrary, the microbiome depletions can lead to pathogenic microorganisms taking over and to a general decrease of microbial diversity, causing to the establishment of a dysbiotic condition. Dysbiotic microbiome has been linked to a large number of human diseases. Several studies have been evaluating the feasibility to modulate human microbiome as therapeutic approach. Current innovative investigations foresee the development and/or optimization of biological strategies aimed to modulate the human microbiome, revert a dysbiotic condition and improve human health. This strategy has been successfully applied also for the modulation of environmental microbiome, primarily in hospital settings but potentially applicable in other built environments that are strongly influenced by human presence. The introduction of probiotics bacteria in cleaning sanitation is based on the concept of "Bidirectional Hygiene" or "Bygiene" (Al-Ghalith and Knights, 2015), according to which, rather than "over-sterilizing" the environment, a more useful approach consists of balancing the pre-existing contamination by restoring beneficial microbial species. *Bacillus* spp. represent non-pathogenic probiotic bacteria, that has been shown to counteract colonization of pathogenic species on surfaces, mainly via the mechanism of competitive antagonism, and secondly trough bacteriocins

production.

In conclusion, it is well known that "*our outer world impacts our inner world and vice versa*", therefore understanding the role of environmental and human microbiome in health and disease, the need to monitor pathogens, and develop control strategies based on microbiome balancing represent research goals of fundamental interest.

Chapter 2

Research Aims

2.1 General overview

The lines of research conducted during my PhD period focused on the role and potential impact of the microbiome both in the environment and in the human body, trying to find out correlations and associations between the environmental and human microbiomes, and between the human microbiome and the human health or disease status.

To this aim, different approaches and studies were undertaken, including on one side the characterization of the microbiome of the hospital environment, due to its critical contribution to the healthcare-associated infections and consequently high impact on hospitalized patient health, and on the other side the characterization of the human microbiome in defined anatomical niches, in order to clarify whether specific microbiome compositions are related to increased risk to develop diseases, especially those virus-induced.

In particular, the hospital, nose, oral and vaginal microbiomes were investigated as detailed below and depicted in Figure 2.1.

2.1.1 The hospital environmental microbiome: pathogen's monitoring and control strategies

The hospital environment significantly contributes to the onset and transmission of the so-called healthcare-associated infections (HAIs), that represents one of the most frequent and severe complications occurring in healthcare settings worldwide, thus, the monitoring of hospital surfaces' contamination is considered of fundamental importance. So far, monitoring tools essentially consisted in microbiological culture-based methods, which however allow the detection of only searched microbes and require time-consuming incubation procedures. By contrast, molecular-based methods, can provide a more rapid and detailed characterization of environmental

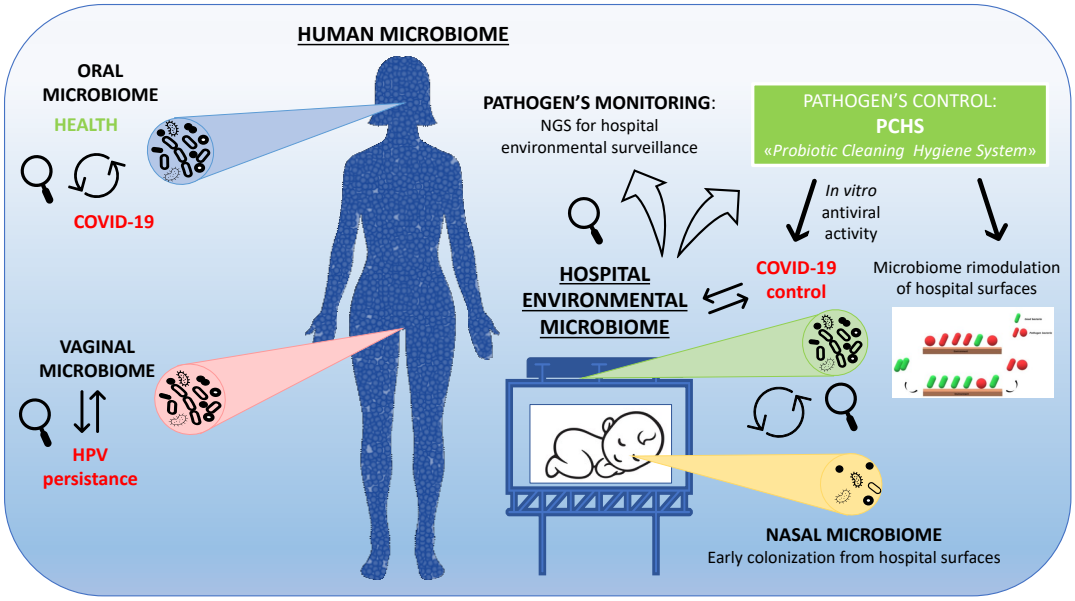


Figure 2.1: The hospital environmental microbiome: pathogen's monitoring and control strategies.

contamination residing in hospital surfaces and open the way to even more comprehensive investigation of complex microbial populations. Based on these considerations, the first aim of our research was to assess the use of Next Generation Sequencing (NGS) technologies for environmental surveillance. Our investigation was conducted in an Italian pediatric hospital (Institute for Maternal and Child Health "IRCCS Burlo Garofolo", Trieste, Italy), to define surface microbiomes in pediatric settings, and to evaluate advantages and disadvantages of the compared technologies, in order to improve environmental surveillance strategies using even more effective methods.

Next, since the risk for contracting HAIs is particularly high in preterm infants (born before 28 weeks) hosted in Neonatal Intensive Care Unit (NICU), the ability of NICU contaminating microbiome to colonize the upper respiratory tract of preterm newborns was explored. To this aim, NGS technologies were used to characterize the bacterial composition of nasal swabs of preterm infants and environmental surfaces microbiome, at the time of delivery and during hospitalization in NICU. In addition, to clarify the origin of newborn nose, the vaginal microbiome of the mothers and the resistome profile were also evaluated by NGS.

Last, based on the proven antiviral activity of PCHS (D'Accolti *et al.*, 2021), the PCHS effectiveness was tested during the COVID-19 pandemics in comparison with chemical disinfection, in the emergency ward of the same children hospital enrolled for the previous studies (Institute for Maternal and Child Health "IRCCS Burlo Garofolo", Trieste, Italy), and monitoring the microbial contamination, including that by SARS-CoV-2, by NGS analysis and other molecular methods.

2.1.2 The human microbiome: investigating the role in health and disease

Since a detailed site-specific map of oral microorganisms (including also eukaryotes and viruses) and their relative abundance is still missing, our first study aimed to obtain a site-specific and comprehensive view of the oral microbiome of young adult healthy individuals (healthy oral microbiome, HOM), using the Whole Genome Sequencing (WGS) technology. The AMR features of the HOM was also characterized, to define its resistome in healthy conditions.

Second, since the dysbiosis of the oral microbiome has been associated with several local and systemic diseases, and SARS-CoV-2 uses oropharynx as the primary site of replication, the WGS analysis approach was used to characterize the oral microbiome of COVID-19 patients, to evidence the eventual association between virus-induced disease and the microbial environment of the oral cavity. In parallel, the development of inflammation and/or local secretory immune response (IgA) was also assessed, to better understand the features of the oral environment that could potentially promote SARS-CoV-2 infection, and to identify potential markers for the risk of developing a severe infection.

Last, our studies wanted to characterize the impact of the vaginal microbiome on the HPV persistence in women undergoing LEEP (Loop Electrosurgical Excision Procedure) treatment because of CIN2/CIN3 HPV-related precancerous lesions. The vaginal microbiome dysbiosis has in fact been reportedly evidenced in several pathological conditions, including cervical cancer, but no conclusive information was available on its role. Moreover, the hypothesis that chronic inflammation may promote carcinogenesis has been reported (Mhatre *et al.*, 2012). The preferred treatment for high-grade CIN consists of LEEP procedure, that has been associated with a significant decrease in the risk of viral persistence at 6 months. However, no information is available on the impact of surgical treatment on vaginal microbiome and cytokines profile. Based on these considerations, our study was aimed to investigate the role of vaginal microbiome, including non-bacterial microorganisms, together with inflammatory microenvironment, in a cohort of CIN2/CIN3 women undergoing LEEP treatment.

Chapter 3

Materials and Methods

3.1 Sample collection

3.1.1 Environmental sample collection

The collection of environmental samples from the surfaces was performed at the Institute for Maternal and Child Health "IRCCS Burlo Garofolo" (Trieste, Italy), after obtaining the approval from the Institutional Scientific Board of the hospital. Environmental samples were collected from the following wards: Pediatric Clinic (PC), Pediatric Surgery (PS), Pediatric Oncology (PO), Neonatal Intensive and Sub-Intensive Care Unit (NICU and NICUs), children's Intensive Care Unit (ICU), Surgical Rooms (SR: Orthopedics, Gynecology and Oculistics), Delivery Room (DR), and Emergency Rooms (ER). Up to three rooms were monitored for each enrolled ward, during 2 and 5 consecutive sampling campaigns (respectively for microbiome characterization in all wards and for the analysis of the impact of PCHS on microbiome profile in ER). Samples collection was performed 7 hours after cleaning, a time after which the contamination levels regrowth because of recontamination (Vandini *et al.*, 2014; Caselli *et al.*, 2016b, 2018). Different critical surfaces were sampled based on ward characteristics. Sampling points included floor (for all ward types), bed footboard (for all wards), sink (for all wards), operating bed/operating table (in SR and DR wards), incubator (in NICU wards). Each target surface was simultaneously sampled by two different methodologies, based on subsequent microbiological or molecular analyses (Comar *et al.*, 2019).

Sampling for microbiological analyses was performed by using Replicate Organism Detection and Counting (RODAC) contact plates containing specific culture media: TSA medium (Merck Millipore, Milan, Italy) was used for total bacteria count, Baird Parker medium (Merck Millipore, Milan, Italy) for *Staphylococcus* spp., MacConkey medium (Merck Millipore, Milan, Italy) for Enterobacteriaceae spp, Herella medium (Lickson, Milan, Italy) for *Acinetobacter* spp., Cetrimide medium (Incofar,

Modena, Italy) for *Pseudomonas* spp., *Clostridium difficile* selective medium (Lickson, Milan, Italy) for *Clostridium difficile*, BEA medium (Incofar, Modena, Italy) for *Enterococcus* spp. and lastly Sabouraud medium (Merck Millipore, Milan, Italy) for mycetes isolation.

Sampling for molecular analysis was performed by sterile rayon swabs (Copan, Brescia, Italy), pre-moistened in sterile Tryptic Soy Broth (TSB) or saline solution, and rubbed on a surface of 100 cm^2, delimited by a sterile 10x10cm disposable plastic template (Copan, Brescia, Italy). Swabs were then put in 5 mL TSB or 0.4 mL saline, depending on the subsequent analysis type, immediately refrigerated, and transported to the laboratory.

3.1.2 Patients' samples collection

Samples collected from patients included:

1. **Nasal swabs from preterm newborns:** a total of 55 nasal swabs were collected from preterm infants hosted in NICU from November 2018 to January 2019, at the Burlo Garofolo Hospital (Trieste, Italy). Samples were collected at the time of birth (group N0, 30 samples), after 9 days (group N9, 18 samples), and after 13 days (group N13, 7 samples). Briefly, anterior nasal swabs were collected by nurses and placed in a sterile medium, using eSwabs and liquid transportation medium (Copan, Brescia, Italy). Immediately after collection, swabs were sent to the laboratory and stored at -80 °C until analysis. Samples were defrosted and vortexed (3x 30 seconds), total DNA was extracted from 300 µL of sample by automatic extractor Maxwell CSC DNA Blood Kit (Promega, Madison, WI, USA), according to the manufacturer's instruction, with a final elution volume of 100 µL.

2. **Vaginal swabs from pregnant women just before delivery:** 20 samples were collected at the DR of Burlo Garofolo Hospital, by using a 200 mm polyethylene Cervix brush device (Rovers Medical Devices B.V., The Netherlands) under speculum examination, by a 360° rotation of the brush. Samples were then suspended in 1.5 mL of sterile TE buffer and stored at -80 °C until analysis. Vaginal swabs were processed and extracted as preterm newborns nasal samples.

3. **Vaginal swabs from HPV-positive women:** 85 samples were collected at the Center of Preventive Gynecology of the University Hospital of Ferrara, after approval by the local Ethic Committee (Comitato Etico Unico della Provincia di Ferrara, Azienda Ospedaliero-Universitaria) (Protocol N. 170394). The study design consists of an oriented observational, prospective, cohort study.

All enrolled women signed a written informed consent prior to enrollment. The recruitment phase of the study was completed in 30 months. Women HPV status was determined by Cobas 4800 HPV Test (Roche Diagnostics, Monza, Italy), allowing detection of hrHPV-16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68); PAP- test (cytologic screening that allows the detection of abnormal cervical cells); colposcopy (examination of the epithelial surfaces of the lower ano-genital tract performed by binocular microscope with 40x magnification). Cervico-vaginal samples were collected by sterile rayon swabs both before LEEP treatment and during follow-up control at 6-month from treatment. Swabs were immediately transferred in a 1.5ml sterile collection tube, containing 0.4 ml of sterile phosphate buffered saline (PBS). All collected samples were maintained refrigerated (2-8 °C), transported to the laboratory for analysis and processed within three hours. In parallel, vaginal washing were collected by washing the vaginal cavity with 5 ml of sterile saline, vaginal secretions were aliquoted in sterile 1.5 ml microtubes, and transported refrigerated to the laboratory, where they have been immediately frozen in liquid nitrogen and kept at -80 °C until use. Cervico-vaginal swabs were vortexed (3x 30 seconds) and centrifuged 14000 x g for 10 minutes at 4 °C. Supernatants and pellets were immediately frozen il liquid nitrogen and kept at -80 °C until use. The total DNA was extracted from the cervico-vaginal swab samples by Exgene Cell SV Kit (Gene All, Tema Ricerca, Bologna, Italy), following the modified protocol already mentioned. Total DNA extracted was quantified by Nanodrop (Thermo Fisher, Milan, Italy) spectrophotometric reading at 260/280 nm. DNA quality was checked by PCR amplification of the human beta-actin gene and bacterial 16S rRNA gene (*PanB* PCR).

4. **Oral samples from healthy subjects:** to characterize the healthy oral microbiome (HOM) and its resistome, oral samples were collected from different oral niches as follows:

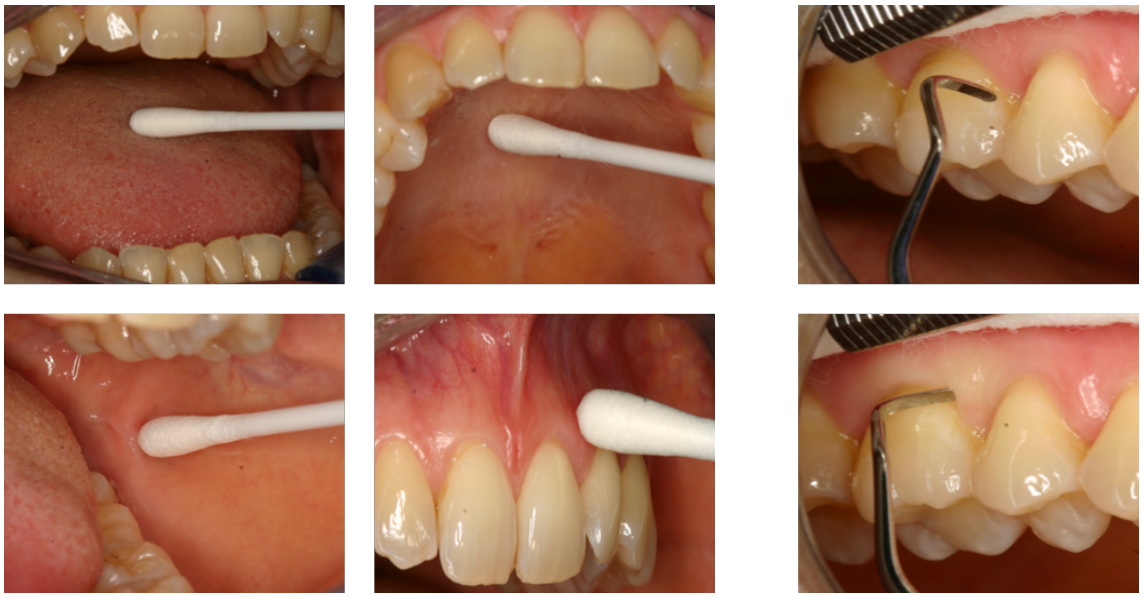
   Saliva : subjects were asked to spit the saliva collected in the mouth for at least 1 min into a sterile 50 ml tube (average volume range of 2-5ml). Saliva after rinsing with 15 ml sterile PBS was also collected.

   Soft tissue sites : samples were collected by sterile rayon swabs (Copan Italia S.p.A., Brescia, IT) and included tongue dorsum (1 cm^2 area at the center of the tongue for 5 sec), hard palate (swabbed entirely for 10 sec), buccal mucosa (swabbed left and right area 10 sec each), keratinized gingiva (swabbed for 10 sec). After collection, swabs were immediately transferred in a 1.5 ml sterile microtube containing 0.5 ml of PBS.

   Hard tissue sites : supragingival plaque was collected from 6 index teeth

(2 molars, 2 premolars, 2 incisors); the index tooth were isolated with cotton rolls, gently dried with air, then a Gracey curette was used to remove all the supragingival plaque from the mesial surface of the selected tooth. The curette tip was immersed in 0.5 ml of sterile PBS in a 1.5ml microtube for 4-5 seconds to ensure transfer of materials to solution, then the other index teeth were immediately sampled using the same procedure and collected in the same microtube. Subgingival plaque was sampled from the same selected 6 index teeth, only after removing all residual supragingival plaque. Similarly, plaques from index teeth were collected with a sterile Gracey curette and pooled in a second microtube containing 0.5 ml of PBS. Any specimens with marked bleeding, based on clinical evaluation, was discharged.

All collected samples were maintained refrigerated (2-8 °C), transported to the laboratory for analysis and processed within one hour. Figure 3.1 represents some of the different soft tissues and hard tissues sampled. Oral samples were vortexed and pelletized by centrifugation at 4 °C (14000 x g for 10 minutes). Supernatant fractions, containing extracellular soluble components, and pelletized corpuscular fractions were collected separately and frozen at -80 °C until use. Total DNA was extracted from pellets by using the Exgene Cell SV Kit (Gene All, Tema Ricerca, Bologna, Italy), following the manufacturer's instruction, adjusted to optimize the extraction from Gram-positive bacteria. The amplifiability of total extracted DNA



(a) **(b)**

Figure 3.1: Sampling of soft tissues with sterile rayon swabs of tongue dorsum, hard palate, buccal mucosa, and keratinized gingiva, in clockwise direction (3.1a). Sampling of hard tissues with sterile Gracey curettes of supragingival plaque and subgingival plaque (3.1b).

was checked by amplifying 10 ng of DNA by two qualitative PCR reactions, targeting the eukaryotic human beta-actin gene and the bacterial 16S rRNA gene (pan bacterial PCR, *panB*), as previously described (Caselli *et al.*, 2016b, 2018).

5. **Oral samples from COVID-19 patients:** to characterize the oral microbiome of COVID-19 patients, samples of saliva after rinsing were collected. Oral rinses were collected in 5 ml of sterile PBS, putting the rinse into a second collection tube containing 0.1% sodium dodecyl sulfate (SDS), for the rapid virus inactivation immediately after rinsing. Straight after collection, samples were refrigerated at 2-8 °C and processed within four hours. Oral samples were pelletized as described above, Total Nucleic Acids (TNAs) were extracted from pellets by using Maxwell RSC Viral TNA Purification Kit (Promega, Milan, Italy), and DNA amplifiability was checked by human beta-actin, pan bacterial and pan fungal (targeting mycetes ITS gene) PCRs (Soffritti *et al.*, 2021).

3.2 Microbiological analyses of environmental samples

Collected RODAC plates were incubated at 30 °C for 24–48 h (bacteria), or at 25 °C for 72 h (mycetes). After the required incubation times, Colony forming units (CFU) were enumerated. Plates containing $\geq$ 200 CFUs were counted as 200 CFUs, following the guideline INAIL-2017 (Giovinazzo *et al.*, 2017). Samples collection was performed in duplicate, resulting in a total of 216 samples obtained from surfaces of different wards (Comar *et al.*, 2019), and 152 total samples from ER areas, monitored before and after PCHS introduction (Soffritti *et al.*, 2022).

3.3 Molecular analyses of environmental and human samples

Collected swabs placed in TSB medium were incubated at 37 °C for 24h, then microbial cells were pelletized (14,000 $\times$ g for 5 min) and stored until use at -20 °C as previously described (Caselli *et al.*, 2018). Microbial pellets obtained after amplification were extracted by the QIAmp UCP Pathogen Mini Kit (Qiagen, Hilden, Germany). Manufacturer's instructions were modified by adding a pre-lysis treatment with lysozyme (solution 5 mg/ml), to enhance lysis of Gram-positive bacteria, as previously reported (D'Accolti *et al.*, 2019). Microbial DNA was used to assess the resistome profile of sampled surfaces by a real time-quantitative PCR (qPCR) microarray (Comar *et al.*, 2019).

Table 3.1: Thermal conditions of Antibiotic R Genes qPCR microarray.

Step	Thermal conditions	Cycles number
Enzyme activation	95 °C 10 min	1 cycle
Denaturation	95 °C 15 sec	40 cycles
Annealing/Elongation	60 °C 2 min	

Collected swabs placed in saline were immediately frozen at −80 °C. The total DNA from swabs collected in saline was extracted from each sample (300 µL) by the Exgene Cell SV Kit (Gene All, Tema Ricerca, Bologna, Italy), with a final elution volume of 100 µL. DNA samples were processed by customized qPCR microarray (to detect and quantify the microbial types present at the moment of sampling) and by NGS (to define the bacteriome profile).

Swab samples in saline (collected in duplicate) were also used to extract total nucleic acids by Maxwell RSC Viral TNA Purification Kit (Promega, Milan, Italy), with a final elution of 100 µl, to analyze the presence of the human coronavirus SARS-CoV-2 genome.

3.3.1 Antibiotic Resistance Genes qPCR Microarray

To characterize the resistome of the microbial population in the collected environmental samples, the total DNA extracted from swabs in TSB was analyzed by the Antibiotic Resistance Genes qPCR microarray (Cat. no. 330261 BAID-1901ZRA, Qiagen, Hilden, Germany), able to detect and quantify simultaneously 87 resistance (R) genes, as previously described (Vandini *et al.*, 2014; Caselli *et al.*, 2016b, 2018), including aminoglycosides, β-lactams, erythromycin, fluoroquinolones, macrolide-lincosamide-streptogramin B, tetracyclines, and vancomycin, and specific detection of *S. aureus*. One µg of extracted DNA was used per plate (10 ng/well/reaction), by using the QS5 thermocycler (Thermo Fisher, Life Technologies, Milan, Italy), following thermal conditions reported in Table 3.1.

Results obtained by qPCR microarrays were analyzed by the online software provided by the manufacturer (Qiagen, Hilden, Germany), through the comparative $\Delta\Delta$Ct method (Livak and Schmittgen, 2001). The differences are expressed as 'Fold Change', compared to negative controls and control samples.

3.3.2 Customized qPCR Microarray for microbial characterization

To characterize the presence of the most frequent hospital pathogens in the collected environmental samples, the DNA samples extracted from swabs in saline

were analyzed by a customized qPCR microarray (BAID-00047RA Qiagen, Hilden, Germany), simultaneously detecting and quantifying the following 14 microbes: *S. aureus*, *S. epidermidis*, *E. faecalis*, *E. faecium*, *E. coli*, *K. pneumoniae/Enterobacter*, *A. baumannii*, *P. mirabilis*, *P. aeruginosa*, *C. perfringens*, *C. difficile*, *A. fumigatus* and *C. albicans*. *Pan Bacteria* (*panB*) and *Pan Mycetes* (*panM*) reactions were also included as controls. Seven ng of DNA per well/reaction were used for the analysis, using the QS5 instrument (Thermo Fisher, Life Technologies).

To characterize the microbial contamination of nasal swabs from preterm newborns, the extracted DNA samples were analyzed by a customized microarray providing simultaneous identification of 22 bacteria and mycetes typically associated with HAIs (D'Accolti *et al.*, 2019). The array (BAID-00142 Qiagen, Hilden, Germany) included the following target species: *Acinetobacter baumanii*, *Aspergillus fumigatus*, *Candida albicans*, *Citrobacter freundii*, *Clostridium difficile*, *Clostridium perfrigens*, *Enterobacter cloacae/Klebsiella oxytoca*, *Enterococcus faecalis*, *Enterococcus faecium*, *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus vulgaris* and *mirabilis*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *epidermidis*, *Streptococcus agalactiae*, *anginosus*, *prneumoniae*, *pyogenes*, *infantis/oralis*, and *salivarius*. Ten ng of DNA per well/reaction were used for the analysis, using the QS5 instrument (Thermo Fisher, Life Technologies).

3.3.3 qPCR Microarray for vaginal microbiome

Total DNA extracted from cervico-vaginal swabs was analyzed by a qPCR microarray allowing the identification and quantification of 90 species usually present in the lower female genital tract (Microbial Vaginal Flora Arrays, catalog n. BAID-1902ZRA-24; Qiagen, Hilden, Germany) included bacteria, mycetes and protozoa, such as *Chlamydia trachomatis*, *Enterococcus faecalis*, *Fusobacterium nucleatum*, *Gardnerella vaginalis*, *Lactobacillus* (*crispatus*, *gasseri*, *iners*, *jensenii*, *salivarius*, *vaginalis*), *Leptotrichia amnionii*, *Mycoplasma genitalium*, *Mycoplasma hominis*, *Neisseria gonorrhoeae*, *Sneathia sanguinegens*, *Treponema pallidum*, *Ureaplasma parvum*, *Ureaplasma urealyticum*, *Veillonella parvula*, *Candida* spp., *Trychomonas vaginalis*, *Toxoplasma gondii*. A total amount of 1 µg of extracted DNA was analyzed for each microarray, corresponding to 10 ng/well/reaction. Thermal conditions of PCR amplification are the same described in Table 1. The relative quantification of each individual microbial parameter was calculated by specific Qiagen software and interpreted with $\Delta\Delta$Ct method (Livak and Schmittgen, 2001).

Table 3.2: Primers sequences of V1-V3 qPCR and Nested V3 qPCR.

Target Gene	Primers	Sequence	Size
V1-V3 region qPCR	27FYM	5' – AGR GTT YGA TYM TGG CTC AG – 3'	500 bp
	U534R	5' – ATT ACC GCG GCT GCT GG – 3'	
V3 region Nested qPCR	B338F_P1-adaptor	5' – ACT CCT ACG GGA GGC AGC – 3'	200 bp
	U534R_A_barcode	5' – ATT ACC GCG GCT GCT GG – 3'	

Table 3.3: Thermal conditions V1-V3 qPCR and Nested V3 qPCR.

Step		Thermal conditions	Cycles number
Initial Denaturation		95 °C 5 min	1 cycle
Amplification	**V1-V3 PCR**	95 °C 30 sec, 59 °C	27 cycles max
	V3 PCR	57 °C 30 sec, 72 °C 45 sec	13 cycles max
Elongation		72 °C 10 min	1 cycle
Hold		4 °C	∞

3.3.4 Next Generation Sequencing (NGS) Analyses

To characterize the bacteriome profile in the collected environmental, nasal and vaginal samples, the DNA samples extracted from swabs in sterile medium were analyzed by NGS, by sequencing the V3 region of bacterial 16S rRNA gene. Briefly, a qPCR with EvaGreen® Dye (Fisher Molecular Biology, Waltham, MA, USA) was performed to amplify bacterial species, using U534R primer and the degenerated primer 27FYM, targeting the V1-V3 region. Then a Nested PCR was carried out, using B338F_P1-adaptor and U534R_A_barcode primers, targeting the V3 region of the 16S rRNA gene, with a different barcode for each sample linked to the reverse primer (Sundquist *et al.*, 2007). Primer sequences of V1-V3 qPCR and Nested V3 qPCR are reported in Table 3.2. Negative control, including no DNA template, were also included to samples analysis. PCR reactions were performed using Kapa HiFi Hotstart ready mix 2X (Kapa Biosystems, Massachusetts, MA, USA) and BSA 400 ng/μL, following thermal conditions presented in Table 3.3.

PCR amplification products from each sample (100 ng) were mixed to generate the pooled library at a final concentration of 100 pM, according to manufacturer's instruction. The Ion OneTouch™ 2 System (Life Technologies, Gran Island, New York, NY, USA) was used to prepare template with the Ion PGM Hi-Q View OT2 200 (Life Technologies, Gran Island, New York, NY, USA). Sequencing was performed with the Ion PGM™ System technology using the Ion PGM Hi-Q View sequencing kit (Life Technologies, New York, NY, USA). The sequence data were processed using Quantitative Insights Into Microbial Ecology (QIIME 2 2020.2) software, that allows the analysis of high-throughput community sequencing data (Bolyen *et al.*, 2019). High quality sequences (Q > 25) were demultiplexed and

filtered with default parameters, except for the length (150 bp). Sequences with ambiguous bases or a homopolymer length > 8 were removed. Operational Taxonomy Units (OTUs) were clustered and taxonomy assignment at the genus level was performed against the reference taxonomy database SILVA V.132 (Quast *et al.*, 2013) with a similarity threshold of 97%.

3.3.5 Whole Genome Sequencing (WGS) Analyses

The DNA extracted from oral samples (100 ng per sample) was analyzed by WGS, at the NGS Service of the University of Ferrara. WGS libraries were prepared with NEBNext Fast DNA Fragmentation and Library Prep Kit (ThermoFisher Scientific, Milan, Italy), following manufacture's protocol. Sequencing was performed by using the Ion GeneStudio S5 platform, with an Ion chip 540 (ThermoFisher Scientific, Milan, Italy). Low-quality sequence data removal was performed directly on the Ion S5 GeneStudio sequencer, as part of in-built processing. Briefly, the Torrent Suite software (Thermo Fisher Scientific, Milan, Italy), installed in the sequencer, automatically clips adapter sequences and trims low-quality bases from the 3' end of each read. Reads with quality less than Q20 were also discarded. Additionally, PRINSEQ (PReprocessing and INformation of SEQuence data) open-source application (Schmieder and Edwards, 2011) was used to remove reads with lengths of less than 100 nucleotides. Reads aligned to the human genome (hg19 version) were excluded by using the Bowtie2 algorithm.

The taxonomic assignment has been performed using Kraken2 (Pubmed ID: 24580807) and a database consisting of archaea, bacteria, fungi, protozoa, and viruses. Raw sequencing data and bioinformatics analyses have been deposited in the European Nucleotide Archive (ENA) website (accession number PRJEB42999).

3.3.6 Analysis of SARS-CoV-2 presence

The presence of SARS-CoV-2 RNA genome in environmental samples from ER of Burlo Garofolo Hospital was assessed by using the NeoPlexTM COVID-19 Detection Kit (GeneMatrix, Seongnam, Kyonggi-do, South Korea), targeting the viral N and RdRp genes, following the manufacturer's instructions.

Total Nucleic Acids extracted from oral rinse samples of COVID-19 patients were analyzed by Droplet Digital PCR (ddPCR) for the detection and quantification of SARS-CoV-2, using the SARS-CoV-2 ddPCR Kit (Bio-Rad Laboratories, Milan, Italy). The test includes three sets of primers/probes into a single assay multiplex, enabling the simultaneous detection of viral targets, regions of SARS-CoV-2 nucleocapsid genes (N1 and N2), and Human RNase P, used as control to normalize the virus counts. A total amount of 100 ng of extracted TNA per sample were analyzed,

following the manufacturer's instructions for use.

3.4 Analysis of sIgA by ELISA

Oral rinse from COVID-19 patients were evaluated for the presence of anti-SARS-CoV-2 secretory IgA (sIgA), by specific Enzyme-Linked Immunosorbent assay, CE-IVD ELISA assay kit (Euroimmun, Lubeck, Germany). The assay is designed to detect IgA directed against the virus S1 protein, and has been previously utilized for IgA detection in serum/plasma samples and ocular fluids (Caselli *et al.*, 2020c). Preliminary assays were performed to evaluate a proper samples dilution, enabling optimal IgA detection and correct differentiation between positive samples and controls. Oral rinse samples were diluited 1:5 in saline solution, and each sample was analyzed in triplicate. Absorbance was measured using a Multiskan FC spectro-photometer (ThermoFisher Scientific, Milan, Italy). According to supplier's instructions, sample positivity was expressed as the Ratio (R) between absorbance value detected in samples and that obtained in the calibrator sample (supplied in the kit as ready-to-use control and added to each essay).

$$R\ value = \frac{\mathrm{OD}_{450nm}\ \mathrm{test}}{\mathrm{OD}_{450nm}\ \mathrm{cal}}$$

Samples were considered negative if R values were < 0.8, weakly positive if R values resulted comprised between 0.8 and 1.1, and strongly positive if R values were ≥ 1.1.

3.5 Pro-inflammatory cytokines analyses

The presence of pro-inflammatory cytokines was evaluated in the oral rinse samples from COVID-19 patients and in the vaginal washing from CIN2/CIN3 hrHPV-positive women.

In particular, for oral rinse, IL-6, IL-17, TNFα and GM-CSF were analyzed by specific ELISA assays (Invitrogen, Thermo Fisher Scientific, Milan, Italy). Absorbance was read on a Multiskan FC spectro-photometer (ThermoFisher Scientific, Milan, Italy) at 450 nm wavelength. Cytokine concentration was obtained by relative quantification with assay-specific standard curve.

For vaginal washings, 50 µl of undiluted vaginal samples were first analyzed by a multiplex cytokine array (Multi-Analyte ELISArray, catalog n. MEH-004A; Qiagen, Hilden, Germany), allowing the simultaneous detection of 12 different cytokines/chemokines: IL1α, IL1β, IL2, IL4, IL6, IL8, IL10, IL12, IL17α, IFNγ, and TNFα. Subsequently, based on the multiplex ELISA results, individual ELISA as-

says were performed for each cytokine/chemokine detected in the vaginal washing samples, including IL1α, IL1β, IL6, IL8, and TNFα (Invitrogen, Thermo Fisher Scientific, Milan, Italy). The assays were performed following the manufacturer's instructions and using 50 µl of undiluted sample per well in duplicate.

3.6 Statistical Analyses

Statistical analyses were performed using parametric Student's t-test and considering as statistically significant a p value at least < 0.05. Bonferroni correction for multiple comparisons was applied for analysis of microarray data (a Pc value <0.05 was considered significant).

NGS data were statistically analyzed QIIME 2 (2020.2). Weighted and unweighted UniFrac distance matrices (Lozupone *et al.*, 2006) were used to assess Beta diversity (between sample-diversity comparison), and presented with principal coordinates analysis (PCoA). Analyses of Similarities (ANOSIM) and Kruskal–Wallis tests were performed to compare the community composition in the group considered (N0-N9-N13, N0-AP, N9-AT, N13-AT), assuming a statistically significant FDR p value of at least < 0.05. For microarray results, statistical analyses were performed using parametric (Student's t test) and non-parametric (Mann–Whitney) tests, considering a statistically significant p value of at least <0.05.

Microbiome data were expressed as relative abundance of each taxonomic unit at genus or species level. The null hypothesis was tested by Kruskal-Wallis test. Alpha and Beta diversity were used to describe the microbiome diversity between sampled sites and subjects. Alpha diversity was obtained by measuring the Shannon H' diversity index and used to describe microbiome diversity between clinical samples. Beta diversity was evaluated by Weighted UniFrac index.

Chapter 4

Results

4.1 The Hospital Environmental Microbiome

4.1.1 Introduction of NGS in environmental surveillance for HAI control

The study was conducted at the Italian pediatric hospital Institute for Maternal and Child Health "IRCCS Burlo Garofolo" (Trieste, Italy), after obtaining approval from the Institutional Scientific Board, and enrolling the following wards: Pediatric Clinic (PC), Pediatric Surgery (PS), Pediatric Oncology (PO), Neonatal Intensive and Sub-Intensive Care Unit (NICU and sNICU), children's Intensive Care Unit (ICU), Surgical Rooms (SR: Orthopedics, Gynecology and Oculistics) and Delivery Room (DR). Two sampling campaigns were performed for each enrolled ward. Environmental samples were collected 7 hours after cleaning, a time chosen based on our previous studies on hospital contamination, showing that this time point is representative of the contamination level in a 24-h period. Different hard surfaces were sampled based on the characteristics of the individual wards: floor for all ward types; bed footboard, operating bed or incubator for inpatients wards, ICU, SR and NICU, respectively; sink or operating table for inpatients rooms or SR/DR, respectively. The same points were simultaneously sampled following two different methodologies according to subsequent microbiological or molecular analyses.

Conventional microbiological analyses were performed by sampling surfaces with RODAC plates, and 216 total microbiological samples (108 surfaces in duplicate) were collected and analyzed. The results showed a different level of contamination in the different wards (4.1a), with high presence of pathogens (4.1c), particularly *Staphylococcus* spp. (4.1e). PC showed the highest level of contamination, with a median value of 40,842 CFU/m^2 (95% CI 30,412–54,664). The searched pathogens (including *Staphylococcus* spp., Enterobacteriaceae, *Klebsiella* spp., *Acinetobacter* spp., *Pseudomonas* spp., *Clostridium difficile*, *Enterococcus* spp., *Candida* spp., *As-*

pergillus spp.), represented the 69.5% of the total mean microbial contamination (sum median value of 12,000 CFU/m^2, 95% CI 2526–40,545). *Staphylococcus* spp. were the most abundant pathogens observed (median CFU 12,985/m^2, 95% CI 2526–39,662). PS harbored a slightly lower level of microbial contamination (29,474 CFU/m^2, 95% CI 25,762–47,619), with pathogens fraction corresponding to 15,579 CFU/m^2 (95% CI 2631–31,580), also in this case highly represented by *Staphylococcus* spp. (median 12,000 CFU/m^2, 95% CI 2105–30,188). sNICU showed levels of contamination similar to PS, with a total median value of 23,158 CFU/m^2 (95% CI 19,387–38,485), a median pathogens' load of 23,158 CFU/m^2 (95% CI 1579–42,515), and a median *Staphylococcus* spp. count of 17,474 CFU/m^2 (95% CI 1263–27,673). The median contamination of PO corresponded to 13,894 CFU/m^2 (95% CI 11,948–26,392), with a median pathogens' level of 4210 CFU/m^2 (95% CI 28,56–13,354), mostly related to *Staphylococcus* spp. (median value 4210 CFU/m^2; 95% CI 526–13,224). ICU and NICU showed similar contamination levels, considerably lower compared to PC, PS, sNICU and PO, having a median total value of 9474 CFU/m^2 (95% CI 5234–45,082) and 9895 CFU/m^2 (95% CI 6.346–48,882), respectively. The median pathogens' load corresponded to 7158 CFU/m^2 in ICU (95%CI 1053–17,723), and to 7579 CFU/m^2 in NICU (95% CI 105–57,343), mainly referred to Staphylococcal component, corresponding respectively to 6947 CFU/m^2 in ICU (median value; 95% CI 1053–16,871), and 7579 CFU/m^2 (median value; 95% CI 105–26,435) in the NICU ward. As expected, SR and DR resulted the less contaminated wards, with a median value of 1263 CFU/m^2 (95% CI 1459–5348) and 842 CFU/m^2 (95% CI 0–8793), respectively. Median pathogens load resulted of 0 CFU/m^2 in SR (95% CI 0–1842), and 210 CFU/m^2 in DR (95% CI 0–2889), with a median number of staphylococci corresponding to 0 CFU/m^2 in SR (median value; 95% CI 0–1821), and 210 CFU/m^2 in DR (median value; 95% CI 0–2889). No statistically significant differences were observed among PC, PS and sNICU, whereas PO, NICU and ICU resulted significantly less contaminated ($p < 0.01$). SR and DR, as expected, were significantly different both compared to the PC–PS–sNICU group ($p < 0.0001$) and to the PO–NICU–ICU group ($p < 0.001$) (Comar *et al.*, 2019).

Among tested surface types, floor and sinks resulted the highest contaminated surfaces (4.1b) (p = ns), bed footboards resulted significantly less contaminated ($p < 0.01$), and minimal levels of contamination were found on operating beds, surgery tables and incubators ($p < 0.0001$). Potential pathogens represented up to 90% of the total contamination, mostly represented by *Staphylococcus* spp. (4.1d and 4.1f). *Staphylococcus* spp. resulted the most prevalent microbial genus, which was detected in 81% of samples (175/216). Among this, however, the species *S. aureus* was identified uniquely in 1/216 samples, suggesting that the large majority of staphylococci contaminating the tested surfaces were coagulase-negative strains.

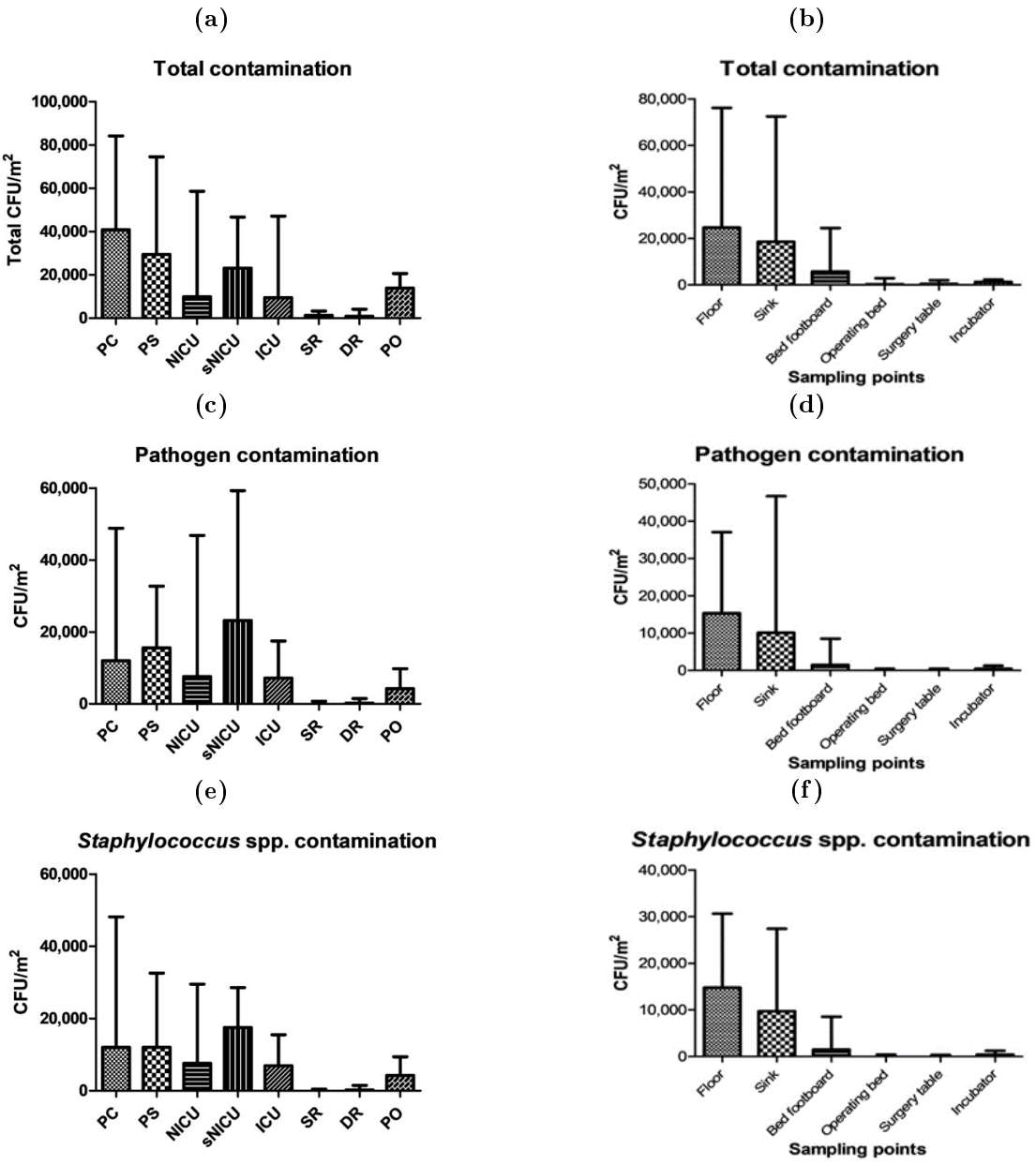


Figure 4.1: Microbial contamination analyzed by conventional culture-based methods. Total contamination (CFUs obtained on TSA medium) (4.1a, 4.1b), pathogen contamination (sum of CFUs obtained on selective media) (4.1c, 4.1d), staphylococcal contamination (CFUs obtained on selective BP medium) (4.1e, 4.1f) are presented. Results of microbial contamination are classified according to type of ward (4.1a, 4.1e) or sampled surfaces (4.1b, 4.1f). All results are expressed as median value of CFU/m^2 with interquartile range. PC, Pediatric Clinic; PS, Pediatric Surgery; NICU, Neonatal Intensive; sNICU, sub-Intensive Care Unit; ICU, children's Intensive Care Unit; SR, Surgical Rooms; DR, Delivery Room; PO, Pediatric Oncology (Comar *et al.*, 2019).

Enterococcus spp. were detected in 28/216 samples, only from floors and sinks, from PC, sNICU and PS rooms. *Acinetobacter* spp. were isolated from 16/216 surfaces, prevalently from floors and sinks from ICU, sNICU and PS wards. *C. difficile* was isolated in 9/216 samples from floors and sinks of PC and sNICU, whereas Enterobacteriaceae were scarcely detected (only found in 2 sinks and 2 floors from PC and PS wards), concomitantly with *Klebsiella* spp. (in only one of the sinks samples). *P. aeruginosa* was identified uniquely in two sink samples from PC rooms. Regarding mycetes, *Candida* spp. were evidenced in 17/216 samples from sinks of PC and sNICU wards, whereas *Aspergillus* spp. were never detected (Comar *et al.*, 2019).

Molecular analyses were performed both by customized qPCR arrays and by qPCR microarrays. The first allowed the simultaneous identification and quantification of 14 pathogens (*S. aureus*, *S. epidermidis*, *E. faecalis*, *E. faecium*, *E. coli*, *K. pneumonia/Enterobacter*, *A. baumannii*, *P. mirabilis*, *P. aeruginosa*, *C. perfringens*, *C. difficile*, *A. fumigatus* and *C. albicans*. The second provided identification and quantification of 87 R genes of the whole contaminant population (resistome). The results (Figure 4.2) showed the presence of *Staphylococcus* spp. in 107/108 samples (99%), and *S. aureus* was detected in 60/108 samples (55%). Enterococcus genus was detected in 86/108 samples (49 resulted positive for *E. faecalis* and 37 for *E. faecium*, *K. pneumonia/Enterobacter* was evidenced in 84/108 collected samples, often in association with *E. coli* (53 samples), *A. baumannii* (26 samples), *P. mirabilis* (13 samples), *P. aeruginosa* (82 samples), *C. perfringens* and *difficile* (37 and 20 samples, respectively). In addition, also mycetes resulted more represented, compared to conventional methods, in particular *C. albicans* was detected in 42/108 samples, and *A. fumigatus* in 18/108 samples (Figure 4.2b).

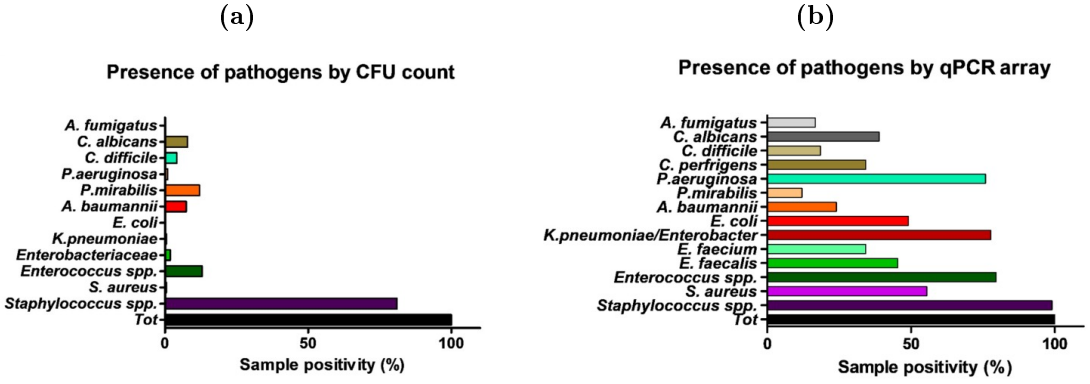


Figure 4.2: Comparison of culture-based analysis (CFU count, 4.2a) and molecular analysis (customized qPCR assay, 4.2b) in the efficiency of detection of microbial contamination in all analyzed samples. Results represent the percentage of detection of the indicated families/genera/species on the total collected samples (216 RODAC plates and 108 swab samples) (Comar *et al.*, 2019).

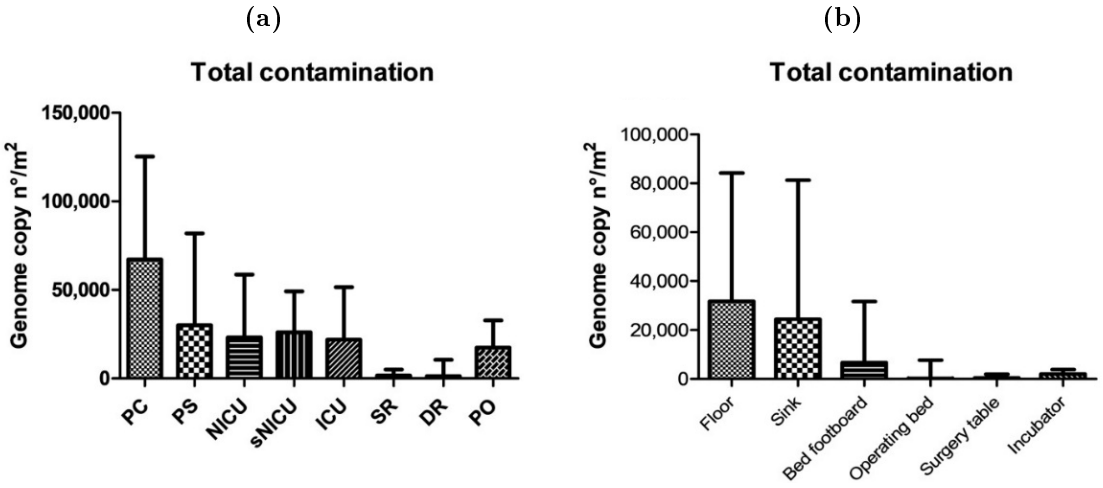


Figure 4.3: Microbial contamination quantified by qPCR molecular assays. Microbial contamination in the analyzed wards (4.3a) and surfaces (4.3b) are expressed as median value of genome copy number/m^2, with interquartile range (Comar *et al.*, 2019).
Abbreviations: PC, Pediatric Clinic; PS, Pediatric Surgery; NICU, Neonatal Intensive; sNICU, sub-Intensive Care Unit; ICU, children's Intensive Care Unit; SR, Surgical Rooms; DR, Delivery Room; PO, Pediatric Oncology.

The results also confirmed that PC and PS had the highest levels of contamination, whereas SR and DR were less contaminated (Figure 4.3a). Statistically significant differences were observed between PC/PS wards and the group of wards NICU/sNICU/ICU/PO ($p < 0.05$). Finally, SR and DR were significantly different from both the first ($p > 0.0001$) and the second group of wards ($p < 0.001$). Floor and sink were confirmed the most contaminated surfaces, bed footboard resulted less contaminated, and the other tested surfaces reported very low levels of microbial contamination, as resulted from microbiological analyses (Figure 4.3b). Overall, the molecular results evidenced higher values of microbial contamination per m^2, compared to CFU counts, likely due to higher sensitivity of molecular compared to cultural assays, or to the fact that molecular methods detect microbes without distinction between living and dead ones, resulting in potential overestimation of contamination compared to cultural methods, which detects only viable microbes.

The characterization of the microbial population resistome, performed by qPCR microarray, highlighted the presence of several R genes in the surface microbiome of the analyzed wards (Figure 4.4). Methicillin-resistance gene (*mecA* was detectable in all wards, except for DR, together with other R genes conferring resistance against macrolides (*ermA*, *ermB*, and β-lactams, including carbapenems (*VIM*-group, *OXA* group). *MsrA* and *ermC* (respectively providing the resistance against macrolides and erythromycin) were also highly represented, whereas ICU wards resulted particularly harbored by *IMP-5* group and NDM gene (New Delhi metallo-β-lactamase). Overall, resistome analysis results were aligned with the contamination level, with wards harboring high levels of R genes (PC, PS) and contamination, and other (SR

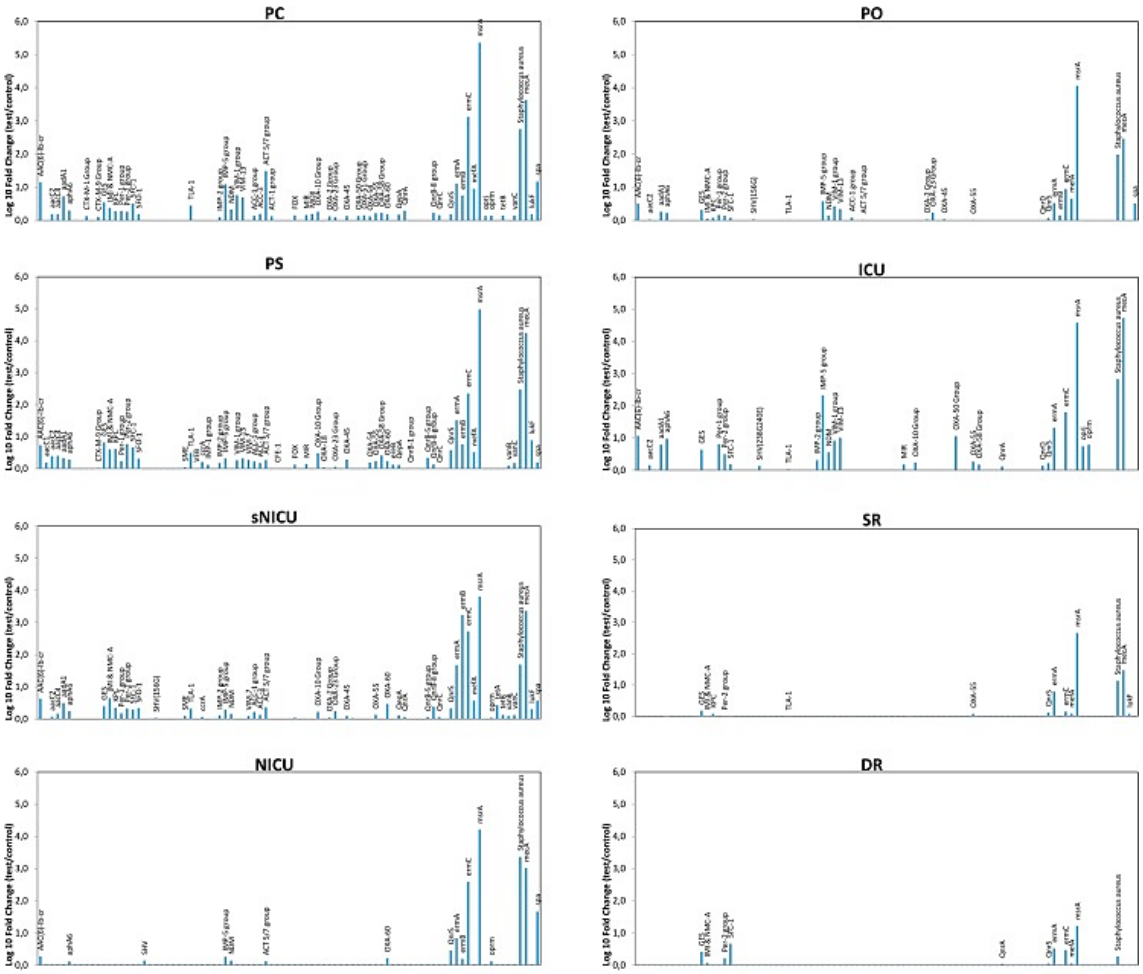


Figure 4.4: Resistome characterization of the contaminating microbiome in the analyzed wards, detected by qPCR microarray. Results are expressed as the mean value of Log_{10} fold change compared to negative controls, for each indicated resistance gene (Comar *et al.*, 2019).
Abbreviations: PC, Pediatric Clinic; PS, Pediatric Surgery; NICU, Neonatal Intensive; sNICU, sub-Intensive Care Unit; ICU, children's Intensive Care Unit; SR, Surgical Rooms; DR, Delivery Room; PO, Pediatric Oncology

and DR) with low levels both of contamination and drug-resistance genes detected (Comar *et al.*, 2019). No statistically significant differences were detected in the resistome profile of PC, PS, ICU and sNICU, although PC and PS harbored higher amount and levels of R genes, compared to ICU and sNICU. By contrast PO, ICU and NICU grouped together showed significantly lower resistance ($p < 0.05$) compared to PC, PS, ICU and sNICU. Finally, SR and DR did not differ significantly from each other, but harbored a lower number and level of R genes both compared to PO-ICU-NICU group ($p < 0.001$) and to the PC-PS-ICU-sNICU group ($p < 0.0001$) (Comar *et al.*, 2019).

The microbial communities contaminating the same hospital surfaces were in parallel analyzed by NGS technology. The results evidenced a high presence of *Cutibacterium* spp. (formerly *Propionibacterium*, not evidenced by cultural and molecular qPCR assays, which was detected in 102/108 samples. *Staphylococcus* spp. was also highly frequent (100/108 samples), followed by *Streptococcus* spp. (89/108 samples), *Corynebacterium* spp. (81/108 samples), *Pseudomonas* spp. (81/108 samples), *Paracoccus* spp. (76/108 samples), *Acinetobacter* spp. (71/108 samples), and *Rothia* spp. (64/108 samples). The predominant communities in analyzed wards are reported in Figure 4.5. *Cutibacterium* spp. (18% in SR and 11% in DR) and *Staphylococcus* spp. (18% in NICU), showed the highest relative abundance percentages. In NICU, *Rothia* spp., *Cutibacterium* spp., *Streptococcus* spp., and *Escherichia–Shigella* spp. were also identified in all the tested surfaces (6/6 samples), resulting 1%-5% of relative abundance. In SR, *Staphylococcus* spp. and *Pseudomonas* spp. were both observed in 16/18 samples (8% and 3% of mean relative abundances), and *Delftia* spp. was detected in 13/18 samples (6% abundance). *Staphylococcus* spp. and *Streptococcus* spp. were the bacteria with the highest values of relative abundance in PS, PC and sNICU (6%, 11% and 18% respectively). In addition, *Acinetobacter* spp., *Pseudomonas* spp., *Cutibacterium* spp. and *Corynebacterium* spp. were also frequently found in sNICU wards. In ICU, the main bacterial contaminants were represented by *Acinetobacter* spp., *Cutibacterium* spp. and *Pseudomonas* spp., which were detectable in all the tested ward surfaces (relative abundance values ranged 2% -4%). In PO the main contaminant was *Cutibacterium* spp. (mean relative abundance 8%), followed by *Staphylococcus* spp. and *Paracoccus* spp. (both 5% of relative abundance) (Comar *et al.*, 2019).

By grouping the samples according to the type of surface (Figure 4.6), *Cutibacterium* spp. showed the highest relative abundance, representing the major contaminant of operating tables (8/8 samples, mean relative abundance 23%) and operating beds (8/8 samples, mean relative abundance 19%). The other bacteria detected at high relative abundance on the operating tables included *Delftia* spp. (6/8 operating tables, mean relative abundance 12%), *Staphylococcus* spp. (7/8 samples, mean relative abundance 5%), *Streptococcus* spp., and *Nitrareductor* spp. (4% in 2/8 tables and 6% in 5/8 tables, respectively). In operating beds *Pseudomonas* spp. (7/8 samples), *Delftia* spp. and *Nitrareductor* spp. (6/8 samples), *Corynebacterium* spp. and *Staphylococcus* spp. (5/8 samples), and *Streptococcus* spp. (4/8 samples) were also frequently observed, with relative abundance percentages ranging from 1% to 7% (Comar *et al.*, 2019).

The main contaminants of the floors were *Staphylococcus* spp. (36/36 samples, mean relative abundance 8%), *Cutibacterium* spp. (35/36), *Corynebacterium* spp. (32/36), and *Streptococcus* spp. (32/36). In sinks, *Streptococcus* spp. resulted

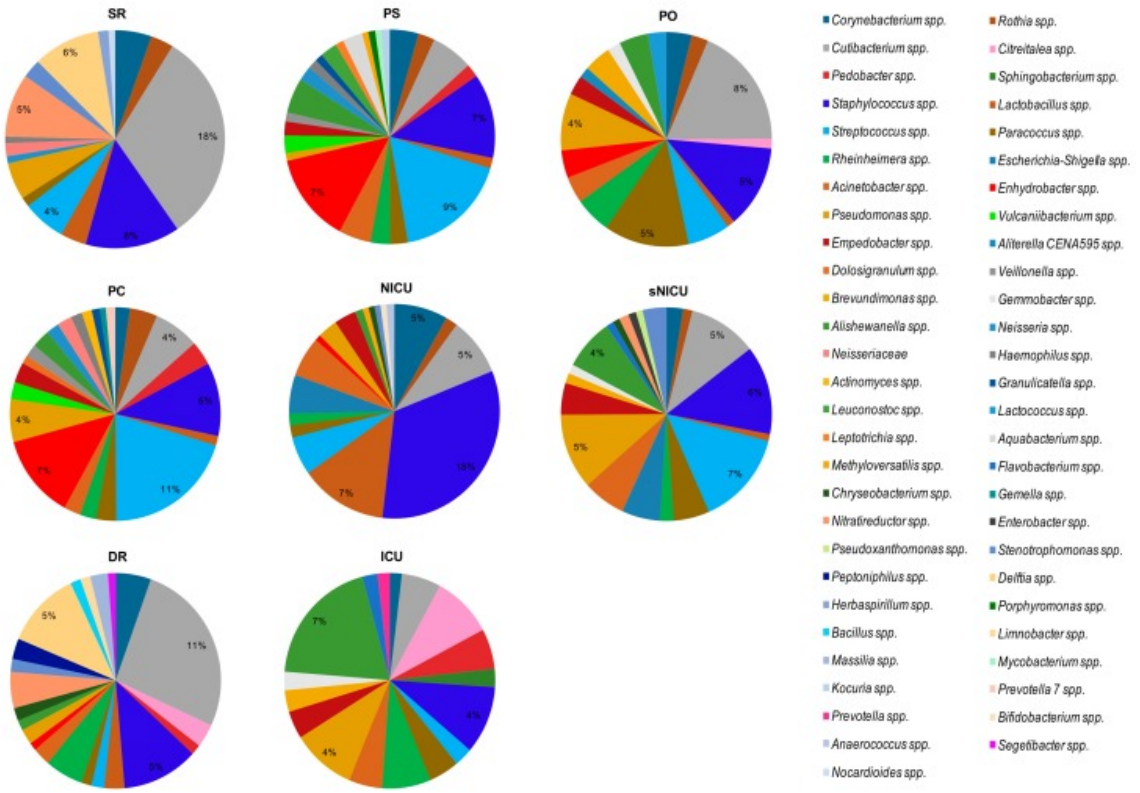


Figure 4.5: The predominant bacterial communities in analyzed wards, as detected by NGS analysis. Data are expressed as mean relative abundance values (Comar *et al.*, 2019). *Abbreviations:* PC, Pediatric Clinic; PS, Pediatric Surgery; NICU, Neonatal Intensive; sNICU, sub-Intensive Care Unit; ICU, children's Intensive Care Unit; SR, Surgical Rooms; DR, Delivery Room; PO, Pediatric Oncology.

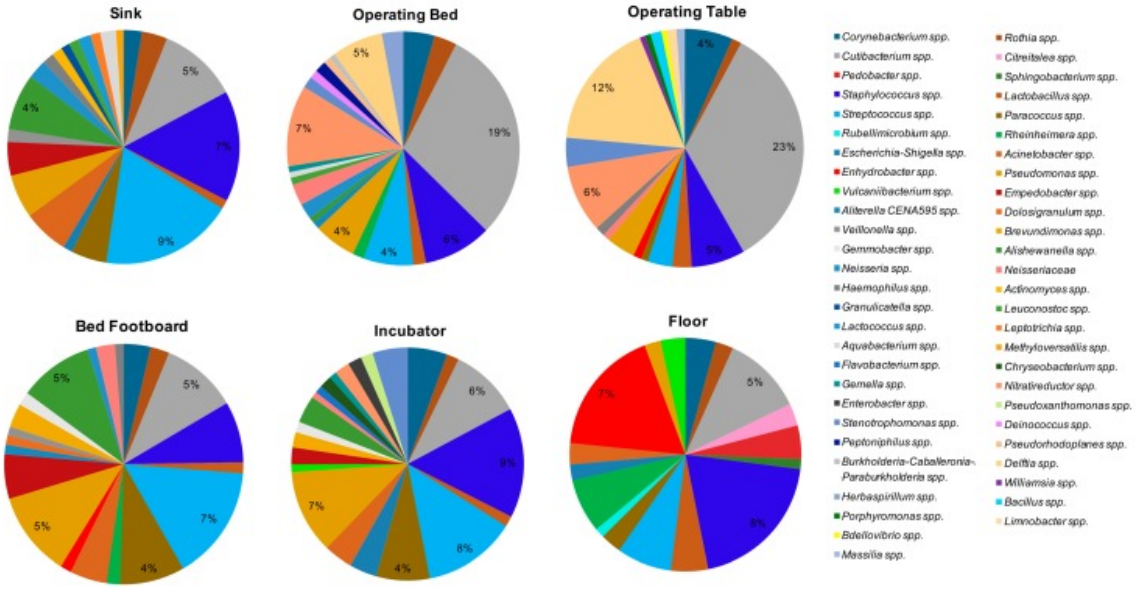


Figure 4.6: The predominant bacterial communities on tested surfaces, as detected by Next-Generation Sequencing (NGS) analysis. Data are expressed as mean relative abundance values (Comar *et al.*, 2019).

the most abundant (9%, in 27/28 sinks), together with *Staphylococcus* spp. (7%, in 27/28 samples), *Cutibacterium* spp. (24/28 samples) and *Pseudomonas* spp. (22/28 samples). In the incubators the main contaminants were *Cutibacterium* spp., and *Streptococcus* spp. (both found in 8/8 incubators), *Pseudomonas* spp., and *Staphylococcus* spp., *Streptococcus* spp. was also the bacterium showing the highest mean relative abundance (7%) among the bed footboards samples.

The microbial diversity (β-diversity) of the microbial communities colonizing the different wards or surfaces was evaluated by unweighted and weighted UniFrac distance matrices, and results were represented by Principal Coordinates Analysis (PCoA). Statistical significance of differences was assessed by one-way Analysis of Similarity (ANOSIM) test applied to the UniFrac distance matrices, showing significant differences for both the weighted ($p = 0.01$, $R = 0.21$) and the unweighted UniFrac ($p = 0.01$, $R = -0.47$) in different surfaces and different wards (weighted $p = 0.01$, $R = 0.13$; unweighted $p = 0.01$, $R = 0.14$) (Figure 4.7). Among surfaces, particular clustering was noted with the floors and the operating tables (Comar *et al.*, 2019).

Hospital contamination and colonization of preterm newborn's nasal cavities

The bacterial composition of nasal swabs of preterm newborns, at birth (group N0) and after admission to the NICU (group N9 after 9 days, and group N13 after 13 days of permanence in the ward) was analyzed by NGS and customized qPCR microarrays. Results were then compared to those obtained with microbial profiling of ward surfaces, at the time of delivery and during the period of hospitalization (Cason *et al.*, 2021). The microbial profile of nasal swabs (Figure 4.8) highlighted the presence of several bacterial genera, including *Corynebacterium* spp., *Staphylococcus* spp., *Streptococcus* spp., *Escherichia-Shigella* spp., *Acinetobacter* spp., *Pseudomonas* spp., *Klebsiella* spp., *Enterobacter* spp., *Lactobacillus* spp., *Cutibacterium* spp., *Stenotrophomonas* spp., *Haemophilus* spp., *Gemella* spp., and *Rothia* spp. The newborns colonized by *Corynebacterium* spp., *Staphylococcus* spp., *Streptococcus* spp., *Escherichia-Shigella* spp., *Klebsiella* spp., and *Enterobacter* spp., increased during time, from N0 to N13. By contrast, *Cutibacterium* spp. and *Pseudomonas* spp. were more present in infants at the time of birth (N0) (Figure 4.8a). *Corynebacterium* spp., *Staphylococcus* spp., and *Escherichia-Shigella* spp. relative abundance increased from N0 to N13: *Corynebacterium* spp. (N0: 2%; N9: 5%; N13: 11%), *Staphylococcus* spp. (N0: 6%; N9: 37%; N13: 44%), and *Escherichia-Shigella* spp. (N0: 7%; N9: 0%; N13: 29%) (Figure 4.8b). The differences were statistically significant for *Staphylococcus* spp. ($p = 0.047$) and *Escherichia-Shigella* spp. ($p = 0.047$), by Kruskal–Wallis test (Cason *et al.*, 2021).

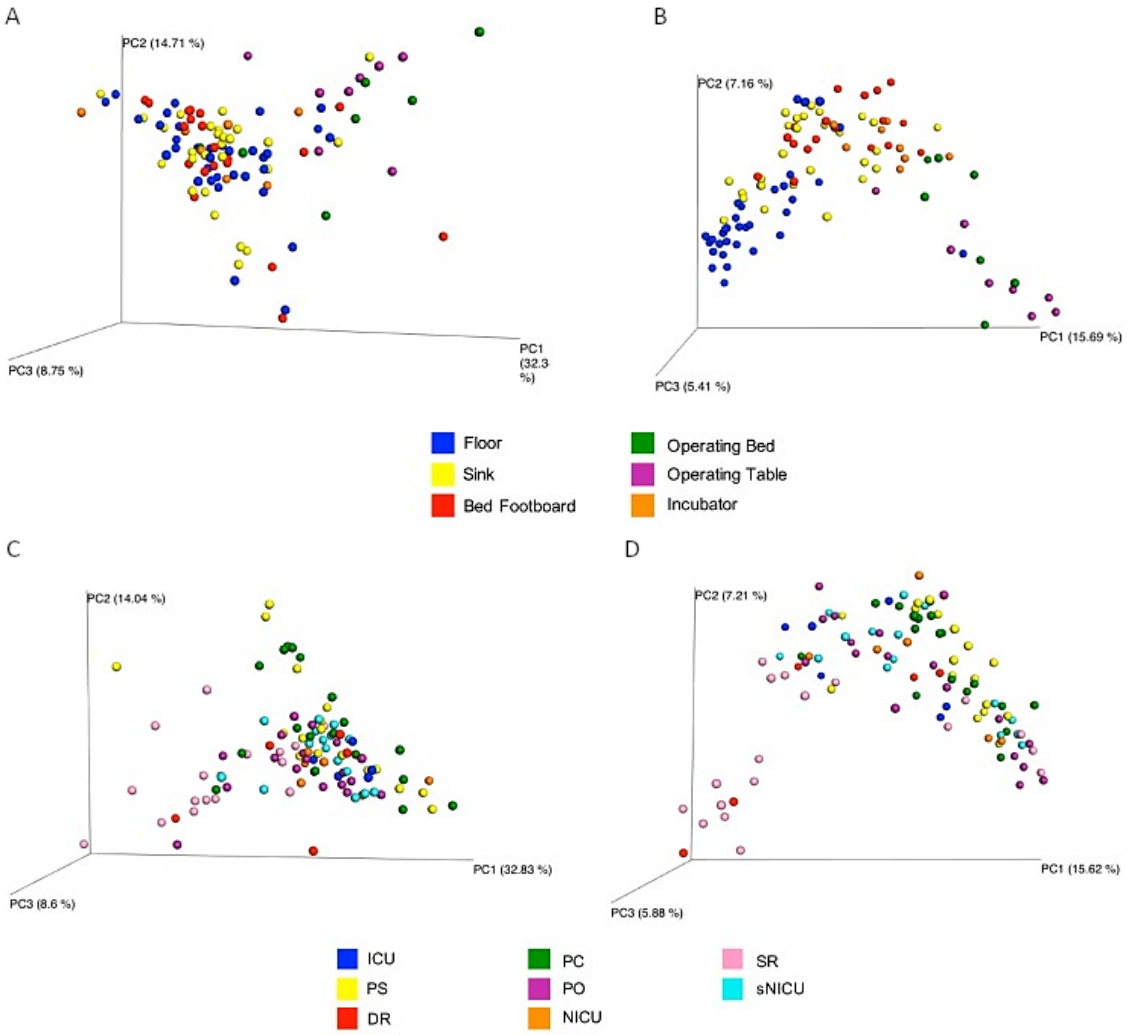
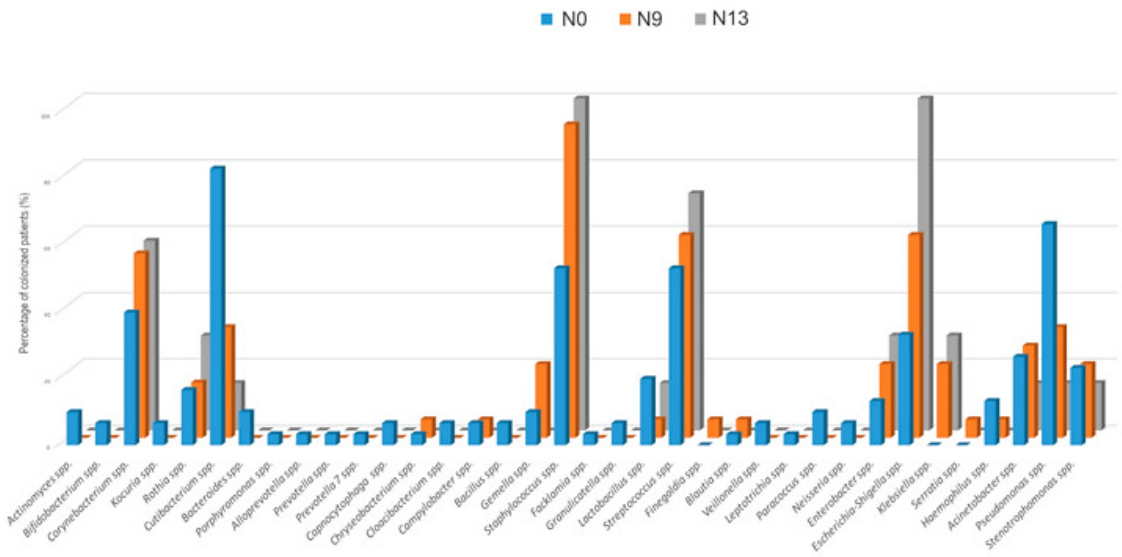


Figure 4.7: Results of UniFrac-based Principal Coordinates Analysis (PCoA) on tested surfaces (4.7A, 4.7B) and wards (4.7C, 4.7D). PCoA plot showing the clustering of bacterial communities according to the type of surface or wards analyzed. Weighted (4.7A, 4.7C) and unweighted, (4.7B, 4.7D) UniFrac-based PCoA. Each dot represents a sample (Comar *et al.*, 2019).
Abbreviations: PC, Pediatric Clinic; PS, Pediatric Surgery; NICU, Neonatal Intensive; sNICU, sub-Intensive Care Unit; ICU, children's Intensive Care Unit; SR, Surgical Rooms; DR, Delivery Room; PO, Pediatric Oncology.

(a)



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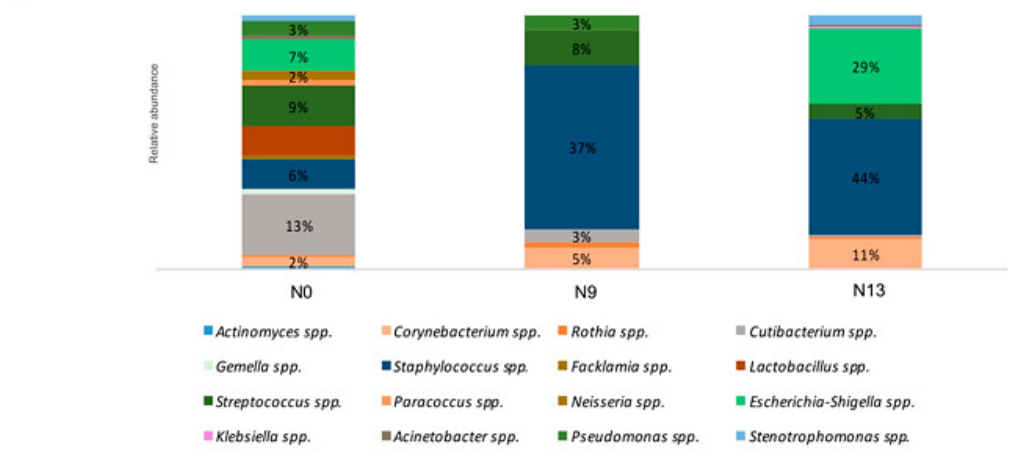

Figure 4.8: The most abundant bacterial communities of nasal swabs from newborns at time of birth (N0) and after two follow-ups (N9, N13). Percentage of colonized patients for each genus (4.8a), mean of bacterial relative abundances for each group (4.8b). N0: samples collected at time of birth. N9: samples collected after 9 days of permanence in NICU. N13: sample collected after 13 days of permanence in NICU (Cason *et al.*, 2021).

We assessed the contribution of mothers' vaginal microbiome to the nasal microbial colonization in newborns, by analyzing by NGS 20 vaginal swabs from mothers just before delivery. *Lactobacillus* spp. represented the predominant colonizer of vaginal flora (86% of mean relative abundance), but *Streptococcus* spp. (6%), *Prevotella* spp. (5%), *Gardnerella* spp. (1%), *Porphyromonas* spp. (1%), and *Mobilincus* spp. (1%) were also detected. Interestingly, in N0 group *Lactobacillus* spp. was observed with a relative abundance of 6% in 20% of newborns, *Prevotella* spp. with 3%, and *Streptococcus* spp. with 9% in 53% of the N0 (Cason *et al.*, 2021).

In parallel, the nasal microbiome of N0, N9 and N13 groups were compared with the environmental microbiome of the delivery room (DR) (N0 vs. DR), and of the NICU wards (N9 vs. NICU and N13 vs. NICU), respectively. The bacterial diversity was compared using the unweighted and weighted UniFrac distance matrices and the results are visualized by PCoA, both with weighted and unweighted UniFrac. Overall, ANOSIM test evidenced high diversity between N0 and DR groups in terms of bacterial composition, while the N9 and N13 vs. NICU groups showed more overlaps in terms of bacterial composition and relative abundances (Figure 4.9) (Cason *et al.*, 2021).

The microbial profiles of DR and NICU were compared to those observed in nasal swabs of N0, N9 and N13 patients (Table 4.1). Interestingly, in DR, *Cutibacterium* spp. represented the most frequent microorganisms found in the newborns nose at birth, in the 83% of subjects. Instead, in NICU, *Staphylococcus* spp. predominated both in N9 (94%, 12/13) and in N13 groups (100%, 7/7). The microbiome distribution of the DR in comparison with the nasal swabs of N0 newborns is reported in Figure 4.10A. *Cutibacterium* spp. resulted the most abundant in nasal swab (13%), and it was detected at higher values in the medical trolley (23%) compared to the other points examined (6% on floor and 5% in footboard). *Staphylococcus* spp. showed a relative abundance of 6% in nasal swabs and represented the primary contaminant of the floor of DR, with a relative abundance of 11%. *Lactobacillus* spp. and *Corynebacterium* spp., both with a relative abundance of 4% in the beds' footboards, showed a rate of noise colonization of 6% and 2%, respectively. The environmental microbiome of the NICU, compared with the nasal microbiome of newborns at N9 and N13 (Figure 4.10B), evidenced the higher abundance of *Staphylococcus* spp., both in nasal swabs of N9 babies (37%) and in the N13 group (44%). The major NICU sources of Staphylococcal contamination appeared to be the sink (10%), the footboard and the floor (9%). *Escherichia-Shigella* spp. was detected in 29% of the N13 group, and in the NICU was found mainly on the floor (4%). *Streptococcus* spp. was present at similar amounts in noses (N9: 8%, N13: 5%) and surfaces (floor and sink: 5%, footboard 8%). *Corynebacterium* spp. showed an average relative abundance of 5% in the N9 group and 11% in the N13 group, while

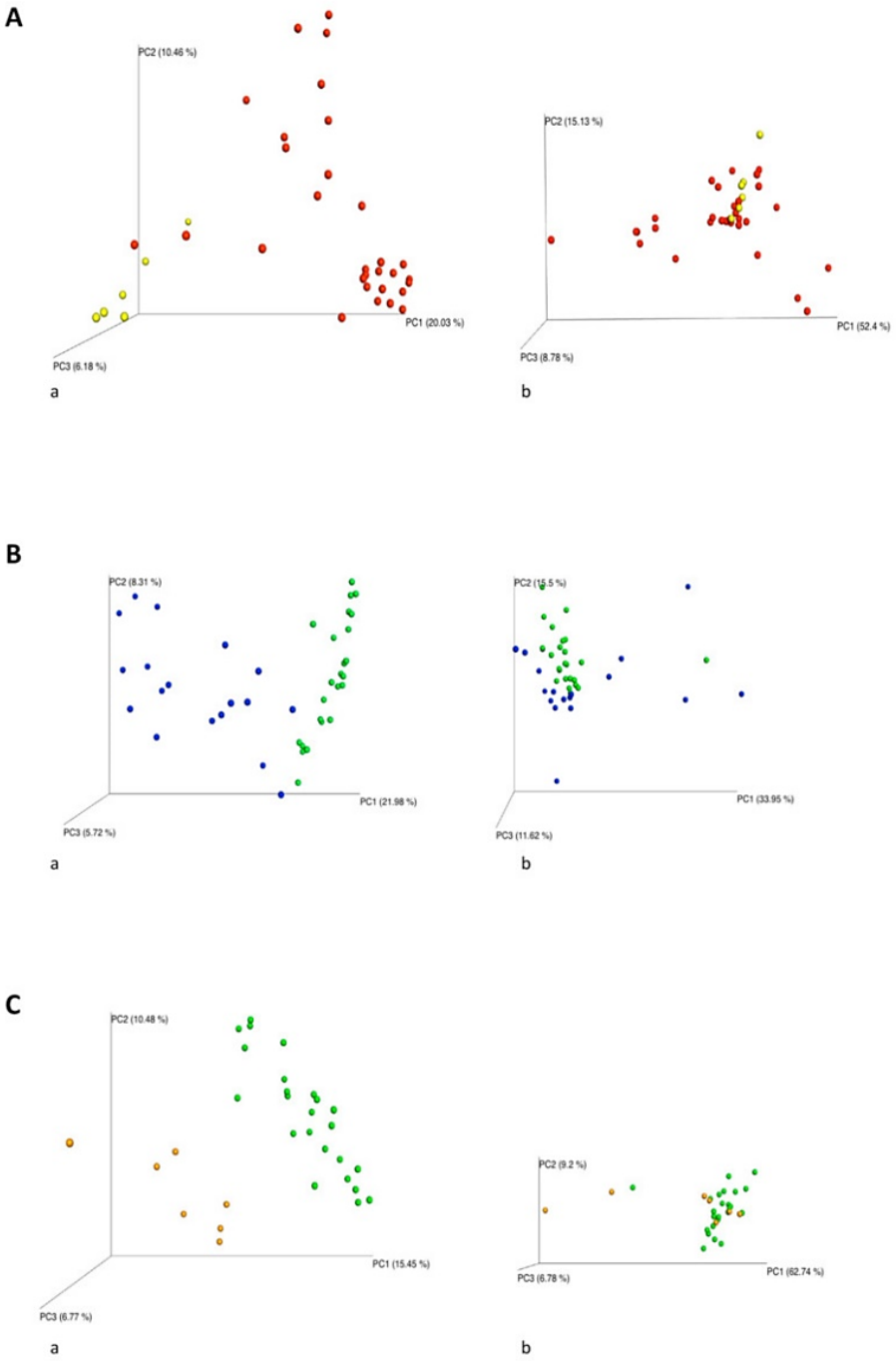

Figure 4.9: Unweighted (a) and weighted (b) UniFrac-based PCoA plots, each dot represents a sample. Comparisons: Nasal swabs collected at time of birth (N0) (red dots) vs. environmental samples from the Delivery Room (DR, yellow dots) (4.9A). Nasal swabs collected after 9 days of permanence in NICU (N9, blued dots) vs. environmental samples from the ward NICU (NICU, green dots) (4.9B). Nasal swabs collected after 13 days of permanence in NICU (N13, orange dots) vs. environmental samples from the ward NICU (NICU, green dots) (4.9C) (Cason *et al.*, 2021).

Table 4.1: Main bacterial genera of environment compared with nasal swabs. Data are expressed as percentage of colonized patients for each genus (Cason *et al.*, 2021). Abbreviations. DR: Delivery room; NICU: Neonatal Intensive Care Unit; N0: nasal swabs collected at time of birth; N9: samples collected after 9 days of permanence in NICU. N13: sample collected after 13 days of permanence in NICU. /: No detection.

Main Bacterial Genera of DR	N0 Colonized Patients (%)	Main Bacterial Genera of NICU	N9 Colonized Patients (%)	N13 Colonized Patients (%)
Staphylococcus spp.	53%	*Staphylococcus* spp.	94%	100%
Streptococcus spp.	53%	*Streptococcus* spp.	61%	71%
Cutibacterium spp.	83%	*Cutibacterium* spp.	33%	14%
Corynebacterium spp.	40%	*Corynebacterium* spp.	56%	57%
Escherichia-Shigella spp.	33%	*Escherichia-Shigella* spp.	61%	100%
Acinetobacter spp.	67%	*Acinetobacter* spp.	33%	14%
Pseudomonas spp.	67%	*Pseudomonas* spp.	33%	14%
Stenotrophomonas spp.	23%	*Stenotrophomonas* spp.	22%	14%
Rothia spp.	17%	*Rothia* spp.	17%	19%
Lactobacillus spp.	20%	*Lactobacillus* spp.	6%	14%
Chryseobacterium spp.	3%	*Chryseobacterium* spp.	6%	/
Paracoccus spp.	10%	*Paracoccus* spp.	/	/
Actinomyces spp.	10%	*Enterobacter* spp.	22%	29%
Gemella spp.	10%	*Bifidobacterium* spp.	/	/
Bacillus spp.	7%	*Alishewanella* spp.	/	/
Neisseria spp.	7%	*Brevundimonas* spp.	/	/
Facklamia spp.	3%	*Gemmobacter* spp.	/	/
Empedobacter spp.	/	*Empedobacter* spp.	/	/
Anaerococcus spp.	/			

in NICU surfaces did not exceed 3% of relative abundance. Other bacterial genera present in both nasal swabs and the NICU environment were *Pseudomonas* spp. (S: 3%, footboard: 7%, floor: 2%, sink 5%) and *Stenotrophomonas* spp. (N13: 4%, footboard: 3%, sink: 1%) (Cason *et al.*, 2021).

The identification of bacteria up to species level by customized qPCR microarray confirmed the results obtained by NGS and evidenced a progressive increase of potential pathogens in the nose of newborns over time (N13 > N9 > N0). Within the genus *Staphylococcus*, the main species were *S. aureus* (N0: 3%, N9: 33%, N13: 43%) and *S. epidermidis* (N0: 27%, N9: 89%, N13: 100%). Within the genus *Streptococcus*, the most frequently detected species were *S. pneumoniae*, *infantis*, *oralis*, and *salivarius*, with higher identification rates in the N13 group. This method also allowed the identification of the fungus *C. albicans* in group N9 (6%) and N13 (43%), but not in group N0. Other microorganisms increasing in N9 and N13 compared to group N0 included *E. faecalis* (N0, 20%; N9, 55%; N13, 85%), *Escherichia-Shigella* (N0, 20%; N9, 38%; N13, 71%), *K. pneumoniae* (N0, 0%; N9, 44%; N13, 85%), *K. oxytoca/E. cloacae* (N0, 23%; N9, 67%; N13, 86%), *P. aeruginosa* (N0, 7%; N9, 38%; N13, 43%), and, although to a less extent, *A. baumannii* (N0, 13%; N9, 11%; N13, 28%). Comparative quantitation between newborn groups evidenced significant increases over time (N13 vs. N0) of *S. epidermidis, K. pneumoniae/oxytoca, Escherichia-Shigella* (about 3 logs; $p < 0.01$), *E. faecalis, S. aureus, P. aeruginosa, S. pneumonia/infantis/oralis/salivarius*, and *C. albicans* (between 1 and 2 logs, $p < 0.01$).

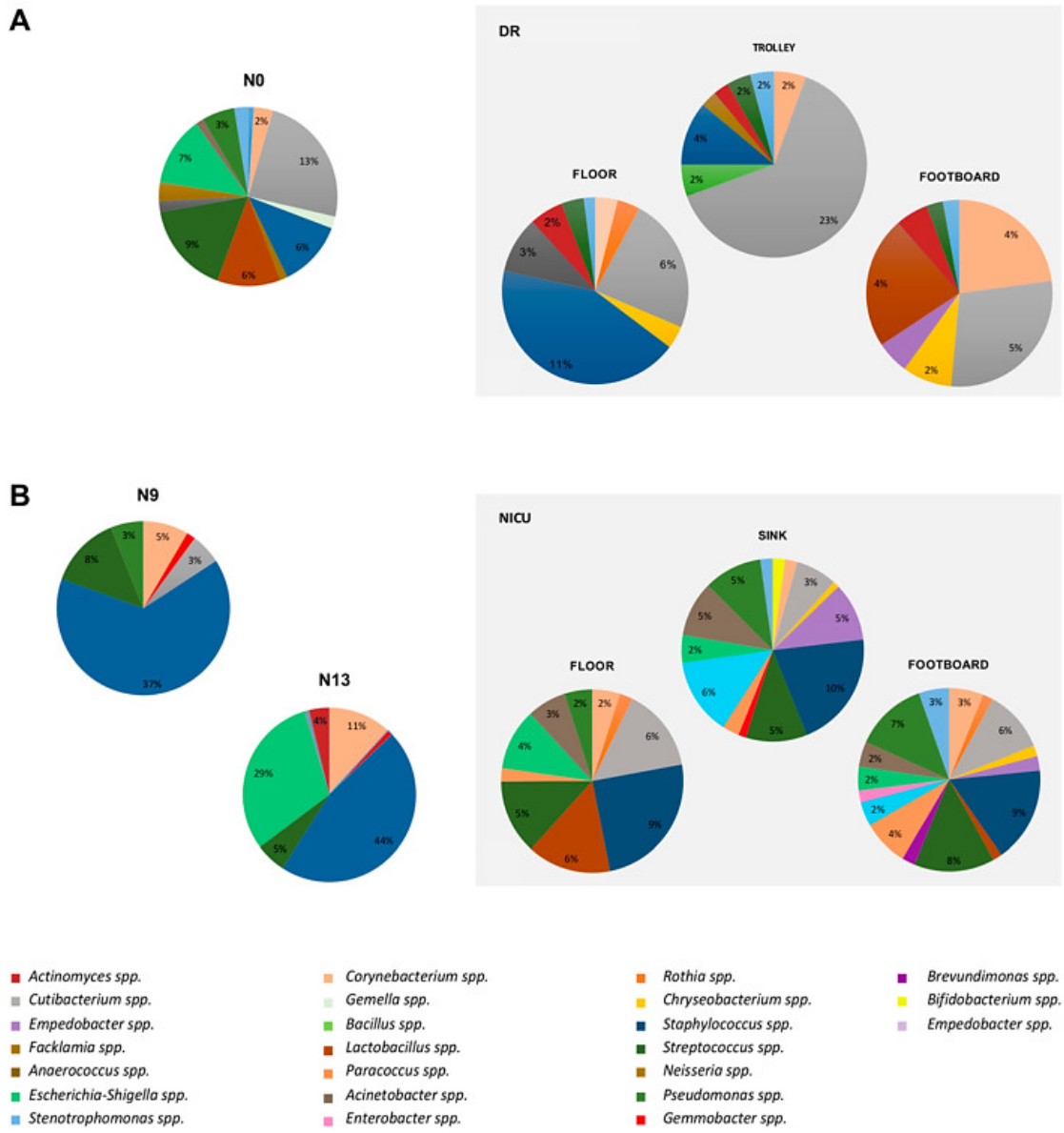

Figure 4.10: The predominant bacterial communities of nasal swabs from newborns, compared to those of environmental surfaces. Results were obtained by NGS. Data are expressed as mean relative abundance values. N0 vs. DR. N0: Nasal swabs collected at time of birth (N0) vs environmental samples of Delivery room (DR), divided into type of surface (floor, footboard, and trolley (4.10A). Nasal samples collected after 9 days and 13 days of permanence in NICU (N9, N13) vs. environmental samples of Neonatal Intensive Care Unit (NICU), divided into type of surface (floor, footboard, and sink) (4.10B) (Cason *et al.*, 2021).

Interestingly, the environmental samples of DR and NICU rooms analyzed by the same method revealed elevate levels of *S. epidermidis* (2200 genome copies/100 cm^2) and *K. pneumoniae/Enterobacter* (1833 genome copies/100 cm^2), followed by *P. aeruginosa* (186.7 copies/100 cm^2), *S. aureus* (153.3 copies/100 cm^2), *Enterococcus faecalis/faecium* (47.5 copies/100 cm^2), and *A. baumannii* (20.3 copies/100 cm^2).

Contamination levels were higher on the floor and sink surfaces compared to bed footboard. In the DR, the level of contamination was generally lower compared to the NICU ward, including essentially *Staphylococci* (*S. epidermidis*, 310 copies/100 cm^2; *S. aureus* 3.3 copies/100 cm^2) and *E. faecium* (13.4 copies/100 cm^2), mainly present on the floor and sink.

Finally, the resistome profile of the nasal microbiome, showed a progressive increase of resistant bacteria over time (Figure 4.11), as no R genes were detectable in the newborn nasal microbiome at birth (N0), while after 9 days (N9) several R genes were detected (*AAC(6)-lb-cr*, *SHV* group, *ACT 5/7* group, *OXA-50* group, *ermC*, *msrA*, *oprj*, *oprm*, and *mecA/spa*, which were increased after 13 days of NICU stay (N13) (*SHV* group, *ACT 5/7* group, *CMY-10* group, *LAT*, *MOX*, *OXA-50* group, *QnrS*, *ermB*, *ermC*, *mefA*, *msrA*, *oprj*, *oprm*, *tetA*, *vanC*, and *mecA/spa*. Most of the detected R genes were also present in the NICU environment (Cason *et al.*, 2021).

4.1.2 Impact of a probiotic-based sanitation on ER microbiome during COVID-19 pandemics

Based on the results obtained in previous studies on the ability of PCHS to stably decrease pathogens and their AMR, and to inactivate SARS-CoV-2 virus (Caselli *et al.*, 2016b, 2018; D'Accolti *et al.*, 2021), a study was started aiming to verify the decontaminating efficiency of PCHS in the Emergency Rooms (ERs) of the same pediatric hospital enrolled in the NGS studies. PCHS system (Copma Scrl, Ferrara, Italy) was applied in replacement of conventional chemical disinfection, for a period of 2 months and following the same frequency of application chemical sanitation, twice a day in the early morning and afternoon. All the other Infections Prevention and Control (IPC) procedures were left unaltered, as done in the previous studies (Caselli *et al.*, 2018). In case of SARS-CoV-2 positivity confirmation of subjects admitted to ER, an emergency disinfection with 0.5% sodium hypochlorite (NaClO) was allowed (Soffritti *et al.*, 2022). ER spaces were monitored before and after PCHS introduction and included triage room, corridor, refection room, suspects' room, and three hospitalization rooms. A total of five sampling campaigns were performed before and after PCHS introduction: two campaigns in the pre-PCHS period ($T0_1$, $T0_2$), with a 10-day interval, and three campaigns in the PCHS period (T1, T2, T3),

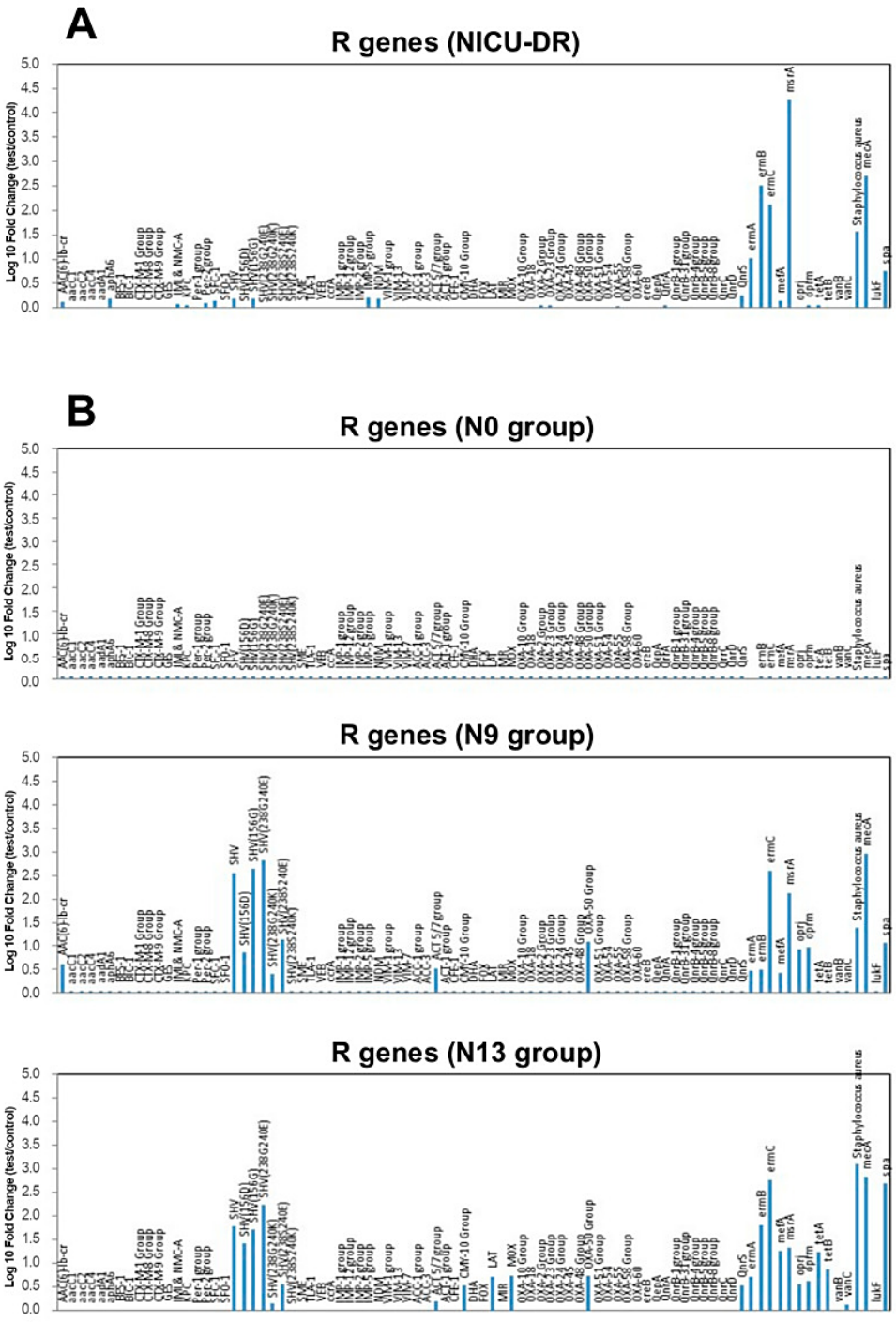

Figure 4.11: Resistome characterization of the microbial population contaminating NICU surfaces (4.11A) and nasal swabs of N0, N9, and N13 newborn groups (4.11B). Results are expressed as Log_{10} fold change of each detected R gene, compared with the negative controls (NTC). The plotted data represent the mean values of duplicate samples obtained in two environmental samplings for NICU environmental duplicate sampled points, and for nasal swabs (Cason *et al.*, 2021).

at 2, 5 and 9 weeks after PCHS introduction, respectively. A total of 152 samples were collected from critical surfaces, namely floor, sink and bed footboard, both for microbiological and molecular microbiome characterization (Soffritti *et al.*, 2022). The surfaces were simultaneously sampled by two different methods, according to subsequent microbiological or molecular analyses.

The results of microbiological analyses are reported in Figure 4.12. A high level of microbial contamination on ER surfaces was reported at the basal level (T0, before PCHS introduction) (Figure 4.12, plot (a)): the total microbial contamination was 29,523 CFU/m^2 (median value, 95% CI 21,533–56,924), highly represented by pathogens contamination that represented over the 89% of the total, with 26,315 CFU/m^2 median value (95% CI 19,155–52,334). The *Staphylococcus* genus was the most prevalent and represented the 85.1% of the total pathogens (22,390 CFU/m^2, 95% CI 16,045–49,554). Mycetes were also detectable, representing about 12.0% of the total pathogens (3,170 CFU/m^2, 95% CI 1,189–9,124). *Bacillus* spp. quote was instead scarcely present at T0, with 991 CFU/m^2 (95% CI 0–1,448), corresponding to 3.3% of the total microbial population. After 2 weeks of PCHS application (T1) (Figure 4.12, plot (b)), *Bacillus* spp. resulted highly increased to 15,418 CFU/m^2 (95% CI 12,498–21,501), representing the 69.9% of the total microbial population measured. The total pathogens load decreased to 6,365 CFU/m^2 (95% CI 4,555–10,201), corresponding to 28.9% of the total microbes at T1, and to 24.2% of the original amount at T0. *Staphylococcus* spp. dropped to 5,598 CFU/m^2 (95% CI 3,478–8,223), maintaining the higher prevalence among pathogens (87.9% of the T1 total pathogens). In addition, also mycetes resulted decreased of 16.8% compared to T0 value, showing 533 CFU/m^2 mean value (95% CI 0–1,109). The trend of pathogens decrease was confirmed after 5 weeks of PCHS application (T2) (Figure 4.12, plot (c)), whereas 9 weeks after PCHS introduction the contamination resulted comparable to the pre-PCHS condition (T0) (Figure 4.12, plot (d)). Pathogen contamination was variable on the different surfaces tested, floor resulted the most contaminated surface, followed by sink and bed footboard (Figure 4.13, plot (a)). In order to investigate the reason of pathogen regrowth during T3 monitoring, we firstly focused on the amount of PCHS-*Bacillus* on treated surfaces (Figure 4.13, plot (b)). Analysis showed an evident decrease of *Bacillus* quotes at T3 compared to T1 and T2 (Soffritti *et al.*, 2022).

To understand the possible causes of *Bacillus* decrease, we evaluated the occurrence of emergency disinfection in the areas included in the study, as disinfection with 0.5% sodium hypochlorite (NaClO) was permitted in case of SARS-CoV-2 positivity confirmation of patients admitted to ER. The results indicated that starting from one week after T2 the concern linked to the increasing number of SARS-CoV-2 contagion led to intensive, almost daily, use of chlorine disinfectants (Figure 4.14),

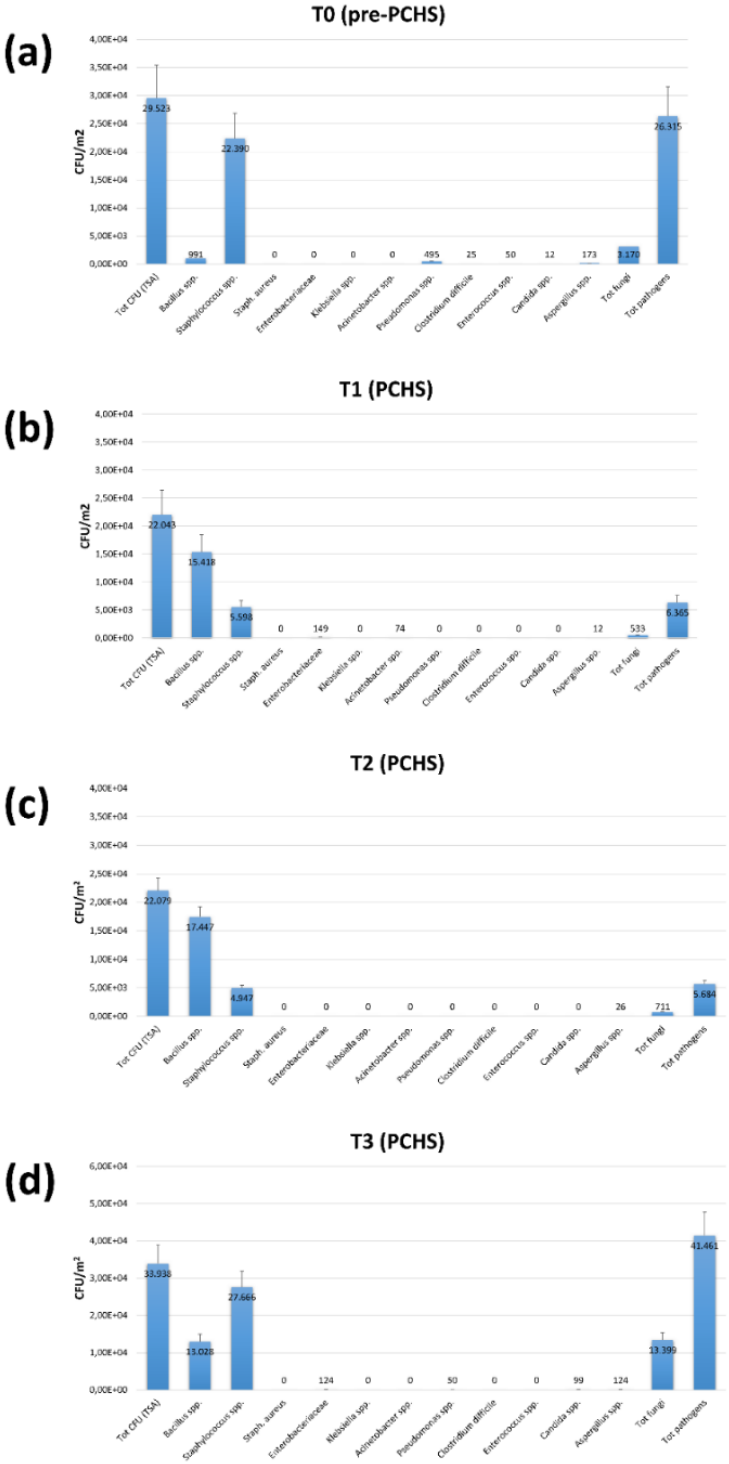

Figure 4.12: Microbial contamination of Emergency rooms in pre-PCHS (T0) (4.12a) and post-PCHS T1-T2-T3 periods (4.12b, 4.12c, 4.12d), by conventional microbiological analyses. Total contamination (CFUs count on TSA general medium), individual pathogens' contamination (CFUs count on each selective medium) and total pathogen contamination (sum of the individual CFU pathogens enumerated on individual selective media) are reported and expressed as median (Soffritti *et al.*, 2022).

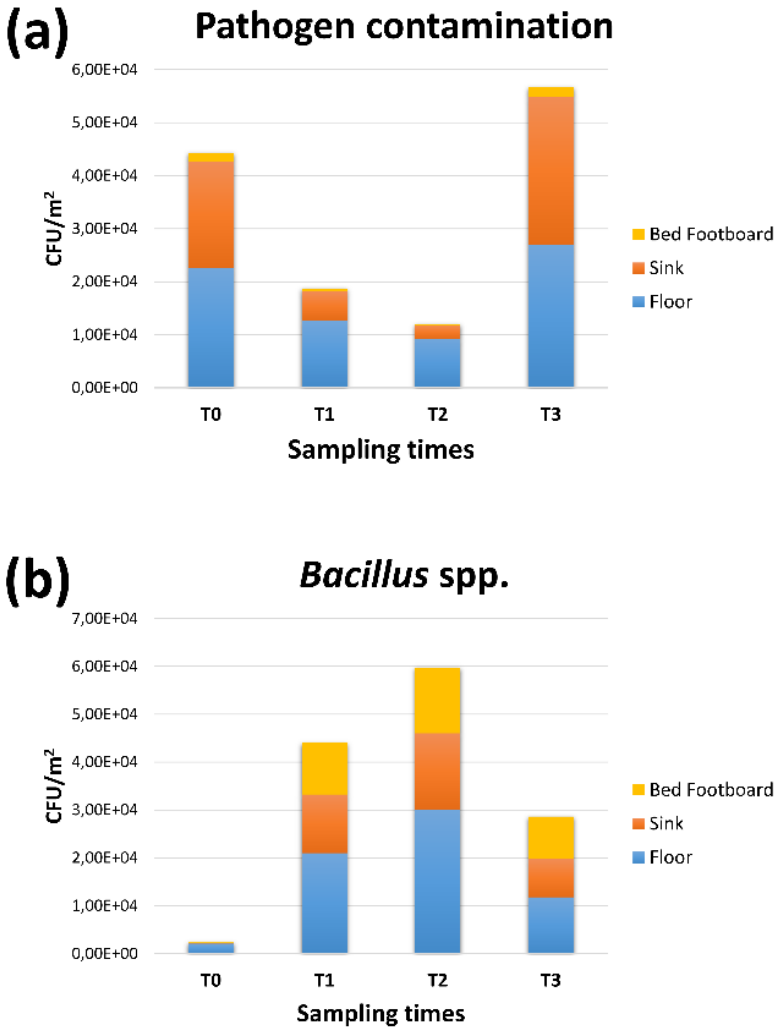

Figure 4.13: Total pathogen contamination (4.13a) and PCHS-derived Bacillus quote (4.13b) in the pre-PCHS and PCHS periods subdivided for the different types of tested surfaces (floor, sink, bed footboard). Results are expressed as the median value (Soffritti *et al.*, 2022).

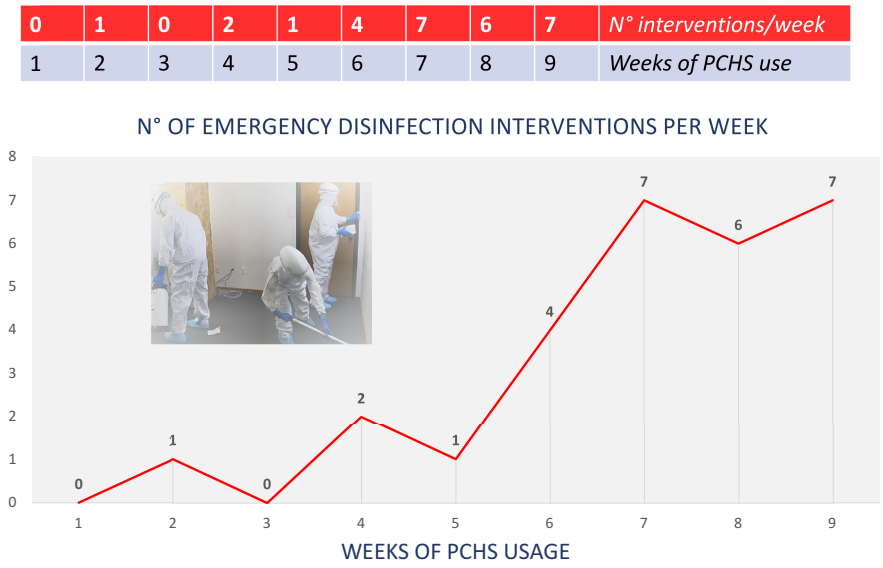

Figure 4.14: Emergency disinfection interventions performed with 0.5% NaClO during the PCHS period in the ER areas. The number of NaClO disinfection interventions per each week are indicated in red, and the number of weeks after PCHS introduction are indicated in black. Modified from Soffritti *et al.* (2022).

likely causing the inactivation of PCHS-*Bacillus* and thus negatively affecting the PCHS action.

Similarly, to what performed in the other wards of the enrolled hospital, the bacteriome profile was also in parallel evaluated by NGS. The results, reported in Figure 4.15, confirmed the decrease of *Staphylococcus* spp. abundance after PCHS treatment (with relative abundance of T1: 9%; T2: 9%; T3: 8%) compared to pre-PCHS period (T0: 19%) ($p < 0.05$). *Streptococcus* spp. also diminished significantly (T0: 8%; T1: 6%; T2: 3%; T3: 2%) ($p < 0.05$), together with the less abundant pathogens *Escherichia-Shigella* spp., showing a 2% relative abundance at T0 ($p < 0.05$). *Acinetobacter* spp. and *Pseudomonas* spp. showed no statistically significant variations over time, whereas *Lactobacillus* spp. showed slightly increasing values of relative abundance over time, although not significant (Soffritti *et al.*, 2022).

Finally, the resistome of the ER microbiome was investigated by qPCR microarray, as described for the other wards, showing that it harbored several resistance genes at T0 (Figure 4.16). In particular, *msrA*, *ermC*, *ermB*, and *mecA* genes (conferring resistance against macrolides, erythromycin, and methicillin) were detected at high level. *AadA1*, *aphA6*, *GES*, *IMI*, *Per-2*, *VIM-1*, *MIR*, *OXA-23*, *OXA-51*, *QnrA*, *QnrB-1*, *QnrD*, and *tetA* genes (related to resistance to streptomycin/spectinomycin, aminoglycoside, β-lactams, fluorochinolones and tetracyclin, respectively) were also detected. The introduction of PCHS induced a significant decrease of resistance genes, as evidenced at T1 ($Pc < 0.01$) and T2 ($Pc < 0.001$)

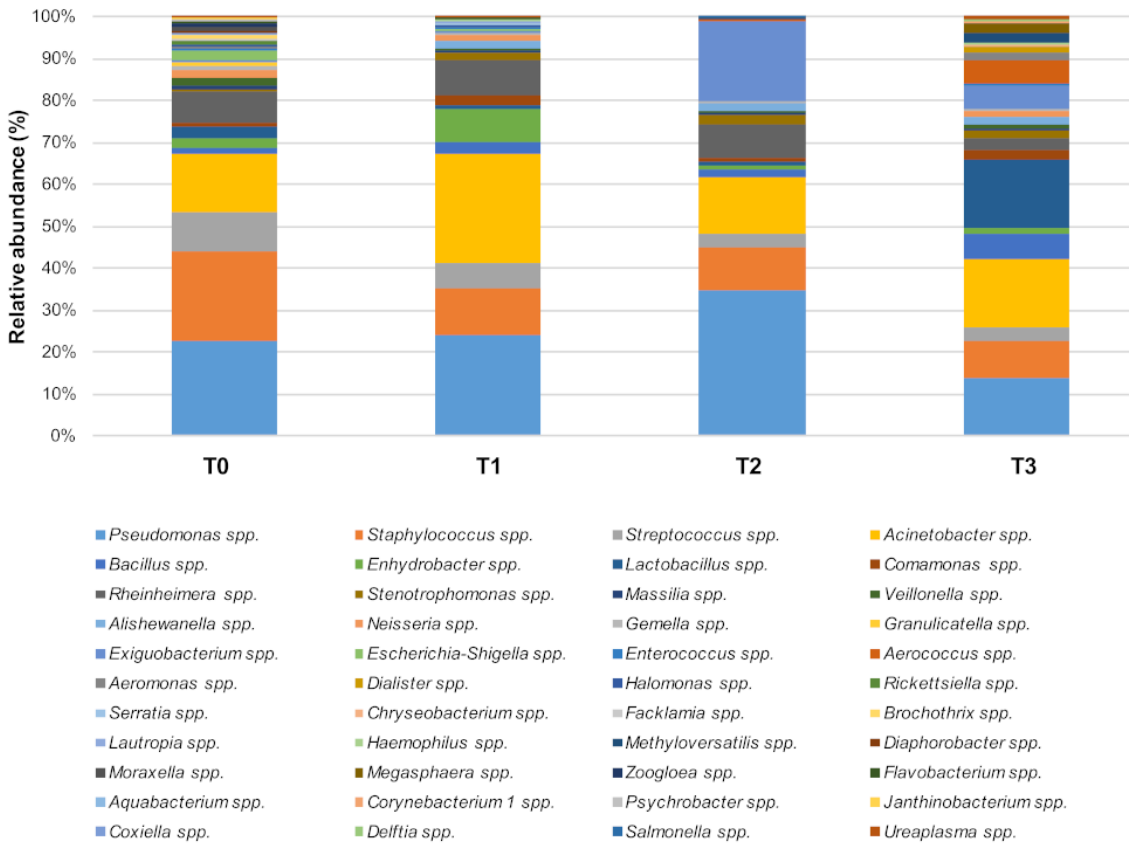

Figure 4.15: Microbiome characterization by NGS analysis of sampled surfaces relative to T0 (pre-PCHS), T1 (2 PCHS-weeks), T2 (5 PCHS-weeks) and T3 (9 PCHS-weeks). The predominant bacterial communities are reported, presented as mean relative abundance values (Soffritti *et al.*, 2022).

times, whereas no significant variations were observed at T3 compared to T0 original values.

Being the study conducted during the COVID-19 pandemics, we also assessed the presence of SARS-CoV-2 genome in the ER areas, by using a specific qRT-PCR. The results showed no environmental sample positive for virus presence, during neither pre-PCHS or PCHS period (Soffritti *et al.*, 2022).

4.2 The Human Microbiome

4.2.1 Defining the healthy oral microbiome

To obtain a site-specific map of healthy oral microbiome (HOM) and resistome characterization, a Single-Arm, Single-Visit, observational study was performed at the Clinical Unit of the University of Ferrara, after approval by the Ethic Committee "Area Vasta Emilia Centro Regione Emilia Romagna" (CE-AVEC). Twenty healthy young adults were enrolled in the study since they fulfilled the inclusion and exclu-

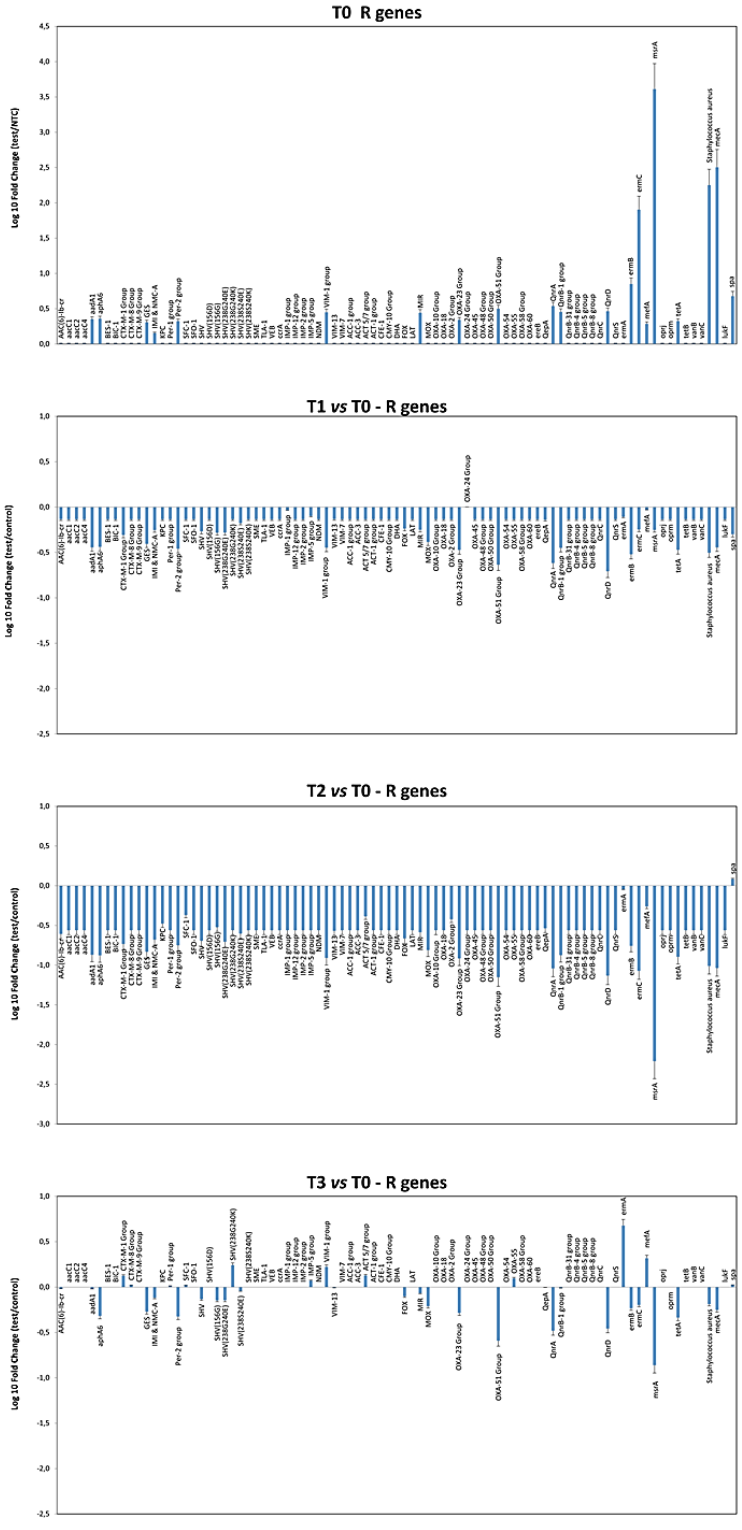

Figure 4.16: Characterization of resistome of the ER microbiome before (T0) and after PCHS introduction (T1, T2, T3). Results are expressed as the mean value of Log_{10} fold change compared to controls, for each indicated resistance gene (Soffritti *et al.*, 2022).

sion criteria listed in Table 4.2 (Caselli *et al.*, 2020b).

The study was performed in accordance with the current standards for observational clinical trials. The study population included 10 male and 10 female subjects, with a mean age of 24.7 years (range 21-30 years). Additional clinical data collected were:

- the number of teeth,
- the Plaque Score (O'Learly PL), which represents the percentage of sites, four per tooth, with dental plaque presence. A condition of excellent oral hygiene is denoted by Plaque Score index <20% (Ghiz *et al.*, 2009),
- the Bleeding on Probing (BOP) index, that represents the percentage of sites, six sites per tooth, showing bleeding during the periodontal probing procedure; the optimal value in healthy subjects should be less than 10%, according to the definition provided during the World Workshop 2017 of the American Academy of Periodontology and European Federation of Periodontology (Chapple *et al.*, 2018). Although HMP protocol considers subjects with mild gingivitis to be included (Mcinnes, 2010), the inclusion criteria of the study were limited to BOP $< 10\%$,
- the type of devices used during oral hygiene practices at home (Oral Hygiene Devices, OHD), including manual or powered toothbrush, and the use of interproximal devices, e.g. floss and interproximal brush,
- for female subjects, the use of oral contraceptives and the monthly period were also considered.

The main features of the study population are listed in Table 4.3.

Overall, the enrolled population presented 28-32 teeth (mean number = 29.85). The plaque score (O'Learly PL) ranged from 8.93% to 52.3% of plaque presence, with a main value of 24.16%. Mean value of BOP index was 3.99%, within a range of 0.6-9.89%, significantly lower than the cut-off value of 10% considered. Among the considered subjects, 55% (11/20) used manual toothbrush and 45% (9/20) used powered toothbrush. Additional interproximal devices were used by 9/20 subjects, six of them combined with manual brushing and three of which in addition to powered brushing (Caselli *et al.*, 2020b).

Collected oral samples from hard palate, keratinized gingiva, mucosa, oral rinse, saliva, sub-gingival plaque, supra-gingival plaque and tongue, were analyzed by WGS, evidencing at least 218 microbial genera and 570 species, characterizing the HOM of the enrolled subjects. In Figure 4.17A, heatmaps represent the genera detected by WGS in each enrolled subject, clustering the specimens derived from each

Table 4.2: List of inclusion and exclusion criteria for subject's enrollment in the study (Caselli *et al.*, 2020b).

INCLUSION CRITERIA	*EXCLUSION CRITERIA*
• *Male and female subjects* • *Age 18-30 years* • *Good general health: free of systemic diseases such as diabetes, HIV infection or genetic disorder, ongoing malignant disease of any type that could interfere with the evaluation of the study objectives* • *Good oral health: free of oral pathologies such as leukoplakia, erythroplakia, oral lichen planus (OLP)* • *Availability for the 6-month duration of the study for an assigned subject* • *Signed Informed Consent Form*	• Pregnant or lactating women • More than 8 missing teeth (with missing teeth, accounted for by third molar extractions, teeth extracted for orthodontic purposes, teeth extracted because of trauma, or congenitally missing teeth) • Presence of orthodontic appliances • Chronic dry mouth (clinically assessed) • Significant halitosis (clinically assessed) • Periodontitis • Untreated carious lesions or oral abscesses • Current or past (within 3 months before enrolment) assumption of medications that may influence oral microbiome (corticosteroids, calcium channel blockers, systemic antibiotics). • Non-surgical and/or surgical mechanical/ manual periodontal debridement within 3 months before enrolment. • Heart diseases or blood pressure alteration that requires a medication • Renal, hepatic, gastrointestinal disease that requires a medication • Diabetes • Presence of any sexually transmitted disease (STD), HIV, HCV infection • Genetic disorders potentially interfering with the evaluation of the study objectives • Chronic obstructive pulmonary disease and asthma • Neoplastic lesions or paraneoplastic syndrome; tumors or significant pathology of the soft or hard tissues of the oral cavity (such as LPO, erythroplakia, leukoplakia, candidiasis) • Current radiotherapy or chemotherapy

Table 4.3: Demographic and clinical features of the study population (Caselli *et al.*, 2020b).
[a]Plaque Score (O'Leary PL, which represents the percentage of sites, four per tooth, presenting plaque; the optimal value should be less than 20%), [b]Bleeding on Probing (BOP) index (representing the percentage of sites, six per tooth, presenting bleeding on periodontal probing; the optimal value should be less than 10%).

Subject ID n.	Teeth n.	Plaque Score[a]	BOP[b]	OHD (Oral Hygiene Devices at home)	
				Toothbrush	Interproximal device
1	29	52,30	9,77	manual	none
2	29	24,14	9,77	powered	none
3	32	19,79	8,33	powered	floss
4	30	12,22	2,22	powered	none
5	31	22,62	1,79	manual	none
6	28	23,21	1,19	manual	floss
7	30	20,00	2,78	manual	none
8	32	28,65	1,56	powered	none
9	31	25,27	5,38	manual	none
10	30	27,78	2,22	powered	floss
11	28	17,26	2,97	powered	none
12	30	22,39	9,89	manual	floss
13	28	30,36	2,98	manual	floss
14	32	27,98	4,17	manual	floss
15	28	8,93	0,60	powered	none
16	32	20,31	1,04	manual	floss
17	31	29,03	2,15	manual	none
18	28	22,02	4,76	manual	floss
19	30	23,89	2,78	powered	floss
20	28	25,00	3,57	powered	none

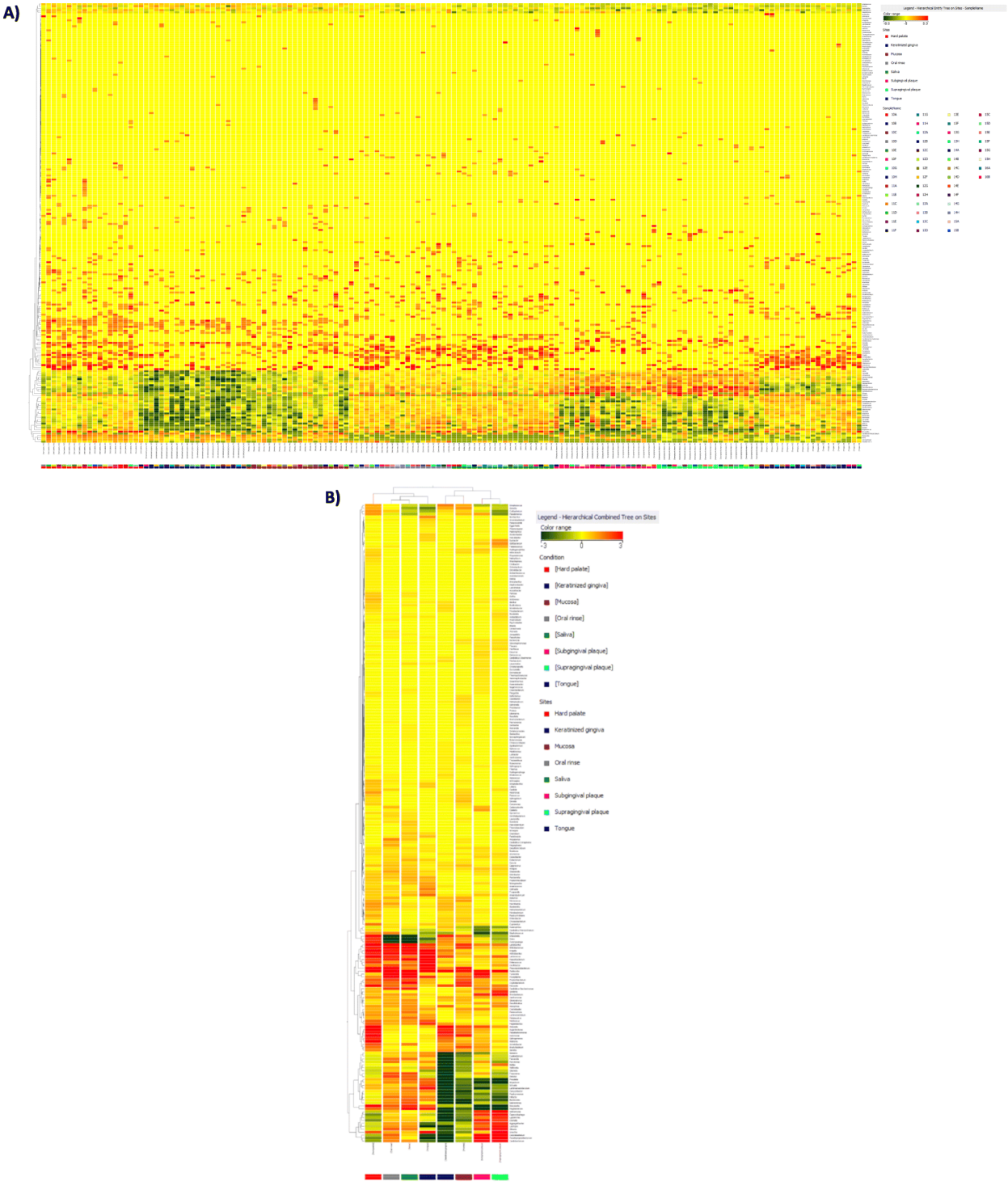

Figure 4.17: Heatmap representation of genera detected by WGS analysis of each enrolled subject, clustered by different sampled sites (4.17A). Heatmap representation of genera detected by WGS analysis grouped by sampled site (hard palate, keratinized gingiva, mucosa, oral rinse, saliva, sub-gingival plaque, supra-gingival plaque, and tongue (4.17B). Hierarchical legends are shown (Caselli *et al.*, 2020b).

sample site. The heatmap clearly evidenced differences among the different oral microhabitats, including saliva and oral rinse. Genera detected by WGS analysis from each subject were grouped by sampled site, as represented in Figure 4.17B, showing again evident differences among oral microhabitats (Caselli *et al.*, 2020b).

A total number of 42 genera were considered as the most abundant bacterial genera. The prevalence of these genera was analyzed for each sampled site, as reported in Figure 4.18, revealing a marked difference in microbial distribution among sampled sites (Caselli *et al.*, 2020b).

In mucosal tissues, *Streptococcus* genus was most prevalent, representing $> 40\%$ of the total genera detected (44% in hard palate, 65% in oral mucosa, and 66% in keratinized gingiva). In the other sampled sites analyzed *Streptococcus* spp. represented 12-23% of the total genera detected (12% in tongue, 13% in supragingival plaque, 15% in subgingival plaque and saliva without rinsing, 23% in oral rinse). *Neisseria*, *Prevotella*, and *Haemophilus* genera were also highly present, ranging from 6 to 29% of the total bacteria detected. The *Rothia* genus (*Actinomycetaceae* family) was also significantly prevalent, ranging from 4 to 24% of the total bacteria (8.4% in tongue, 24.1% in supragingival plaque, 13.4% in subgingival plaque, 6.7% in saliva without rinsing, 7% in oral rinse, 7.2% in hard palate, 4.3% in oral mucosa), although it was no present in keratinized gingiva. As expected, anaerobes (*Actinomyces*, *Veillonella*, *Fusobacterium* were found principally in subgingival plaque specimens, and were generally less represented. On the other hand, *Simonsiella* was almost exclusively observed in hard palate samples, according to previous results obtained by 16S rRNA NGS technique (Aas *et al.*, 2005; Caselli *et al.*, 2020b).

The analysis of microbial abundance at species levels highlighted the prevalence of *Streptococcus mitis* (9.5% of the total detected species), followed by *S. oralis*, *salivarius*, and *sanguinis* (in order of abundance). As expected, *Streptococcus mutans*, widely reported in association with caries development, was scarcely present in HOM, representing only the 0.003% of the total species detected (Takahashi and Nyvad, 2011). Among *Haemophilus* genus, *Haemophilus parainfluenzae* was the most prevalent species, showing a relative abundance of 11.8% of the total species, then *H. haemolyticus* (0.4%) and H. *influenzae* (0.3%). Investigating the other highly abundant genera identified by WGS analysis, *Neisseria*, *Prevotella* and *Rothia*, the species more represented were *Prevotella melaninogenica* (7.8%), *Neisseria subflava* (5.2%) and *Rothia dentocariosa* (4.9%) were the most prevalent species.

Mycetes were scarcely detected in HOM, representing only the 0.004% of the total oral microbiome. *C. albicans* was the prevalent species. Mycetes were mainly detected in samples of hard palate, oral rinse and supra-gingival plaque (Caselli *et al.*, 2020b). In addition, none of the samples from each different site harbored protozoa. This result could be due to the high homogeneity of our study population, in terms

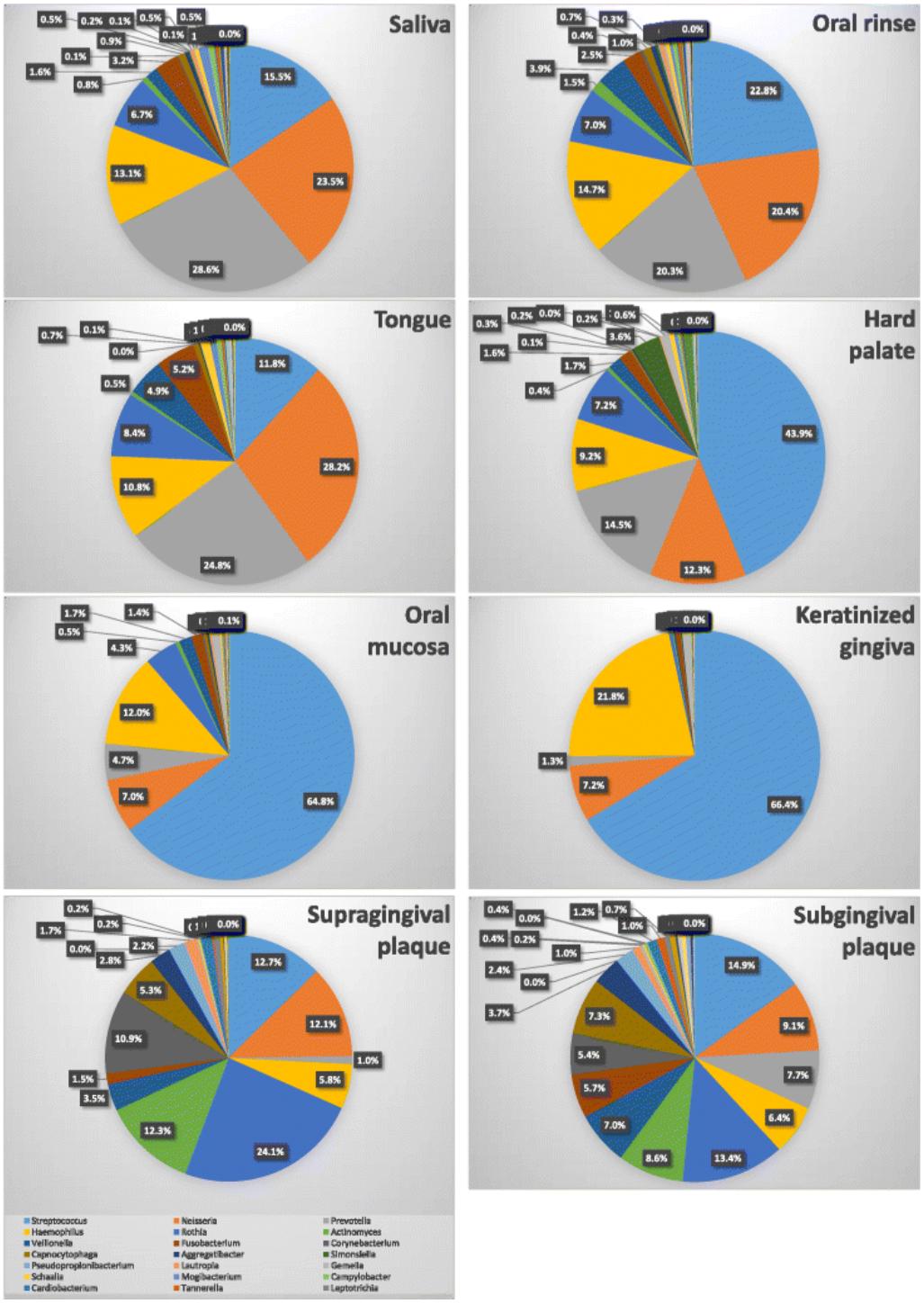

Figure 4.18: Percentage distribution of detected genera harboring the healthy oral cavity, subdivided for the different sampled sites (Caselli *et al.*, 2020b).

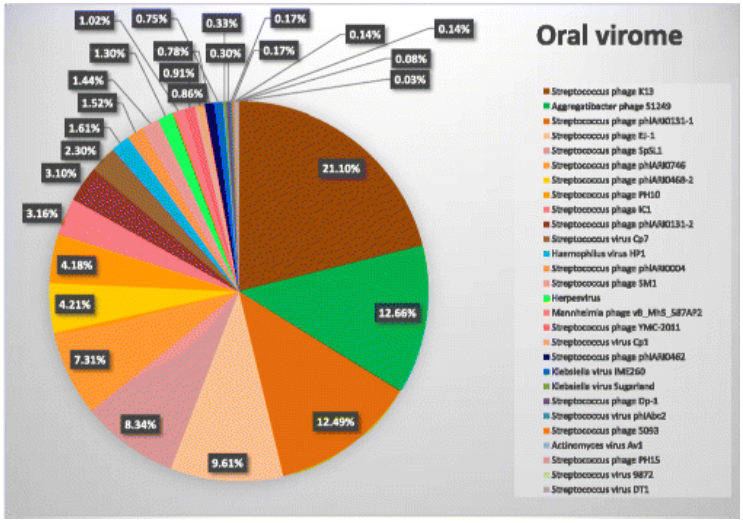

Figure 4.19: Whole virome composition in the oral cavity. Results are expressed as the percentage of counts of each species on the total counts of the whole oral virome in all sampled sites (Caselli *et al.*, 2020b).

of age and general health conditions, which makes oral colonization by protozoa above the detection threshold (10 copies per sample) unlikely.

Interestingly, the viral component of HOM (oral virome) represented the 0.03% of total microbes and was observed in particular in mucosal sites and supragingival plaque (Figure 4.19). Eukaryotic viruses represented 0.0006% of the total microbial counts and consisted exclusively of members of the *Herpesviridae* family. Instead, bacterial viruses were more prevalent, including bacteriophages of the *Siphoviridae*, *Myoviridae* and *Podoviridae* families. Streptococcal phages resulted the most prevalent viruses, in particular *Streptococcus* phage K13, *Aggregatibacter* phage S1249, and many other *Streptococcus* viruses. In addition, phages directed against *Haemophilus, Mannheimia*, *Klebsiella*, and *Actinomyces* were also observed (Caselli *et al.*, 2020b).

Interestingly, comparing the oral virome profile of oral rinses with that of saliva (without rinsing) and of the other sampled sites (Figure 4.20), the virome of oral rinse sites emerged as representative of the whole virome obtained by sampling specific oral niches (soft and hard tissues), at least for the most prevalent species/genera. By contrast, saliva specimens were less informative about whole virome present in the oral cavity, displaying very different abundance values of the most prevalent viral species (Caselli *et al.*, 2020b).

The HOM diversity was investigated by measuring its Alpha (Shannon H' diversity index) and Beta diversity (Weighted UniFrac index) in the different sampled sites and subjects. As expected, results showed the presence of inter-individual differences between subjects (Figure 4.21A), with Alpha diversity values ranging from 3.22 to 5.37. However, Anova test did not evidenced statistically significant differ-

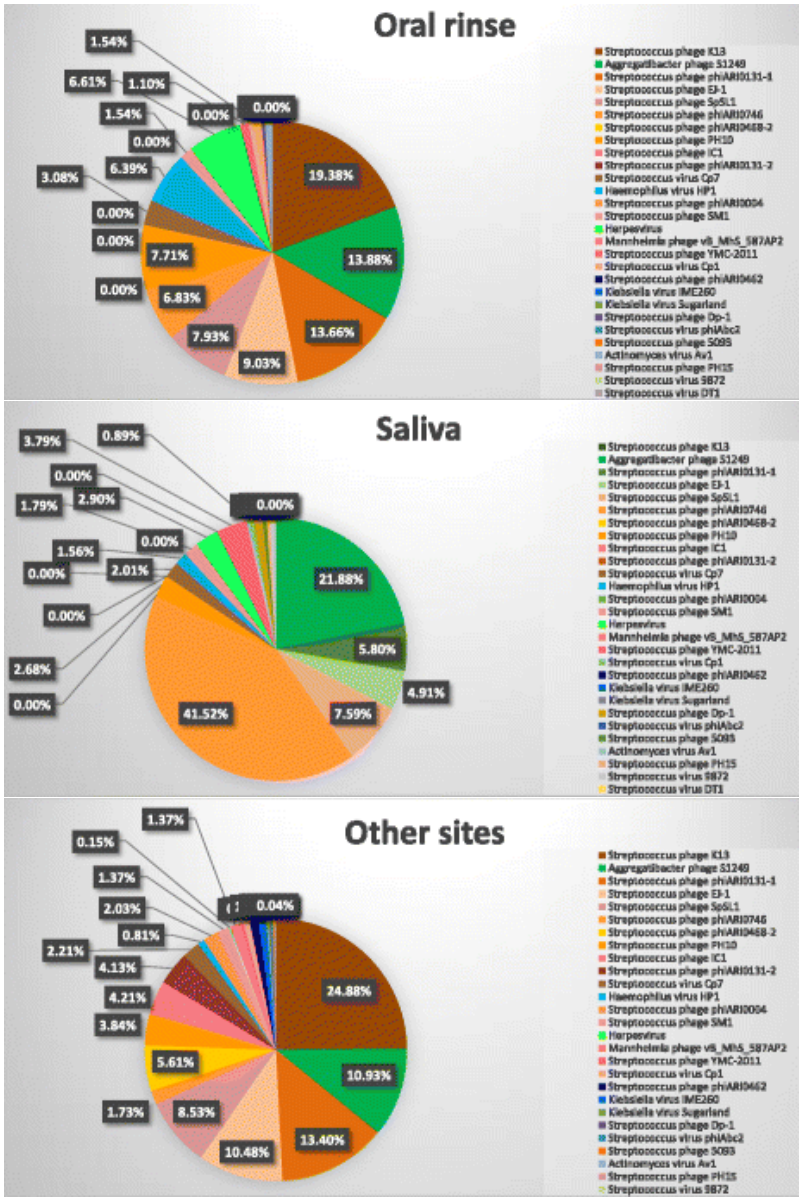

Figure 4.20: Comparison between virome profile observed in saliva, oral rinse and other sampled sites (hard and soft tissues). Results are expressed as the percentage of counts of each species on the total counts of the whole oral virome in all sampled sites (Caselli *et al.*, 2020b).

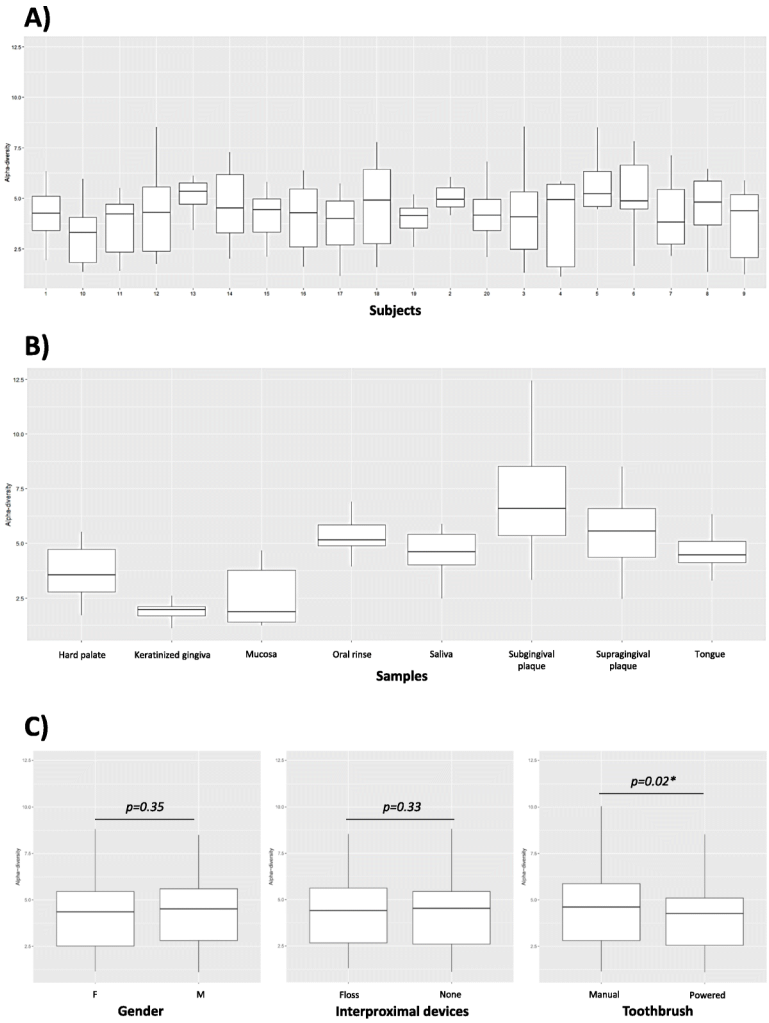

Figure 4.21: Alpha diversity analysis of collected samples, in each enrolled subject (4.21A), specific sampling site (4.21B), and sub-group of participants based on gender, interproximal devices and type of toothbrush used (4.21C) (Caselli *et al.*, 2020b).

ences among subjects ($p = 0.876$), indicating the presence of common characteristics in the HOM profile of healthy subjects. By contrast, the Alpha diversity was significantly different in the different sampled sites (Figure 4.21B). In particular, six different clusters were recognizable ($p < 0.0001$): subgingival plaque (alpha value = 7.03), supragingival plaque and oral rinse (4.56 and 5.29 respectively), tongue and saliva (4.62 and 4.56 respectively), hard palate (3.69), mucosa (2.46), and keratinized gingiva (1.89). Furthermore (Figure 4.21C), the Alpha diversity was not significantly different in participants sub-groups subdivided for gender and type of OHD applied (interproximal devices used, manual or powered tootbrush), except for the comparison between subjects that used manual or powered tootbrush, that clustered in different classes ($p = 0.02$) (Caselli *et al.*, 2020b).

Similarly, Beta diversity analysis evidenced distinguishable patterns corresponding to the different oral micro-habitats, further confirming the significant differences observed for specific sampling sites (Figure 4.22). High similarities were observed

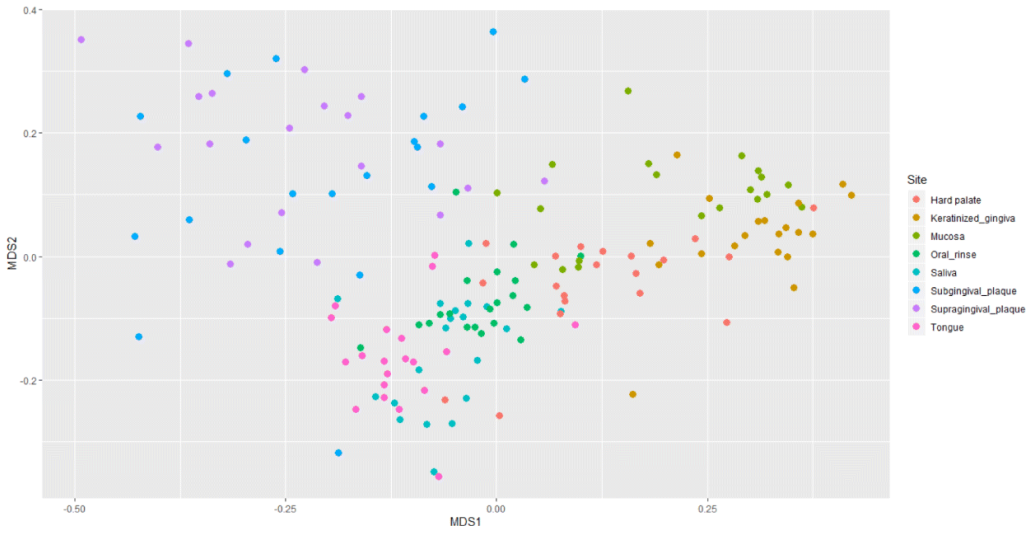

Figure 4.22: Beta diversity in the different site-specific samples from all the study subjects, evaluated by Weighted UniFrac analysis. Multidimensional scaling (MDS) analysis of Weighted UniFrac similarity index are reported (Caselli *et al.*, 2020b).

between supragingival and subgingival plaque, between the oral mucosa and keratinized gingiva, between saliva and oral rinse, and between tongue surface and saliva (Caselli *et al.*, 2020b).

Based on the differences evidenced among the different oral niches, we performed a comparative analysis of microbial genera distribution and relative abundance between saliva, oral rinse, and the other sampled sites, to understand if a unique sample of saliva may be considered representative of the whole oral microbiome. As depicted in Figure 4.23A, similar yet clearly different microbiomes were evidenced in saliva and oral rinse. The most prevalent HOM genera (including *Streptococcus*, *Candidatus*, *Cutibacterium*, *Gemella*, *Pseudomonas*, *Actinomyces*, *Pseudopropionibacterium*, *Aggregatibacter*, *Corynebacterium*, *Staphylococcus*, *Veillonella*, *Parvimonas*, and *Micrococcus* were more represented in oral rinse samples, compared to saliva without rinsing. This was even more evident for less represented genera/species, such as *Candida* spp., and the oral virome.

Taking together (Figure 4.23B), these data confirmed that oral rinse is more representative of the whole HOM compared to saliva (Caselli *et al.*, 2020b).

Due to the importance of AMR spread in the population, and based on the results on oral rinse, the resistome of the healthy HOM was analyzed in those samples, by specific qPCR microarray previously described. The results are represented in Figure 4.24 and showed that HOM harbored several R genes, whose most prevalent were *mefA* (macrolide efflux protein A), *ermB* (macrolide lincosamide streptogramin B, and *tetB* (tetracycline efflux pump), with respectively Log_{10} fold change values of 5.43, 4.60, and 4.19, compared to negative controls (Figure 4.24A). Interestingly, *mefA* and *ermB* were observed to be prevalent in *viridans* group streptococci (VGS),

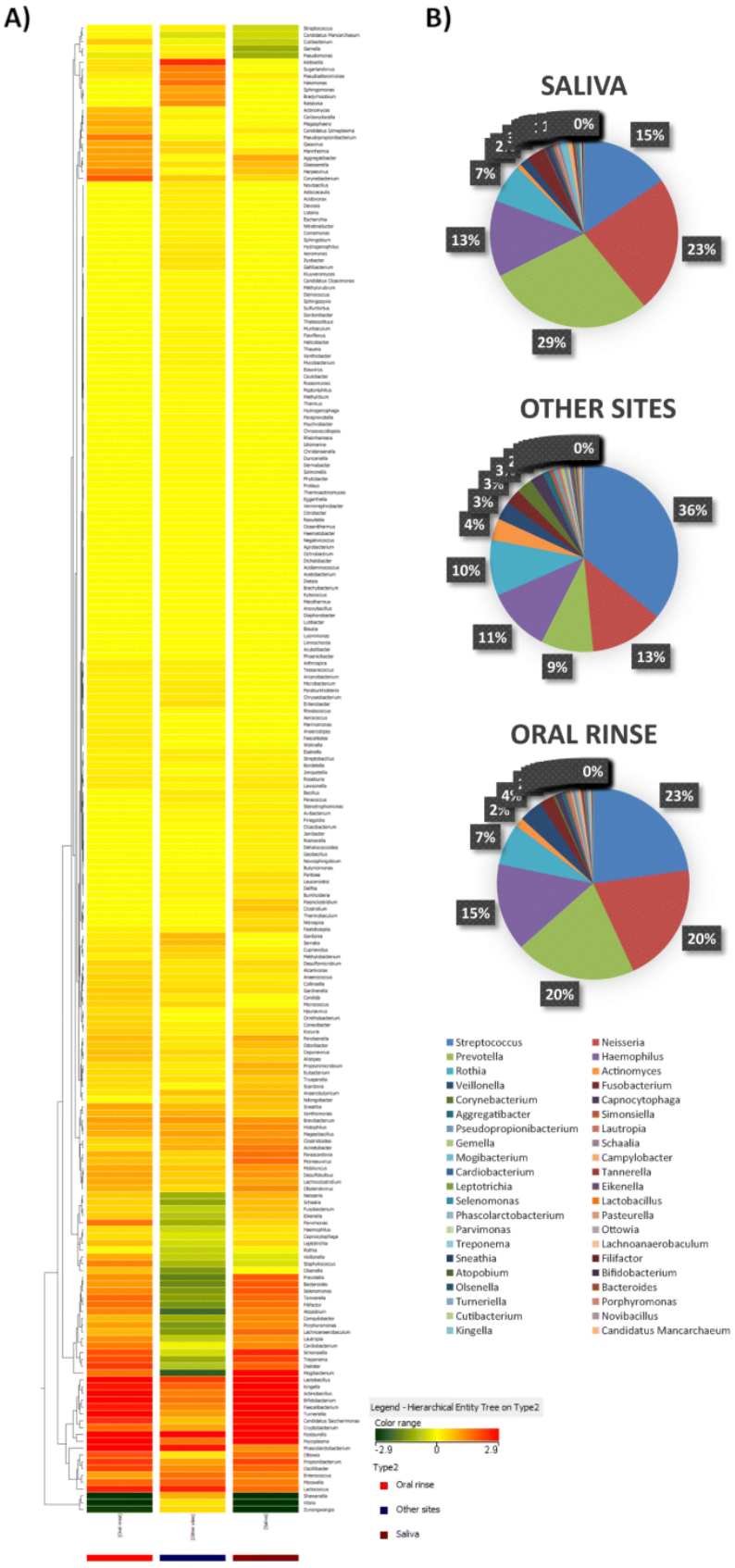

Figure 4.23: Distribution and relative abundance of microbial genera in saliva, oral rinse and other sites. Comparison of mean values detected in saliva, oral rinse, and other sites (hard and soft tissues). Hierarchical legend is also shown (4.23A). Comparison of mean relative abundance values detected in saliva, oral rinse and other sites (hard and soft tissues) (4.23B) (Caselli *et al.*, 2020b).

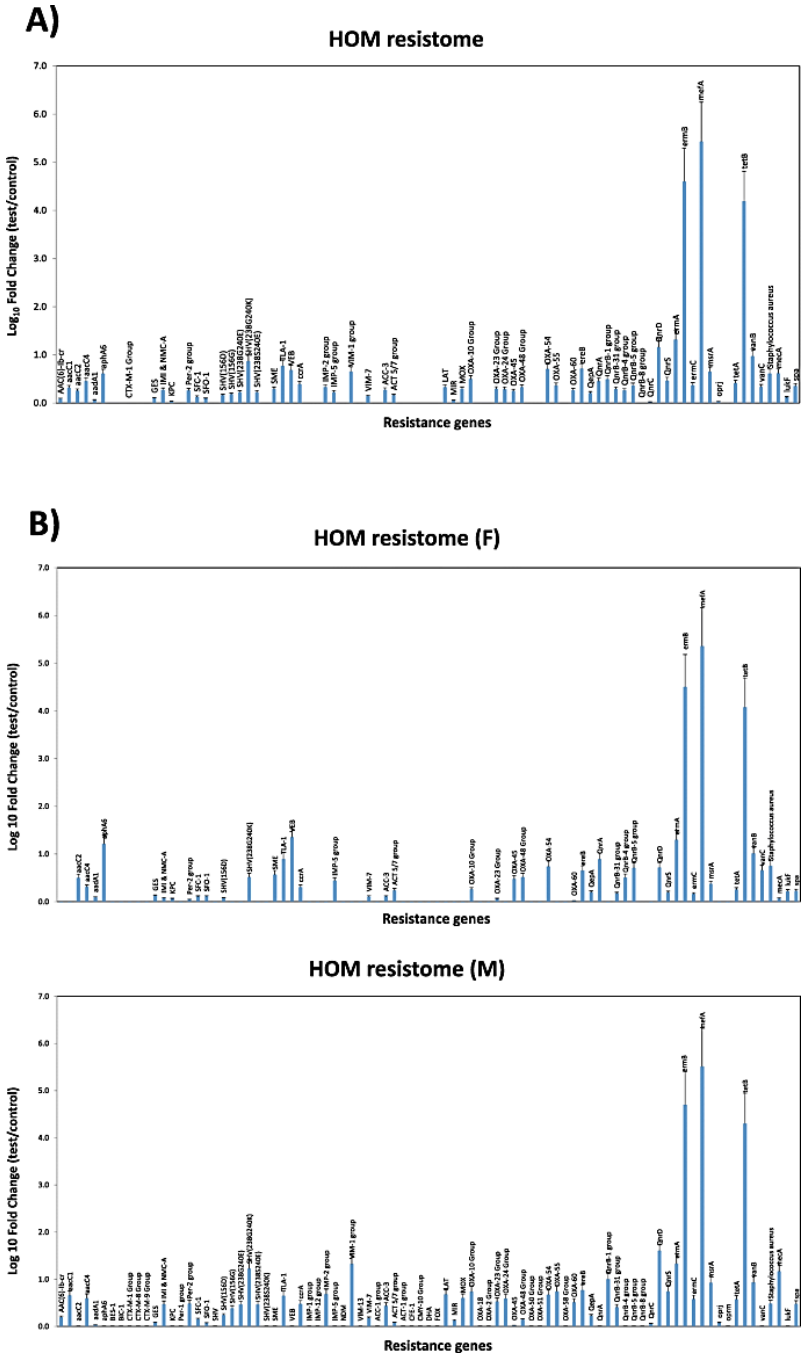

Figure 4.24: Characterization of HOM resistome, analyzed by specific qPCR microarray. Resistome of the whole study population (4.24A), resistome compared between female (F) and male (M) subjects (4.24B). Results are expressed as the mean value ± SD of Log_{10} fold change of oral rinse samples compared to negative controls, for each target resistance gene (Caselli *et al.*, 2020b).

alone or in combination (Zolezzi *et al.*, 2004). The resistance is mediated via an efflux pump able to regulate intracellular macrolide levels. Tetracycline resistance genes, including *tetB*, which coded for an active efflux pump, had been prevalently found in Gram-negative bacteria, however a recent study detected the presence of *tetB* in clinical isolates of *Streptococcus oralis* strains (Arredondo *et al.*, 2019). The presence of *tetB* in Gram-positive bacteria highlights the potential role of *Streptococcus* genus in becoming a reservoir and promoting the dissemination of antibiotic resistance genes in an environment so favorable to horizontal gene transfer as the oral biofilm. In addition, other R genes were detected, although to a less extent, including *ermA* and *SHV*-group genes. The first confers the resistance against macrolides like *ermB* gene, and it is frequently found in oral Gram-positive bacteria (Varaldo *et al.*, 2009). *SHV*-group resistance genes confer resistance against beta-lactams and were also observed in many Gram-positive bacteria, including streptococci. Of note, *QnrD* gene (quinolone-resistance determinant D), a plasmid-mediated fluoroquinolone resistance typically present in Gram-negative bacteria, was also present in the HOM resistome. Interestingly, the HOM resistome of female and male participants (Figure 4.24B) showed non-significant differences in the most prevalent R genes (*ermB, mefA, tetB*, and significant differences in less prevalent genes, including *aac* genes, *SHV*-group, *VIM-1* group, *Qnr* group, *msrA*, *vanC* and *mecA*. Overall, the female gender harbored higher levels of *aac2* and *vanC genes*, compared to the male resistome, whereas *aac1, SHV*-group, *VIM-1* group, *Qnr*-group, *msrA* and *mecA* resulted more prevalent in males than in females subjects (Caselli *et al.*, 2020b).

4.2.2 The oral microbiome in COVID-19 patients

Based on the results on HOM characterization, a cross-sectional observational study was performed, to define the oral microbiome of COVID-19 patients, in order to evidence any association between its profile, the local immune and inflammatory response and clinical disease. Study participants were recruited among the hospitalized patients of the University Hospital of Ferrara, in the COVID-19 and Infectious diseases ward (non-COVID-19 group), after approval by the Ethics Committee Area Vasta Emilia Centro della Regione Emilia-Romagna (CE-AVEC: no. 408/2020/Oss/UniFe, approved on April 21, 2020). Participant subjects were recruited over a four-month period between April and July 2020. Each enrolled subject was included in the study after signing informed consent. Enrollment and collection of clinical and epidemiological information were conducted by clinicians of the two wards included in the study. Inclusion and exclusion criteria were listed in Table 4.4. COVID-19 patients recruited in the study were clinically classified according

Table 4.4: List of inclusion and exclusion criteria for subject's enrollment in the study (Soffritti *et al.*, 2021).

INCLUSION CRITERIA	*EXCLUSION CRITERIA*
• *Male and female subjects* • *Age > 18 years* • *Signed Informed Consent Form* • *Molecular diagnosis of SARS-CoV-2 infection (for COVID-19 group)*	• Pregnant or lactating women • Uncooperative patient (inability to perform oral rinse to collect samples) • Lack of signed Informed Consent Form

to symptoms severity in four categories: 1) Asymptomatic, without symptoms covid-related, hospitalized for a different pathology; 2) Paucisymptomatic, presenting aspecific flu-like symptoms; 3) Symptomatic, with specific respiratory symptoms; 4) Severely symptomatic, subjects needing ventilation. For the control group, subjects hospitalized with non-respiratory diseases and tested SARS-CoV-2 negative, were recruited. Overall, 75 subjects, including 39 COVID-19 patients and 36 controls, were enrolled in the study (Soffritti *et al.*, 2021).

All the COVID-19 patients had confirmed SARS-CoV-2 positivity at hospital admission by nasopharyngeal swab analysis performed by the Hospital microbiology laboratory. COVID-19 subjects included 20 males (51.3%) and 19 females (48.7%), with a mean age of 71.1 ± 18.4 years (range 25–99). Oral rinses were collected from COVID-19 patients after 0-43 days since their first positive SARS-CoV-2 test. At the time of sample collection, 28.2% (11/39) of patients were asymptomatic, 17.9% (7/39) were paucisymptomatic, 55.3% (21/39) were symptomatic, including 2 of them (5.1%, 2/39) presenting severe symptomatology and requiring assisted ventilation. All enrolled COVID-19 patients were treated with hydroxychloroquine and azithromycin at the time of hospitalization (Gautret *et al.*, 2020). Control group consisted of patients tested SARS-CoV-2 negative and admitted for non-respiratory disease, the group included 61% (22/36) males and 39% (14/36) females, with a mean age of 66.5 ± 18.8 years (range 20–94). The main characteristics of the study population, focused on demographic and clinical features were reported in Table 4.5. No statistical differences were evidenced between COVID-19 group and control group, with regard to both age (Kruskal–Wallis test; $p = 0.27$) and gender (Chi-square test; $\chi^2 = 0.734$, $p = 0.39$) (Soffritti *et al.*, 2021).

Oral rinse samples were collected as described for HOM and analyzed for SARS-CoV-2 presence, oral microbiome characterization and local inflammatory and secretory immune responses.

The SARS-CoV-2 genome amount in the oral cavity was analyzed by ddPCR, a very sensitive quantitative method, contrarily to the routinely used diagnostic qualitative assays (Falzone *et al.*, 2020; Suo *et al.*, 2020). The results confirmed the

Table 4.5: Demographic and clinical features of COVID-19 group and control study participants (Soffritti *et al.*, 2021).
(*) Symptom score was: 1, asymptomatic; 2, paucisymptomatic; 3, symptomatic; 4, severely symptomatic. NPS, nasopharyngeal swab. Age and gender distribution significance were assessed, respectively, by Kruskal–Wallis and Chi-square tests.

Subject n°	Control group		COVID-19 group				Age/gender distribution
	Gender	Age	Gender	Age	Days after NPS	COVID-19 symptoms (*)	
1	F	74	M	76	13	3	***Age:***
2	M	72	F	72	4	3	**CTR:** 66.5 ± 18.8 years
3	M	73	F	56	0	1	**COVID-19:** 71.1 ± 18.4 years
4	F	86	M	49	3	1	**CTR vs. COVID-19:** $p = 0.27$, n.s.
5	F	38	F	49	6	2	
6	F	66	M	99	6	2	
7	F	67	F	80	18	4	
8	F	40	F	73	16	3	
9	M	53	F	68	2	1	
10	M	42	F	33	18	2	
11	M	75	F	51	2	2	
12	F	86	M	76	4	3	
13	M	60	M	82	8	3	
14	M	59	F	87	29	1	
15	M	83	M	47	6	1	
16	M	86	F	91	18	3	
17	F	86	M	89	5	3	
18	M	71	F	94	16	3	***Gender:***
19	M	88	M	94	20	2	**CTR:** 22/36 males (61%)
20	M	84	M	80	15	3	**COVID-19:** 20/39 males (51.3%)
21	F	88	M	85	18	3	**CTR vs. COVID-19:** $p = 0.39$, n.s.
22	F	86	F	83	7	3	
23	M	20	M	25	10	3	
24	F	94	F	78	18	4	
25	F	46	F	83	49	3	
26	F	76	F	45	17	3	
27	M	50	M	82	1	1	
29	M	51	F	82	2	1	
30	M	53	M	59	0	2	
31	M	45	M	45	43	1	
32	M	70	M	57	5	3	
33	F	85	F	86	4	3	
34	M	49	F	48	3	2	
35	M	67	M	90	11	3	
36	M	49	F	70	0	1	
37	M	76	M	81	51	1	
38	–	–	M	87	23	3	
39	–	–	M	78	11	1	
	–	–	M	63	18	3	

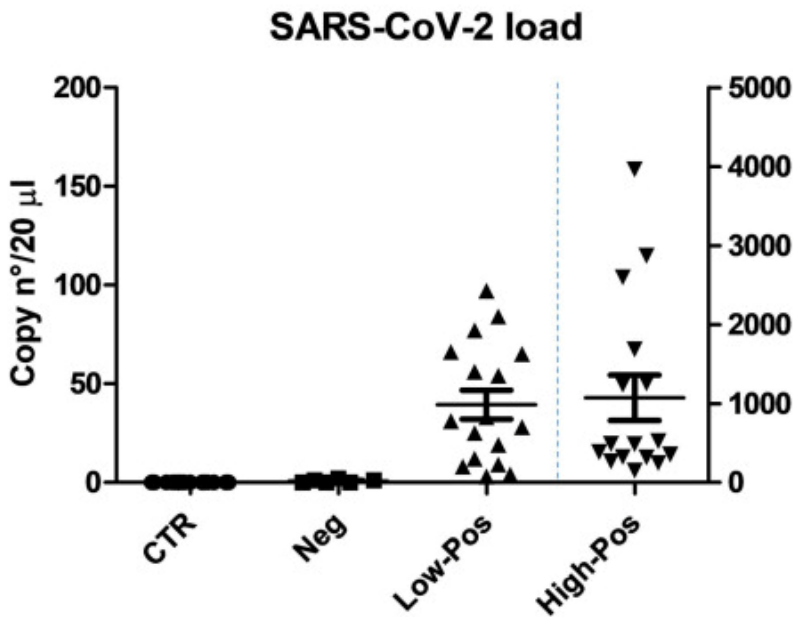

Figure 4.25: SARS-CoV-2 virus load quantified by ddPCR, expressed as genome copy number/20μl of analyzed sample. Left y axis refers to control, negative and low-positive values, whereas right y axis refers to high-positive COVID-19 subjects. Mean value ± SEM is also reported (Soffritti *et al.*, 2021).

absence of SARS-CoV-2 in the control group, whereas COVID-19 group included both positive and negative oral samples (Figure 4.25). In particular, 41% (16/39) of subjects harbored a high SARS-CoV-2 virus load (>101 genome copies in 20 μl of the amplified sample), 43.6% (17/39) harbored lower but detectable amounts of virus (3-100 copies), and 15.4% (6/39 patients) did not present any detectable virus in the oral cavity (<3 copies in 20 μl). Notably, the virus load detected in the oral cavity correlated with symptom severity (Spearman $r = 0.774$; 95% CI 0.608–0.875) ($p < 0.0001$), identifying specific subgroups of COVID-19 patients (Soffritti *et al.*, 2021).

Alpha diversity analysis of COVID-19 oral microbiome evidenced a lower Shannon H' diversity index in COVID-19 group compared to controls ($p = 0.01$) (Figure 4.26A), with higher difference between symptomatic COVID-19 and control subjects (Figure 4.26B). Of note, Alpha diversity values inversely correlated with patient's symptom severity (Spearman $r = -0.431$, 95% CI $-0.666/-0.120$, $p = 0.006$). Alpha diversity was lower in male group compared to female group, which paralleled symptoms severity (Figure 4.26C) (Soffritti *et al.*, 2021).

The microbiome profile appeared clearly altered in COVID-19 compared to control group (Figure 4.27), with several bacteria resulting more abundant in COVID-19 than in controls, including *Streptococcus*, *Veillonella*, *Prevotella*, *Lactobacillus*, *Capnocytophaga*, *Porphyromonas*, *Abiotrophia*, *Aggregatibacter*, and *Atopobium*. On the other side, *Rothia, Haemophilus, Parvimonas, Fusobacterium*, and *Gemella* resulted

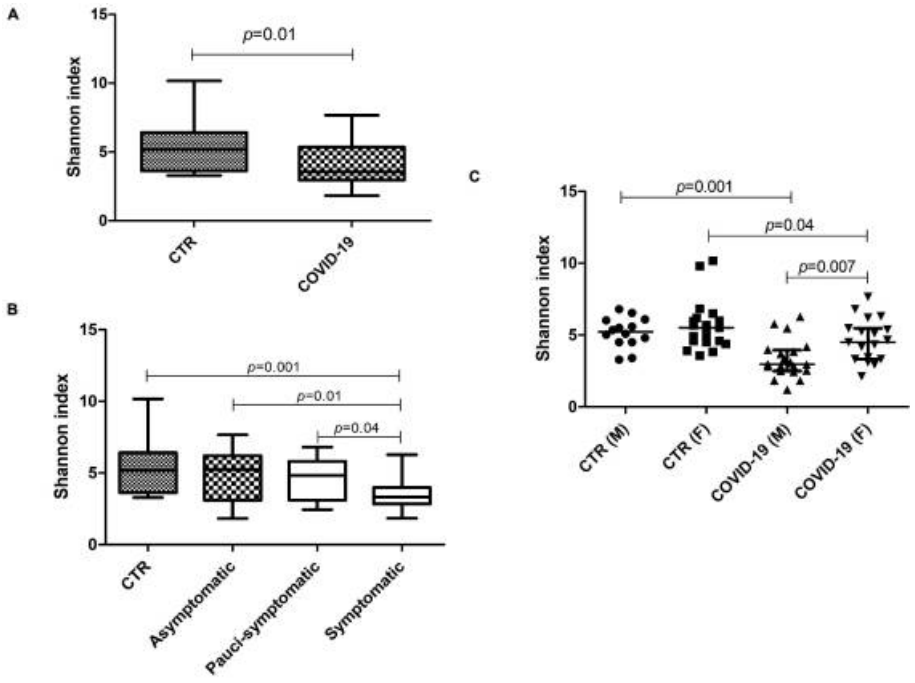

Figure 4.26: Alpha-diversity values in the oral microbiomes of control and COVID-19 subjects. Comparison between control and COVID-19 patients (4.26A), comparison between controls and COVID-19 asymptomatic, pauci-symptomatic, and symptomatic subjects (4.26B), comparison between genders (M, male; F, female) in the control and COVID-19 groups (4.26C). Median values with interquartile range are shown for each test (Soffritti *et al.*, 2021).

less abundant in COVID-19 than controls (Figure 4.27A). Interestingly, *Enterococcus* and *Enterobacter* genera were exclusively observed in COVID-19 patients, and not detectable in control subjects. Analyzing the microbiome composition at the species level, COVID-19 group evidenced a decreased abundance of *Haemophilus parainfluenzae* and *parahaemolyticus*, *Gemella morbillorum* and *sanguinis*, *Parvimonas micra*, and *Neisseria subflava*. By contrast, *Neisseria mucosa*, *Veillonella parvula*, *Lactobacillus fermentum*, *Enterococcus faecalis*, *Atopobium parvulum*, *Acinetobacter baumannii* resulted increased, in comparison to controls. Interestingly, periodontopathogens species were significantly more represented in COVID-19 group, namely *Prevotella melaninogenica*, *jejuni*, *denticola*, and *oris*; *Eikenella corrodens*; *Capnocytophaga sputigena* and *gingivalis*; and *Aggregatibacter aphrophilus* (Soffritti *et al.*, 2021).

High differences between COVID-19 and control groups were observed also for the fungal and viral components of the oral microbiome. In contrast to the decrease abundance of the bacterial component observed in COVID-19 patients, the abundance and species richness of fungal component resulted increased in COVID-19 microbiome compared to controls (Figures 4.28a and 4.28b). *Candida* and *Saccharomyces* spp. were the mainly components of the oral mycobiome in the control group (47% and 52% of relative abundance, respectively). On the other hand,

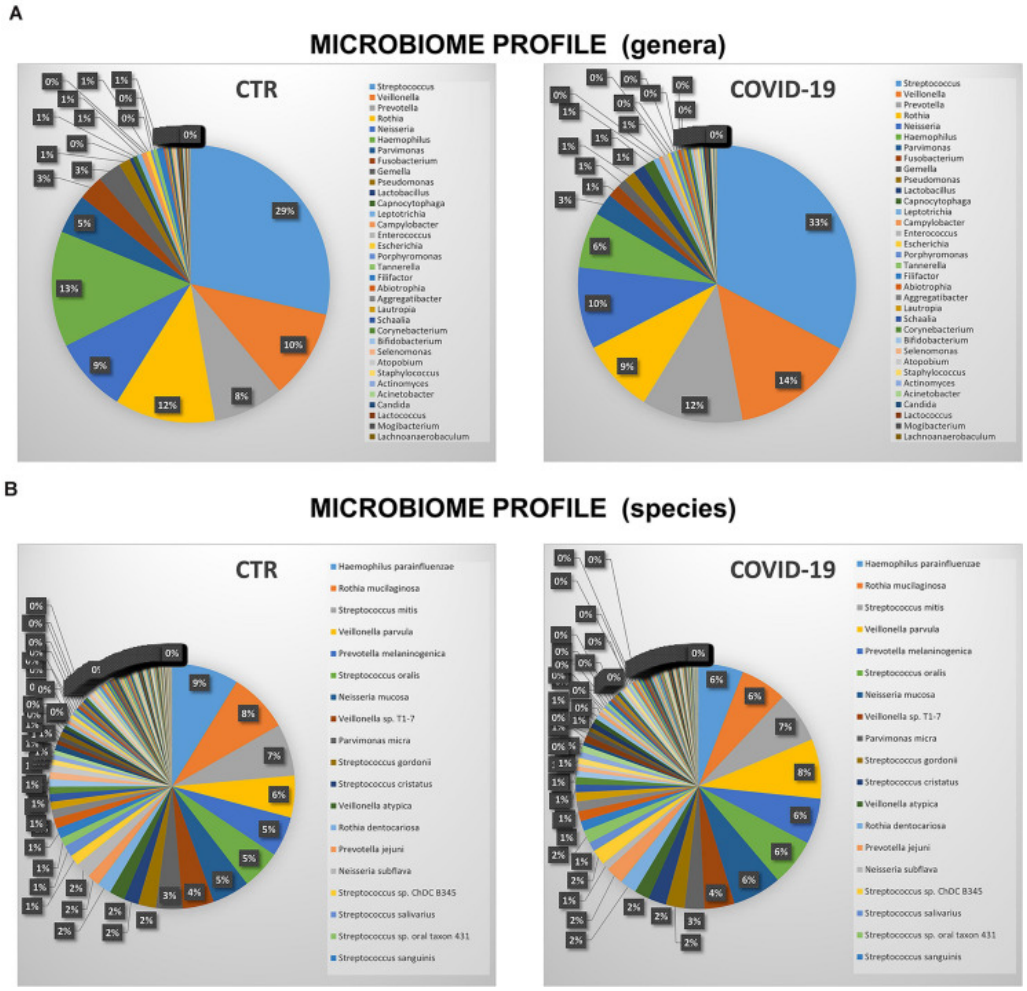

Figure 4.27: Microbiome profile in COVID-19 group and control group. Relative abundance and distribution of detected microbial genera (4.27A). Relative abundance and distribution of detected microbial species (4.27B) (Soffritti *et al.*, 2021).

COVID-19 mycobiome resulted characterized, in addition to *Candida* and *Saccharomyces* spp., by *Aspergillus, Nakaseomyces*, and *Malassezia* spp., although with low relative abundance (4%, 3%, and <1%, respectively). Fungal species identified by WGS analysis were *Candida albicans*, *Saccharomyces cerevisiae*, *Aspergillus fumigatus*, and *Malassezia restricta* species.

Last, also the viral component resulted more abundant in COVID-19 patients compared to controls, increasing from 0.07% of relative abundance in controls to 0.12% in COVID-19 patients (Figure 4.29). *Lymphocryptovirus* and *Simplexvirus* genera of the *Herpesviridae* family were detected both in COVID-19 and control subjects, but Epstein Barr virus (EBV) resulted reactivated in 28.2% (11/39) of COVID-19 patients and in only 5.6% (2/36) of controls. Herpes simplex virus type 1 (HSV-1) and four bacteriophages directed against *Staphylococcus* (Staphylococcus phage ROSA), *Streptococcus* (Streptococcus phage EJ-1 and phage PH10), and *Lactobacillus* (Lactobacillus phage phiadh), resulted also increased in COVID-

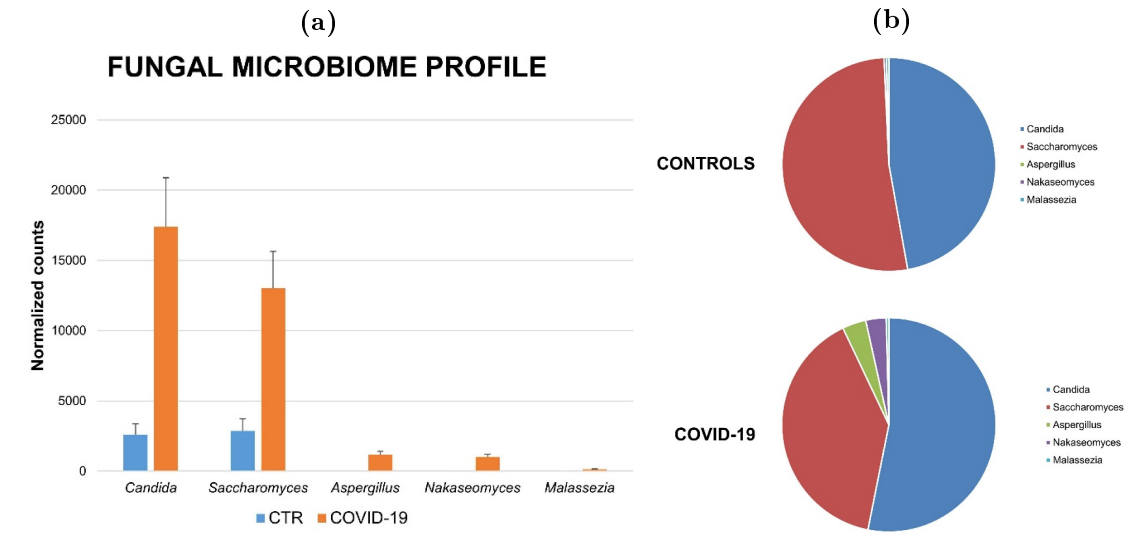

Figure 4.28: Mycobiome profile characterizing the oral cavity of controls and COVID-19 patients. Abundance of fungi expressed as total normalized counts for each individually detected mycetes (4.28a). Percentage distribution of the fungal genera in controls and COVID-19 patients (4.28b) (Soffritti *et al.*, 2021).

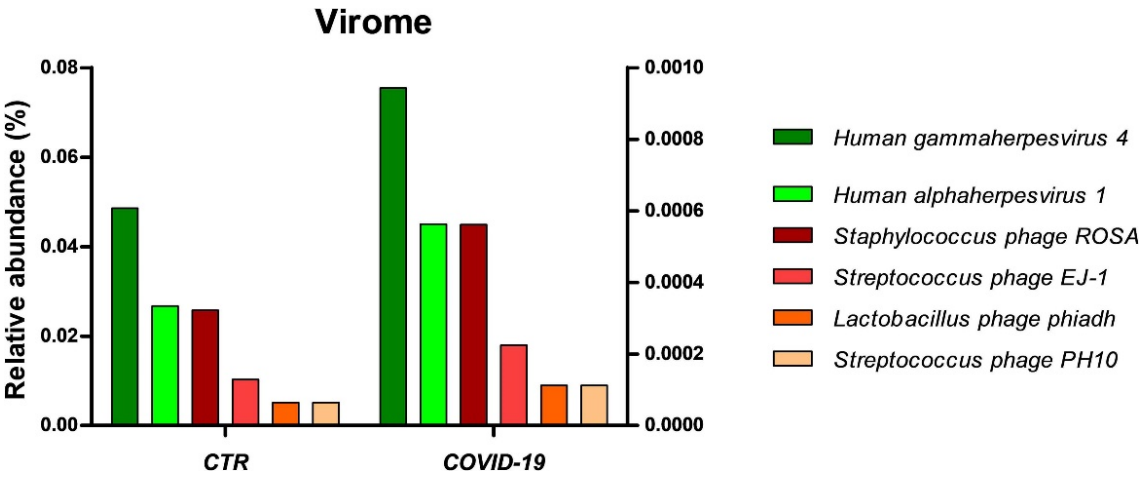

Figure 4.29: Oral virome of controls and COVID-19 patients, expressed as relative abundance (%) (Soffritti *et al.*, 2021).

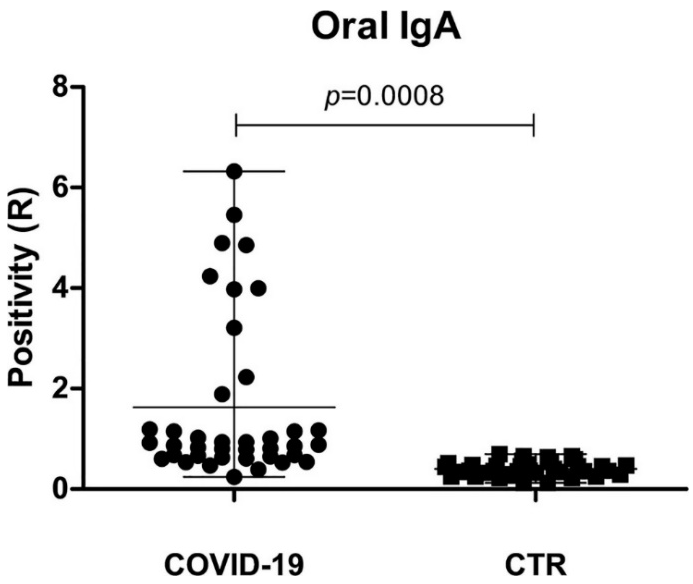

Figure 4.30: Secretory IgA levels in the oral cavity of COVID-19 patients and control group (CTR). The positivity is expressed as ratio R: samples values/value of control calibrator, provided in the essay. Mean values with range are also reported (Soffritti *et al.*, 2021).

19 patients compared to controls (Soffritti *et al.*, 2021).

The local inflammatory and mucosal immune response toward SARS-CoV-2 were also assessed in parallel.

Oral secretory IgA (sIgA) levels in oral rinse samples, analyzed by a specific CE-IVD ELISA assay (Euroimmun, Lubeck, Germany), evidenced the presence of oral sIgA in most COVID-19 patients (25/39, 64.1%), whereas no controls resulted positive for IgA presence ($p = 0.0008$) (Figure 4.30). High levels of sIgA (R $>$ 2.0) were detected in 25.6% (10/39) of COVID-19 patients, whereas 38.5% (15/39) had intermediate sIgA levels (range of R 0.8-2.0), and 35.9% (14/39) showed low (R $\sim$ 0.8 threshold value) or no presence of sIgA. Interestingly, the level of sIgA correlated inversely with symptom severity (Spearman $r - 0.355$; 95% CI $-$ 0.600 to 0.047; $p = 0.02$) (Soffritti *et al.*, 2021).

The oral samples were also analyzed for the presence of inflammatory cytokines included in the so-called "cytokine storm", considered a typical feature of COVID-19 severe disease. To this aim, the presence of IL-6, IL-17, TNFα, and GM-CSF was assessed in the collected oral samples by specific ELISA assays (Thermo Fisher Scientific, Life-Technologies, Milan, Italy). The results showed that IL-6 and IL-17 were significantly higher in COVID-19 patients compared to controls (p= 0.005 and p= 0.02, respectively) (Figure 4.31). Similarly, also TNFα and GM-CSF were increased in COVID-19 group, although the differences compared to control group were not statistically significant. However, the difference became statistically significant when comparing symptomatic COVID-19 subgroup with controls (TNFα p =

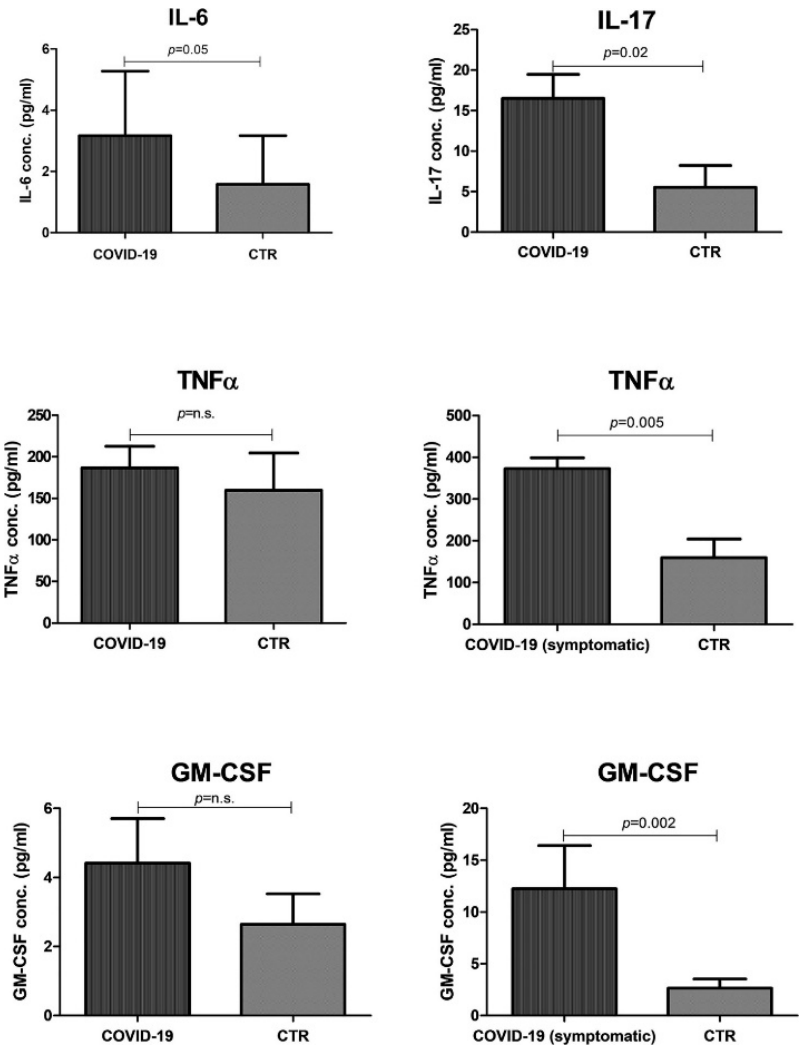

Figure 4.31: Levels of pro-inflammatory cytokines/chemokines (IL-6, IL-17, TNFα, GM-CSF) in the oral cavity of COVID-19 patients and controls (CTR). Comparisons between COVID-19 symptomatic group and controls are also reported for TNFα and GM-CSF. The results are expressed as the mean values ± SEM of the concentration (pg/ml) for each indicated cytokine (Soffritti *et al.*, 2021).

0.005; GM-CSF $p = 0.002$). Of note, inflammation correlated with COVID-19 severity and with oral microbiome dysbiosis, characterized by Alpha diversity ($p < 0.01$) (Soffritti *et al.*, 2021).

4.2.3 The vaginal microbiome in HPV persistence in CIN2/CIN3 women

A total of 85 women affected by CIN2/CIN3 cervical lesions, attending the Center of Preventive Gynecology of the University Hospital of Ferrara and candidate for surgical removal of lesions by LEEP, were recruited in the study (based on the inclusion/exclusion criteria reported in Table 4.6, with the aim to assess the role of vaginal microbiome in hrHPV persistence/clearance after surgical CIN2/CIN3

Table 4.6: List of inclusion and exclusion criteria for subject's enrollment in the study (Caselli *et al.*, 2020a).

INCLUSION CRITERIA	*EXCLUSION CRITERIA*
• *Female subjects, age 30-50 years*	• Pregnant women
• *CIN2/CIN3 diagnosis*	• Innate or acquired immunodeficiency
• *Candidate for LEEP*	• Concomitant neoplastic diseases
• *Availability at 6- month follow-up*	• Chronic inflammatory diseases/infections including aerobic vaginitis and other sexually transmitted diseases
• *Signed Informed Consent Form*	• Corticosteroid therapy
	• Immune therapy
	• Unavailability at 6-month follow-up
	• Lack of signed Informed Consent Form

removal.

The CIN classification was performed by the unique central Pathological Anatomy laboratory of the University Hospital of Ferrara, based on the protocol defined for the CC prevention of Emilia Romagna region (Soloman, 1989; Darragh *et al.*, 2013; GISCI, 2018). Women were asked to participate in the study during the first visit, after histological diagnosis of CIN2 or CIN3 lesion and planning of surgical excision treatment. During this first visit inclusion and exclusion criteria were assessed, and an informed consent was provided to eligible women. In addition, subjects were asked to complete an anamnestic survey, which was aimed to collect data of several risks factors potentially associated to CIN development (age, smoking habits, education level, ethnicity, nationality, use of contraceptives, weight, height, and BMI index).

The epidemiological data of the enrolled cohort of patients are summarized in Table 4.7. Patients with diagnosis of CIN2 and CIN3 lesions represented 48.2% (41/85) and 51.8% (44/85) of the whole population, respectively. As expected, the majority of enrolled patients (72/85, 84.7%) were positive for hrHPV, as determined by routine testing. The 13 hrHPV-negative subjects were excluded from the study. The entire follow-up was completed by 66 patients (66/72, 91.7%).

Two samplings were performed from each patient, the first one at the enrollment before surgical excision (basal) and the second during the follow-up control (after 6 months from treatment). From each subject, one cervico-vaginal specimen and one vaginal washing were collected and used for microbiome analysis and cytokine profile, respectively (Caselli *et al.*, 2020a).

The vaginal microbiome profile was analyzed by a specific qPCR microarray detecting and quantifying simultaneously 90 species usually present in the lower female genital tract, including bacteria, mycetes and protozoa. Microbiome profiling at the

Table 4.7: Characteristics of the CIN2/CIN3 cohort of patients enrolled in the study (Caselli *et al.*, 2020a).
Abbreviations: BMI, Body Mass Index (kg/[m]^2); IUD, Intra Uterine Device; ASC-US, Atypical Squamous Cells of Undetermined Significance; ASC-H, Atypical Squamous Cells, cannot exclude high-grade lesion; L-SIL, Low-grade Squamous Intraepithelial Lesion; H-SIL, High-grade Squamous Intraepithelial Lesion; NTZ, Normal Transformation Zone; anTZ-G1, abnormal Transformation Zone Grade 1; anTZ-G2, abnormal Transformation Zone Grade 2.

Demographic features	**Study population (n, %)**
Total n.	85
Age (mean, range)	38 (30–50)
BMI (mean, range)	22.8 (16.5–34)
Smoke (n, %)	28 (32.9%)
Ethnicity (n, %)	
Caucasian	78 (94.8%)
African	1 (1.2%)
Asiatic	3 (3.5%)
Hispanic	3 (3.5%)
Deliveries (n, %)	
Nulliparous	35 (41.2%)
Parous	49 (57.6%)
Contraceptives (n, %)	31 (36.5%)
Oral	22 (25.9%)
Ring	6 (7.1%)
IUD	3 (3.5%)
Pap test (n, %)	
Negative cytology	5 (5.9%)
ASC-US	2 (2.4%)
ASC-H	14 (16.5%)
L-SIL	40 (47.1%)
H-SIL	24 (28.2%)
Colposcopy (n, %)	
NTZ	6 (7.1%)
anTZ-G1	43 (50.6%)
anTZ-G2	36 (42.3%)
Histology (n, %)	
CIN2	41 (48.2%)
CIN3	44 (51.8%)
hrHPV-positive (n, %)	72 (84.7%)

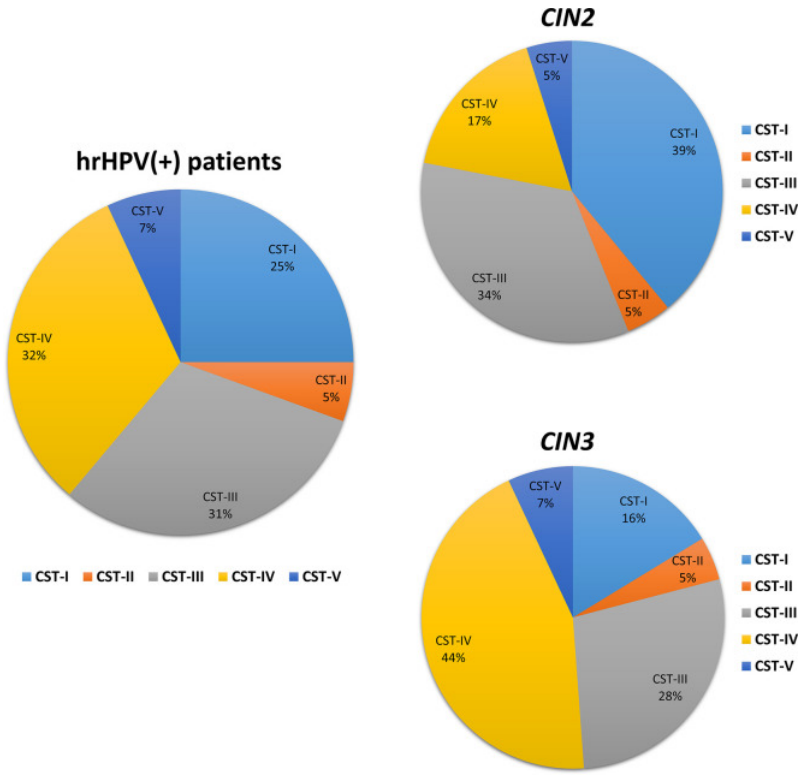

Figure 4.32: Prevalence of Community State Types (CSTs) in the whole group of hrHPV patients and in CIN2, CIN3 subgroups. Microbial profile was analyzed by specific qPCR microarray, able to simultaneously identify and quantify 90 microbial species of the vaginal tract. CSTs were defined according to the prevalence of Lactobacilli or other bacteria (Caselli *et al.*, 2020a).

basal level, prior to LEEP treatment, allowed the classification into the 5 recognized Community State Type (CST) groups (Figure 4.32), showing the prevalence of the CST-IV group (23/72, 32.0%), markedly characterized by vaginal microbiome dysbiosis. CST-III was detected in 22/72 (30.6%) subjects, CST-I in 18/72 (25%) women, and CST-II in 5.5% (4/72) women.

The CST distribution resulted significantly different between CIN2 and CIN3 subgroups, with CST-IV significantly increased in CIN3 group (44%), compared to CIN2 group (17%) ($p < 0.001$), and a concomitant decrease of CST-I in CIN3 vs CIN2 (16% vs 39%) (Caselli *et al.*, 2020a).

As to the prevalence and abundance of bacterial and mycotic species, the CST-I group showed *L. crispatus* dominance and high prevalence of other Lactobacilli, with detectable levels of *F. magna*, *G. vaginalis*, and *U. parvum* (5.84, 21.66, and 13.62-fold change compared to controls, respectively). The CST-II group showed *L. gasseri* dominance, detectable levels of *A. vaginae* and *C.albicans* (respectively 5.54 and 15.54-fold compared to controls), and the almost total absence of other Lactobacilli. The CST-III group showed *L. iners* dominance and other less prevalent Lactobacilli, high abundance of *U. parvum* (68.45-fold changes), and detectable

levels of *F. magna*, *G. vaginalis*, *Peptoniphilus asaccharolyticus*, *Prevotella bivia*, *Streptococcus agalactiae*, *anginosus*, *mitis*, *thermophilus* and *salivarius*. The CST-IV group presented low or no Lactobacilli, and high abundance of microorganisms associated with Bacterial Vaginosis (BV), including *Aerococcus christensenii* (13.01-fold), *Anaerococcus prevotii* (25.38-fold), *A. vaginae* (3428.12-fold), *Leptotrichia amnionii* (152.11-fold), *Mycoplasma hominis* (12.83-fold), *Parvimonas micra* (64.93-fold), *Peptoniphilus asaccharolyticus* (65.58-fold), *Porphyromonas asaccharolitica* (19.28-fold), *Prevotella bivia* (36.99-fold) and *buccalis* (60.64-fold), *Sneathia sanguinegens* (266.03-fold), and *U. parvum* (52.28-fold). Other bacterial species were detectable, although with lower amount, such as *F. magna, F. nucleatum, Mobiluncus curtisii*, *Mobiluncus mulieris*, and *Peptostreptococcus anaerobius* (all resulted about 5-fold change compared to controls). Finally, the CST-V group showed *L. jensenii* dominance, fair amounts of *L. crispatus*, *iners* and *vaginalis*, a high abundance of *Prevotella nigrescens*, and very low presence of species detected in other CSTs clusters. No protozoa were detected in any sample.

The comparison of microbial profile of CIN2 and CIN3 subgroups evidenced significant differences (Figure 4.33). The CIN2 microbiomes were still dominated by *Lactobacillus* spp., although several anaerobic Gram-negative BV-associated bacteria were highly present (particularly *A. vaginae*, *G. vaginalis*, and *U. parvum*), and other less abundant bacteria were detected (*C. albicans*, *F. magna*, *Peptoniphilus asaccharoliticus*, *Peptostreptococcus anaerobius*, *Prevotella bivia*, and *Streptococci* spp.). The CIN3 microbiomes resulted dominated by *G. vaginalis*, *L. iners* (the only *Lactobacillus* species that did not show an evident decrease compared to CIN2 group), *A. vaginae* and *U. parvum*. Other species observed and not frequently detectable in CIN2 patients included *Aerococcus christensenii*, *Anaerococcus prevotii*, *Leptotrichia amnionii*, *Mycoplasma hominis*, *Parvimonas micra*, *Peptoniphilus asaccharolyticus*, *Porphyromonas asaccharolitica*, *Prevotella bivia* and *buccalis*, and *Sneathia sanguinegens* (Caselli *et al.*, 2020a).

Overall, *L. iners* was the most frequently detected species in CIN2/CIN3 patients (76,7% of samples), followed by *G. vaginalis*, *F. magna*, *L. crispatus*, *P. asaccharolyticus*, *P. bivia*, *A. prevotii* and *U. parvum*, that were harboured in more than 50% of subjects (Figure 4.34). Moreover, the CIN3 subgroup was characterized by higher frequency of *G. vaginalis* and *A. vaginae*, compared to CIN2 subgroup (75% vs 63% and 52% vs 29%, respectively). Also, *F. nucleatum*, *P. micra*, *P. disiens/buccalis*, *L. amnionii*, *M. hominis*, *U. urealyticum*, and *C. trachomatis* were increased in CIN3 compared to the CIN2 group. On the contrary, Lactobacillus spp. other than *L.iners* were significantly decreased in CIN3 patients, compared to CIN2, in particular *L. crispatus* (47% vs 70%) and *L. jensenii* (27% vs 48%). In addition, also *F. magna*, *S. epidermidis*, *L. vaginalis*, *L. salivarius*, *P. acnes*, and

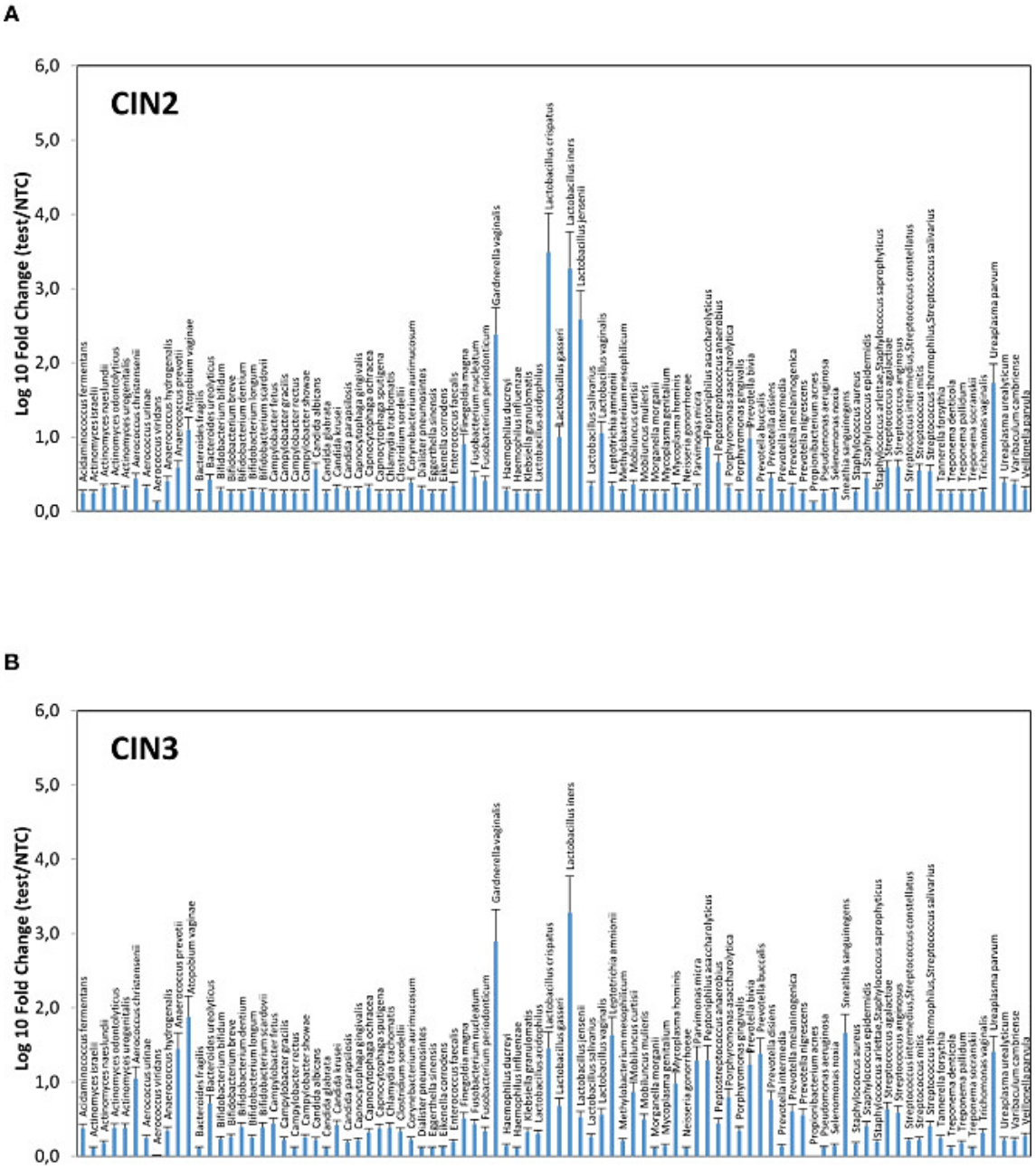

Figure 4.33: Microbiome profiles of the CIN2 (4.33A) and CIN3 (4.33B) subgroups of patients. Results are expressed as the mean value ± SD of Log_{10} fold change compared to negative controls (NTC, no template controls) (Caselli *et al.*, 2020a).

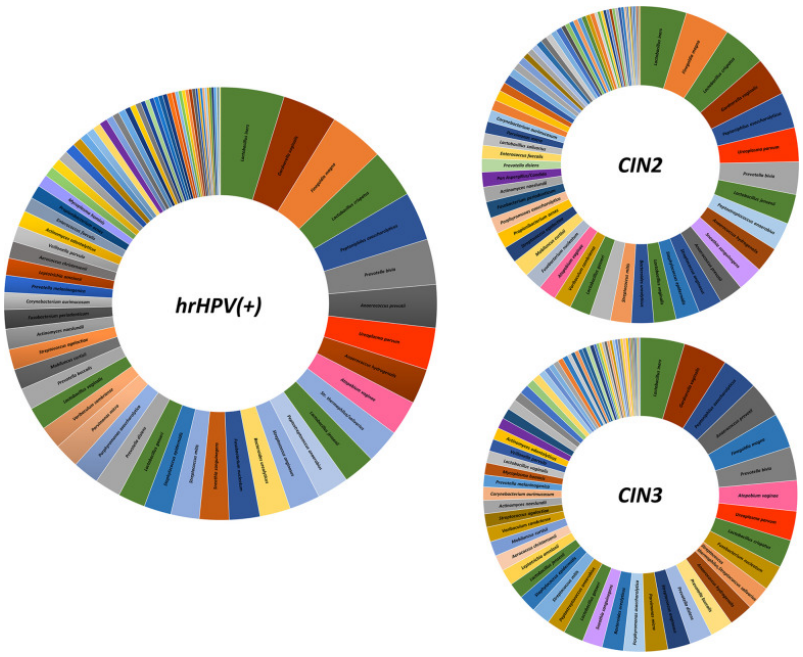

Figure 4.34: Frequency of detection of microbial species in the study population (whole patients hrHPV positive on the left, CIN2 and CIN3 subgroups on the right). Frequency of microbial species is expressed as percentages of detection based on qPCR microarray results (Caselli *et al.*, 2020a).

Candida krusei were less frequent in CIN3 compared to the CIN2 samples. (Caselli *et al.*, 2020a).

Among the subjects that completed the follow-up at 6 months after surgical excision, most women resulted hrHPV-negative (52/66, 78,8%), without any clinical recurrence, but persistent hrHPV infection was detected in 14 patients (14/66, 21.2%), and 4 of them had CIN2/CIN3 recurrent diagnosis (4/14, 28%). Overall, the follow-up group showed significantly diverse CST prevalence, compared to the basal profiles (Figure 4.35), with a significant increase of CST-I prevalence (from 25% to 37%, $p < 0.001$), and a concomitant decrease of CST-IV (from 33% to 15%, $p < 0.0001$). CST-II and CST-V prevalence remained unaltered between follow-up and basal profiles, and CST-III resulted slightly increased, although not significantly (Figure 4.35A). The analysis of microbiome composition at species level evidenced a general increase of Lactobacilli, mainly of *L. crispatus* (increased of 930-fold compared to basal levels), although *L. iners* was still the most prevalent. On the other hand, follow-up microbiomes were characterized by lower presence of *A. vaginae*, *U. parvum*, and *G. vaginalis* (Figure 4.35B).

The CST distribution at follow-up was different also in CIN2/CIN3 (Figure 4.36A), and hrHPV-cleared/persistent (Figure 4.36B) comparisons. The decrease of CST-IV was in fact more evident in CIN3 group (from 44% to 12%, $p < 0.001$), compared to CIN2 group (from 17% to 13%, $p < 0.05$). Increase of CST-I group appeared more evident in CIN3 group (from 16 to 29%, $p < 0.001$), compared to CIN2 group

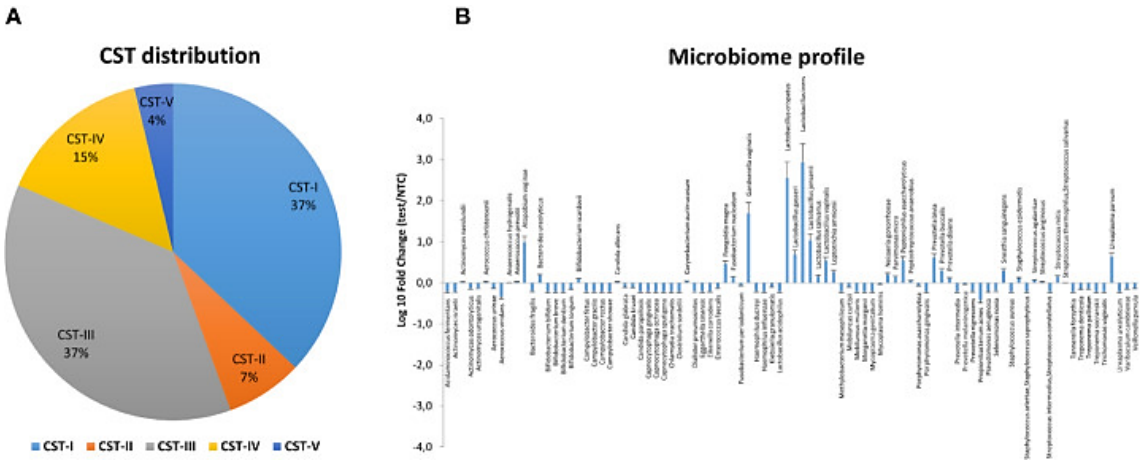

Figure 4.35: Microbiome profile in Follow-up samples in CIN2/CIN3 cohort of study. CST distribution in follow-up patients (66 patients), expressed as the percentage of each CST among all patients who completed the follow-up (4.35A). Microbiome profile of the whole follow-up group of patients (66 patients), results are expressed as Log10 fold change compared to negative controls (NTC, no template controls). Mean values ± SD for each microbial parameter are shown (4.35B) (Caselli *et al.*, 2020a).

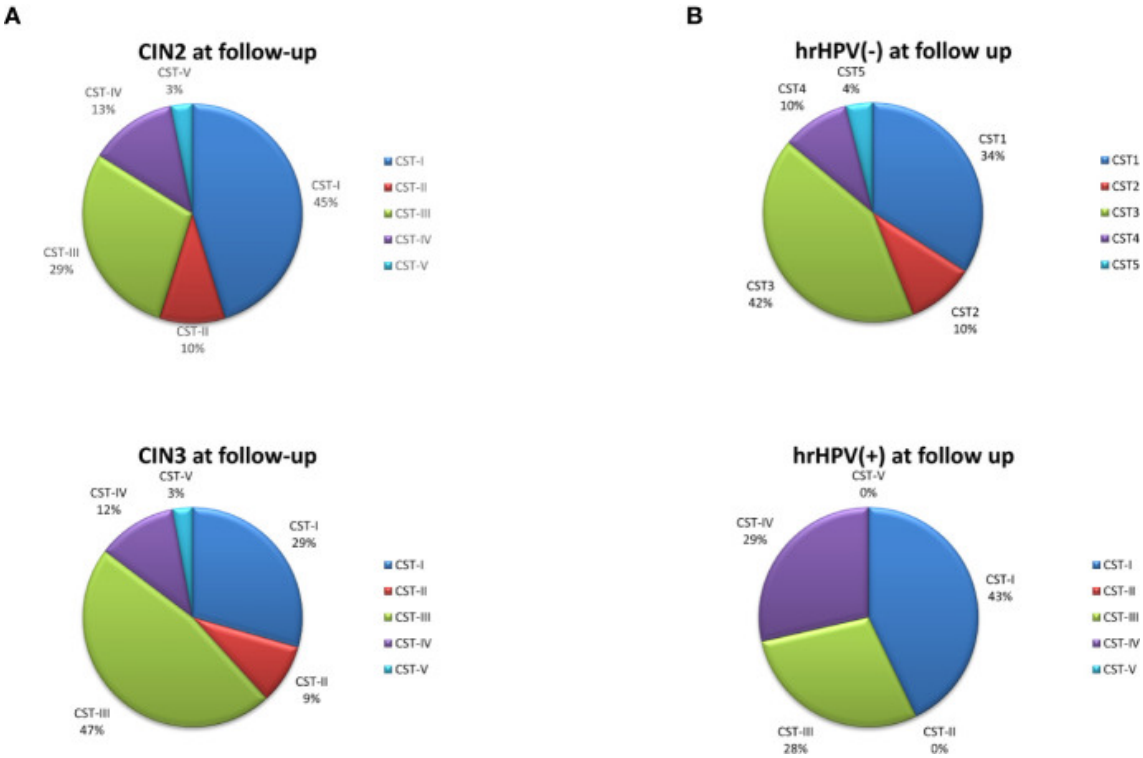

Figure 4.36: CST distribution in follow-up patients, subdivided for lesion degree (CIN2 or CIN3) before intervention (4.36A) and for hrHPV-status at follow-up (4.36B). The results are expressed as the percentage of each CST among the considered subgroups (Caselli *et al.*, 2020a).

(from 39 to 45%, $p < 0.01$). In the same way, CST-III resulted significantly increased in CIN3 (from 28 to 47%, $p < 0.001$), compared to CIN2 subgroup, and not significantly altered in CIN2 subgroup (from 34 to 29%). Concerning the hrHPV-status at follow-up, CST-IV prevalence was decreased in cleared subjects (from 32 to 10%, $p < 0.0001$), while that of hrHPV-persistent group did not show an altered prevalence (32 vs. 29%, $p =$ ns).

The whole microbiome profile of the follow-up group was highly influenced by the hrHPV-negative subgroup, characterized by a significantly increase of *L. crispatus* (19.3-fold compared to the basal amount, $p < 0.001$) and a concomitant decrease of BV-associated species, including *A. vaginae*, *G. vaginalis*, and *U. parvum* (Figure 4.37A), whereas few or no changes were observed in the microbiome profile of hrHPV-positive subgroup (Figure 4.37B).

Patients with persistent hrHPV infection and clinical CIN2/CIN3 recurrence instead evidenced a decrease of *L. crispatus* levels, a slight increase of *S. agalactiae* and no other significant variations (data not shown).

In order to evaluate the vaginal inflammatory microenvironment, vaginal washings were analyzed by a multi-analyte ELISA assay (Qiagen, Hilden, Germany), showing the presence of alteration in IL1α, IL1β, IL6, IL8, and TNFα (Figure 4.38A), that were further quantified by specific ELISA assays (Thermo Fisher, Milan, Italy). The results showed high amounts of proinflammatory cytokines, with no statistically significant differences between CIN2 and CIN3 subgroups (Figure 4.38B). IL8 resulted the most expressed cytokine, with a mean concentration of 1,105 pg/ml (range 284.9–2315 pg/ml). IL6 showed a mean concentration of 178.9 pg/ml (range 1.9–3493 pg/ml), IL1β a mean concentration of 162.3 pg/ml (range 13.4–649.9 pg/ml), IL1α and TNFα resulted less present, with mean value of 29.7 pg/ml (range 6.8–181.9 pg/ml) and 33.4 pg/ml (range 6.3–633.3 pg/ml), respectively. By contrast, differences of cytokine profiles among CST groups were observable (Figure 4.38C), with statistically significant differences IL1β and IL8 in grouped CST-II, III and IV versus CST-I and V ($p < 0.01$), and IL6 and TNFα in the CST-IV profile versus the others ($p < 0.05$) (Caselli *et al.*, 2020a).

Notably, at follow-up the cytokines levels resulted significantly decreased, compared to their respective basal levels ($p < 0.0001$–0.05) (Figure 4.39A). Further subdividing follow-up samples based on hrHPV persistence, it appeared clear that the most significant drop occurred in hrHPV-negative patients, whereas low or no differences were observed in hrHPV-positive patients, compared to basal profiles (Figure 4.39B) (Caselli *et al.*, 2020a).

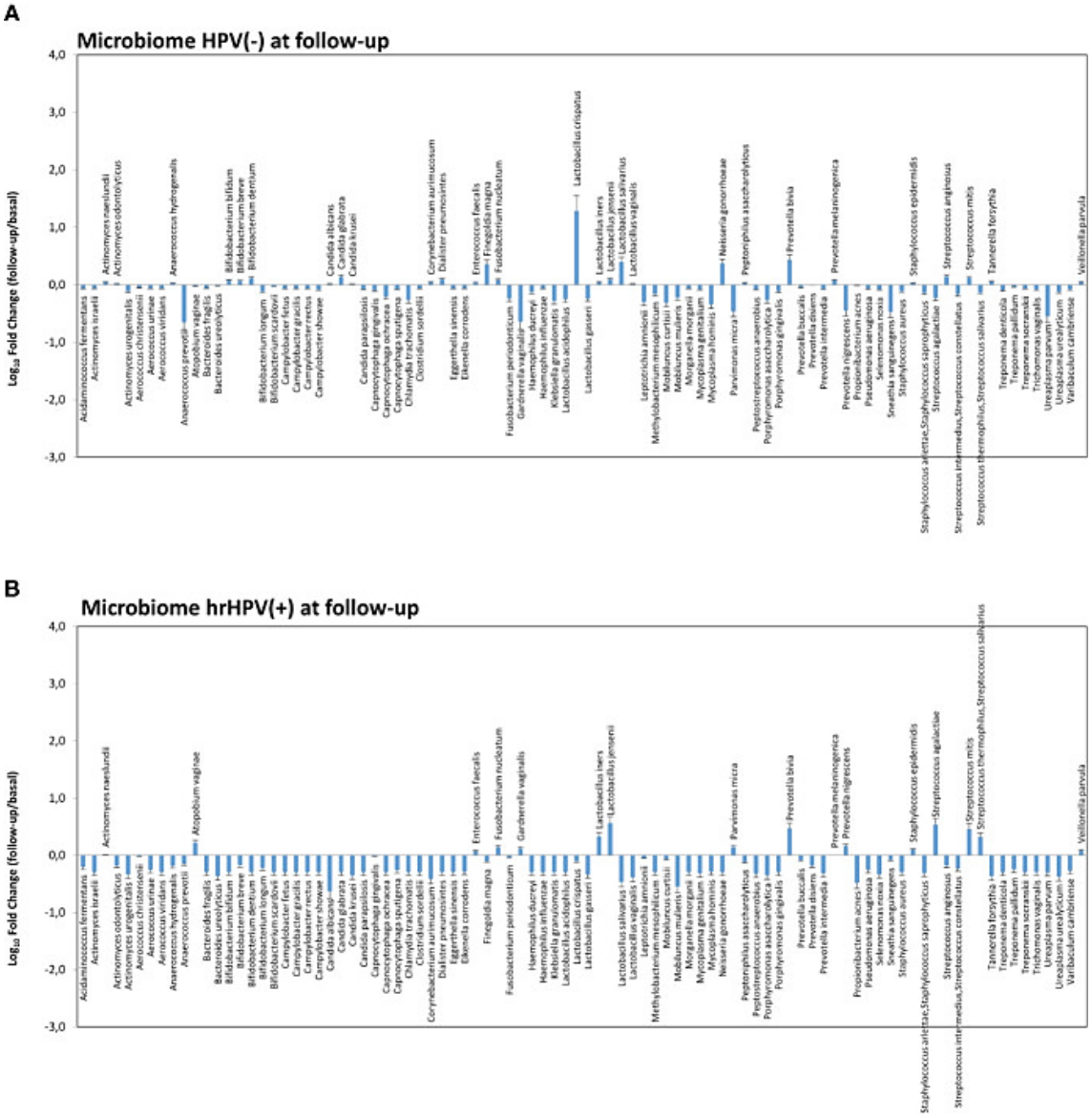

Figure 4.37: Microbiome profile in hrHPV-negative (4.37A) and hrHPV-positive (4.37B) subgroups at follow-up. Results are expressed as the mean value ± SD of Log_{10} fold change compared to negative controls (NTC, no template controls) (Caselli *et al.*, 2020a).

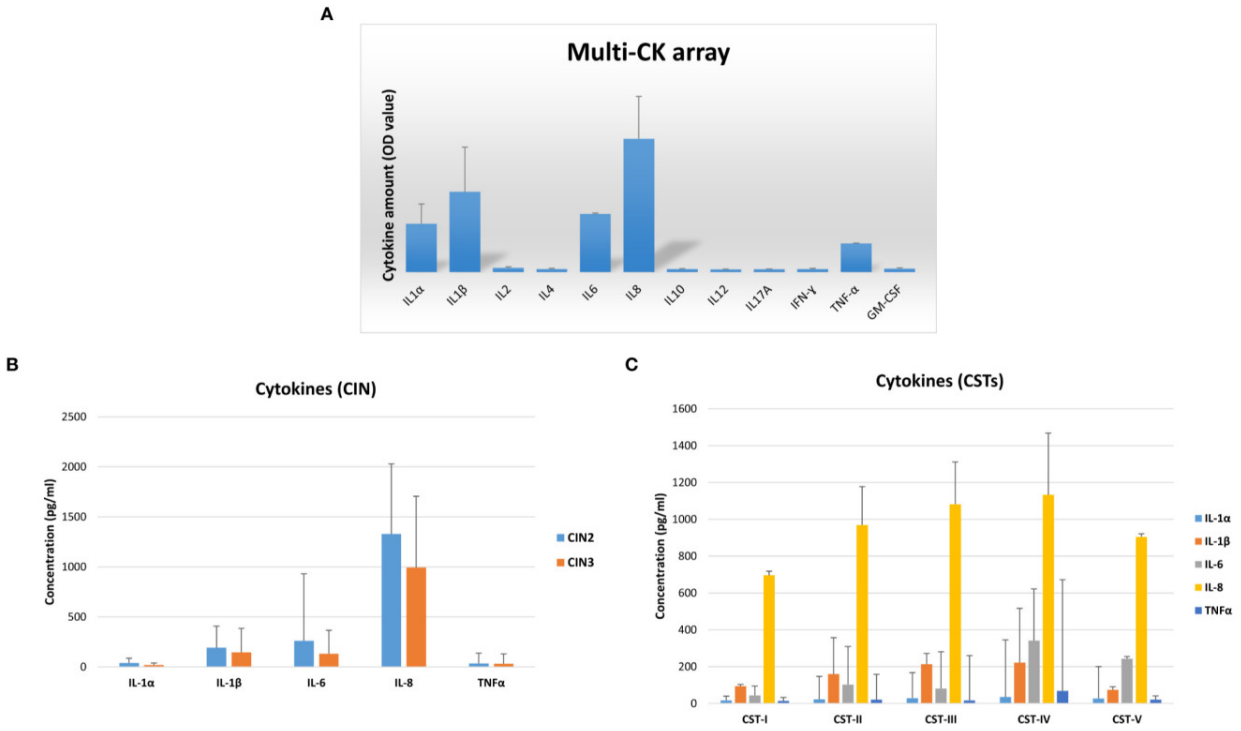

Figure 4.38: Cytokine profile in vaginal washing from the CIN2/CIN3 cohort of patients. Results obtained by multi-analyte cytokine ELISA array, expressed as mean OD values ± SD (triplicate samples) (4.38A). Results of specific ELISA assays, expressed as mean pg/ml ± SD values of duplicate samples, subdivided according to the severity of lesion (CIN2 or CIN3 subgroups) (4.38B), or for CST profiles of patients prior to LEEP intervention (CST-I – CST-V) (4.38C) (Caselli *et al.*, 2020a).

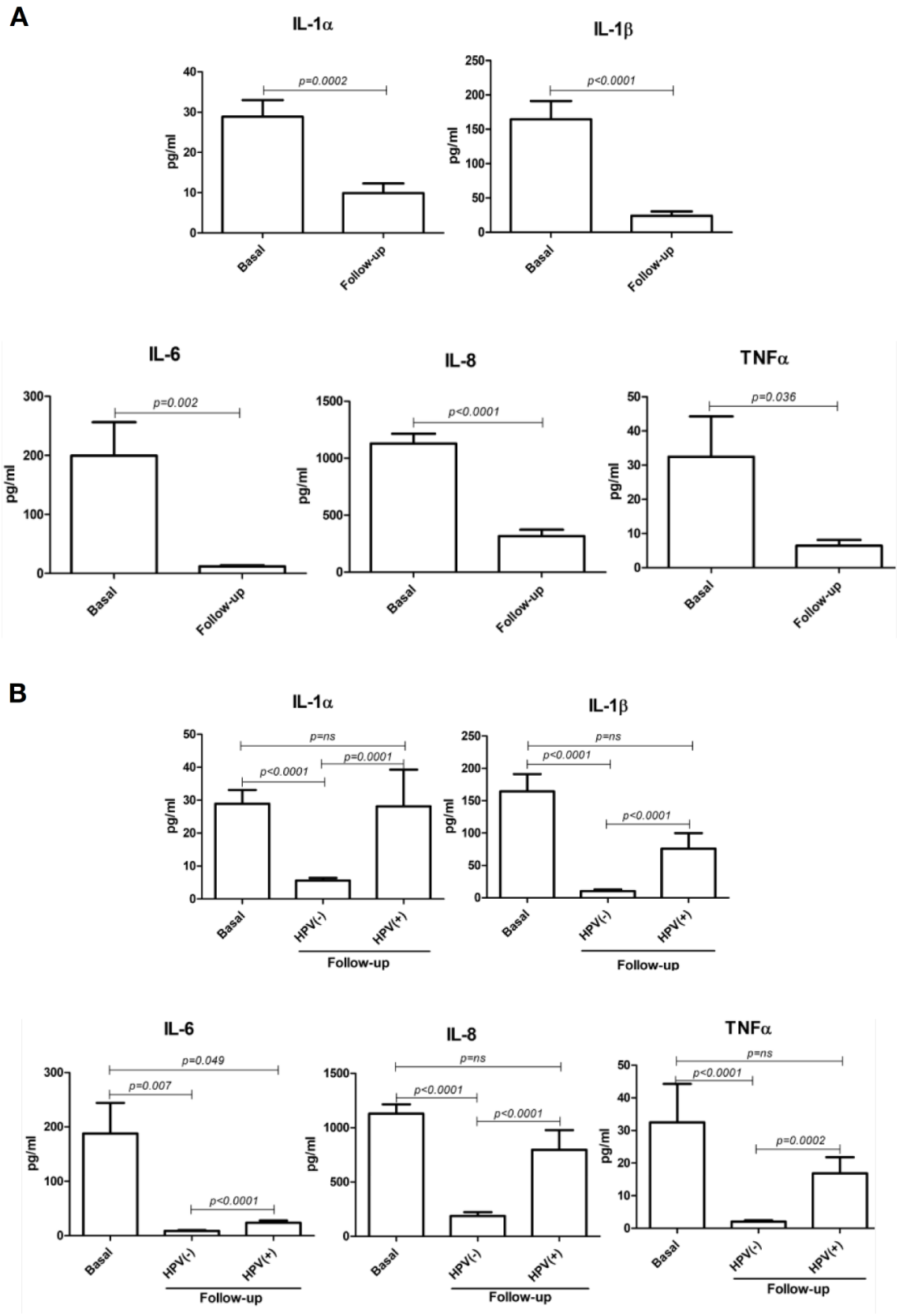

Figure 4.39: Cytokine profile of follow-up samples, compared to basal levels (4.39A). Comparison between basal group and follow-up samples, subdivided in hrHPV-negative and hrHPV-positive subgroups (4.39B). Results are expressed as mean pg/ml ± SD values of duplicate samples (Caselli *et al.*, 2020a).

Chapter 5

Conclusions and Future Perspectives

The deeper investigation of microbiome, driven by rapid advances in molecular technologies, has led to an increasing knowledge of environmental and human microbiomes, and their important connections. The influence of environmental microbiome in the natural colonization of the human body has been observed to occur rapidly after birth. Throughout life, the human microbiome is subject to a continuous interaction with microbes derived from the surrounding habitat. This dynamic interplay could, on the one hand, support the maintenance of a human microbiome eubiotic condition, or, on the other hand, could have a negative impact on human health. For instance, environmental microbiome of hospital surfaces has been widely recognized to promote the spread of HAI characterized by high AMR, that is considered a major threat for human health worldwide. In parallel, concerning human health, the establishment of a dysbiotic microbiome has been observed in association with several disease conditions, highlighting the role of the microbial component colonizing the human body in influencing our health.

Overall, our research interests aimed to shed light on the influence of environmental microbiome on human health, and on the other side, to investigate potential association between human microbiome and disease condition, in particular those viruses induced, using different methodological approaches.

It has been recently observed that the built environment can be considered as "super-organisms", and that its microbiome is constantly influenced by the presence of human occupants and *vice versa*. For hospital settings, the microbiome colonizing the nosocomial surfaces has a crucial role in the onset and transmission of HAIs, that represents one of the most severe complications occurring in hospital settings worldwide. The monitoring of hospital surface contamination is considered of fundamental importance, and the need to implement environmental surveillance systems is recognized (D'Accolti *et al.*, 2019), thus we were interested in evaluating the use of NGS and other molecular technologies for hospital environment microbiome characterization, comparing this methodologies with those microbiological culture-based. Our

results showed that NGS is able to characterize bacterial contamination with higher sensitivity, compared to other methods. On the other hand, NGS method evidenced the presence of skin/mucosa colonizers (e.g. *Cutibacterium* spp., *Streptococcus* spp. and *Corynebacterium* spp.), and other low pathogenic bacteria (e.g. *Paracoccus* spp., *Rothia* spp.) not necessarily associated with infectious risk. Moreover, NGS results are expressed as relative abundance values, being based on the characterization of the whole bacterial population, thus resulting less informative compared to other quantitative methods, such as the culture-based CFU count or qPCR. qPCR results presented in fact a better level of concordance with CFU count, describing more closely the true level of contamination of hospital surfaces and its associated infectious risk. Of note, a main difference between conventional and molecular methods lies in the fact that CFU count only shows living cells, able to grow in selective media, whereas molecular methods revealed all the strains present in a defined sample, thus presenting potential overestimation of pathogen contamination. In future works, it would be useful to use differential extraction methods for the discrimination between alive and dead cells (for example by using propidium monoazide) (Joo *et al.*, 2018), in order to obtain more reliable data of the microbial component that more realistically could transmit infections. A further limitation of NGS is the lack of detection of non-bacterial microbes (mycotic and viral components). However, NGS could be considered a unique tool for environmental surveillance, as it resulted the only technique able to identify non-searched bacteria and its high efficiency enables the identification of even scarcely represented species. Other important aspect to evaluate when monitoring hospital contamination is the resistome profile of microbes contaminating hospital surfaces, especially considering the increasing threat of AMR spread among HAIs-associated pathogens. Based on these considerations, the use of specific qPCR microarrays was demonstrated to efficiently characterize the resistome of contaminating population, and thus providing a useful tool to monitor AMR spread and environmental surveillance. Overall, based on collected data, the results of our investigation suggest that the high-throughput sequencing of 16S rRNA gene might be an effective method for monitoring the whole composition of hospital bacterial microbiome, especially if flanked by molecular PCR methods for species identification and resistome characterization. Such tools might provide a deeper knowledge of the hospital microbiome composition and may represent a potential improvement in the monitoring and control of HAIs transmission.

Since the risk for contracting HAIs has been observed to be particularly relevant in preterm infants (born before 28 weeks) hosted in Neonatal Intensive Care Unit (NICU), the potential influence of NICU contaminating microbiome on preterm newborns' colonization has been a further focus of our research. Based on the potential of NGS use for monitoring hospital environment, this technology has been applied

to characterize the bacterial composition of environmental surfaces microbiome and of nasal swabs of preterm infants, at the time of delivery and during hospitalization in NICU. In addition, the contribution of mother's vaginal microbiome in the nose colonization of newborn, and the resistome profile were also evaluated by NGS. Newborns' nose microbiome after few hours from birth evidenced the presence of several bacterial species, derived both from the vaginal microbiome of mothers (such as *Lactobacillus* spp., and *Streptococcus* spp.) and environmental contaminants of delivery rooms (for example, *Cutibacterium* spp., *Staphylococcus* spp., *Corynebacterium* spp., *Acinetobacter* spp., *Pseudomonas* spp., and others). Interestingly, our results showed that the colonization by specific pathogenic bacteria HAIs-associated increased with the time of permanence in NICU ward. This trend was observed in particular for *Staphylococcus* spp., *Escherichia-Shigella* spp., *K. pneumoniae*, and *K. oxytoca/E. cloacae*, that represented the most abundant bacterial colonizers of NICU floor and footboards. Moreover, the analysis of nasal newborns' resistome profile evidenced the absence of resistance genes at the time of birth, then an increase of R genes detection after the admission in the intensive unit, that paralleled with the colonization from the hospital environment. Taking together, our study points out that environmental microbiome, including drug-resistant species, detected on hospital surfaces can be transmitted by contact or by air particles, and could reach the respiratory tract or other profound tissues of preterm infants. This data further supports the role of hospital environment as a *reservoir* of potentially pathogenic and multi-drug resistant microorganisms, highlighting the importance of an active environmental monitoring of hospital microbiome and the need to optimize new strategies for surface cleaning.

Chemical-based disinfectants, indeed, have been reported to present numerous side effects, including high environmental impact on urban settings, wildlife, and aquatic ecosystems; moreover, their use has been proven to select or induce AMR in pathogens. The massive application of high-level disinfection has been mandatory imposed to counteract COVID-19 pandemic caused by SARS-CoV-2 spread, however presenting potential negative impact on environmental pollution and pathogens AMR. In order to overcome these concerns, the innovative probiotic-based cleaning system (PCHS, Probiotic Cleaning Hygiene System; Copma Scrl, Ferrara, Italy) has been previously proven to stably abate environmental pathogens surface's contamination in hospital settings, without selecting AMR bacteria. The antiviral effectiveness of PCHS system has been recently demonstrated on different enveloped viruses, known to persist long on surfaces. Results showed the ability to inactivate 99.99% of all tested viruses, within 1-2 hours of contact, both in suspension and on surface tests. Interestingly, the antiviral properties of PCHS were maintained even after 24h after application, in contrast to the loss of activity of chemical compounds

(70% EtOH and 0.5% NaClO) within 2 hours from application (D'Accolti *et al.*, 2021).

Based on the positive results obtained on PCHS antiviral activity, we tested the effectiveness of PCHS sanitation *in situ* in hospital environment, during the COVID-19 pandemics. PCHS activity, previously assessed in other hospital adult setting, was also confirmed in the children hospital environment. The system was able to stably remodulate ER microbiome, inducing a >80% decrease of the HAI-associate microbial contaminants and in parallel, a drop of R genes presence of up to 2 logarithms. Our study evidenced the negative influence of bactericidal/sporicidal chemical disinfections on PCHS activity, supporting the predominant role of the probiotic component on the effectiveness of the system. To note, the absence of SARS-CoV-2 on environmental surfaces during the experimentation, despite the presence of positive subjects, confirmed the low chance of fomites transmission of the virus and confirmed the *in vitro* assessed antiviral activity of PCHS.

Overall, results of PCHS effectiveness strongly confirm the potential of this innovative and biological-based approach to modulate the environmental contamination and ensure the control of pathogens spread and AMR transmission. PCHS antimicrobial and antiviral long-lasting activities, both *in vitro* and *in situ* confirmed, together with its affordable and eco-friendly properties, render it a system potentially applicable in several non-sanitary environments, including schools, offices, public transports, and all kinds of highly frequented environments.

The same approach, based on the potential of probiotic use on the remodulation of resident microbiome, has been also suggested as therapeutic approach to address a dysbiotic microbiome condition and restore a eubiotic one, promoting human health as ultimate goal. Based on these objectives, it is considered of fundamental importance the deeply characterization of human microbiome composition, unique in every defined anatomical niche, in order to obtain the most exhaustive "picture" of microbial profile harbored in a state of general health, and consequently investigate microbiome dysbiotic alterations during onset and progression of local or systemic human diseases. In this context, the second main focus of our research involved the study of the human microbiome profile in particular in the oral and vaginal districts in health status, and in association to pathologies, particularly those viruses-induced.

As the first objective, since a detailed site-specific map of oral microorganisms (including also eukaryotic and viral components) and their relative abundance was lacking, our research aimed to obtain a site-specific and comprehensive view of the oral microbiome of young adult healthy individuals (HOM), using WGS technology. In parallel, the AMR features of the HOM were also characterized, to define the microbial resistome profile in healthy conditions. The characterization of oral

micro-habitat in our cohort of twenty healthy subjects evidenced the presence of different microbial communities among the oral microhabitats investigated (hard and soft tissues, saliva and oral rinse), confirming the existence of a core microbiome, that can be considered as a reference for eubiosis definition. No statistically significant differences were observed related to gender and use of hygiene devices, whereas the use of manual toothbrush has been associated with a higher Alpha diversity, compared to the use of powered one. WGS analysis allowed the identification of the mycobiome and virome components of the oral cavity, evidencing a small prevalence of fungi (0.004% of the whole HOM), and by contrast a high presence of viruses in our study population (0.03% of the HOM), mainly represented by human Herpesviruses and phages, prevalently directed against the most prevalent bacterial species found in the oral cavity. Of note, oral rinse samples resulted the most representative of the whole niche-specific microbiomes, compared with saliva specimens; in addition, resistome analyses evidence the presence of several R genes, conferring resistance to macrolide, lincosamides, streptogramin and tetracycline. Overall, our WGS and microarray data, although the limited number of subject enrolled, provide a comprehensive picture of HOM features and its resistome profile in healthy individuals, and could be considered as reference for studies aimed to investigate the role of oral microbiome and its dysbiotic alteration during both oral and systemic diseases.

For this purpose, the WGS analysis approach was further used to characterize the oral microbiome of COVID-19 patients, to evidence the eventual association between virus-induced disease and the microbial environment of the oral cavity. In parallel, the inflammation profile and/or local secretory immune response (sIgA) were also assessed, to better define the characteristics of the oral environment that could potentially support SARS-CoV-2 infection. Metagenomic analysis evidenced very significant differences in the HOM composition between COVID-19 patients and controls, the former showing a decrease in the Alpha diversity and species richness. To note, oral dysbiosis correlated to COVID-19 symptoms severity. Moreover, a higher relative abundance of periodontopathogens species (e.g. *Prevotella*, *Porphyromonas*, *Aggregatibacter* and *Atopobium* was observed in patients, suggesting an association between with these specific bacteria and SARS-CoV-2 infection, in line with those reported for other respiratory viruses. The presence of bacteria belonging to *Enterococcus* and *Enterobacter* genera in COVID-19 subjects may be considered as potential markers of susceptibility for developing a severe infection. In addition, the mycobiome and virome components resulted significantly increased in COVID-19 group, highlighting a potential result of HOM dysbiosis, that could favor, on one hand the colonization of oral mycetes, and on the other hand the activation/reactivation of oral viruses. The higher presence of mycotic and viral colonizers in the oral cavity may increase inflammation status and further impair immune

system ability to counteract viral infections, thus supporting the importance of an interplay between all the component of oral microbiome in determining the health or disease status of the host. The local inflammation and the mucosal immune response were additionally assessed. A significant increase of cytokines levels (IL-6, IL-17, TNFα, GM-CSF) in the oral cavity proved the development of inflammation right at the entry site of the virus and confirmed the importance of oral conditions for the subsequent systemization of SARS-CoV-2 infection. Mucosal sIgA immune response against the virus has been inversely correlated to COVID-19 symptoms severity, as it was observed significantly more abundant in asymptomatic or paucisymptomatic patients, suggesting its potential role in controlling virus penetration via mucosal surfaces to the entire body. Overall, although it is not possible to establish whether the alteration in the microbial community could be the cause or the effect of the SARS-CoV-2 replication, our findings may be useful for the identification of markers predicting the development of symptomatic COVID-19 disease. Our data may open the way to a therapeutic approach based on the rebalance of oral microbiome composition, in order to prevent or ameliorate COVID-19 symptomatology. Toward this direction, in line with our observation of environmental microbiome modulation, the administration of specific probiotic has been previously proposed, with the aim to revert microbiome dysbiosis and counteract virus-induced respiratory diseases (Wang *et al.*, 2016). Therefore, this strategy could represent a possible intervention in COVID-19 treatment and deserves further investigation.

Lastly, our interest focused on the assessment of the impact of the vaginal microbiome on the HPV persistence in women with CIN2/CIN3 lesions who undergone surgical excision. The microbiome profile before LEEP intervention showed an higher prevalence of CST-IV group, compared to that reported in a healthy Caucasian population (Ravel *et al.*, 2011), with a significantly higher prevalence in CIN3 patients compared to CIN2 group. Our results confirmed the association between dysbiotic microbiomes and disease severity. Species identification allowed by microarrays analyses, evidenced the clear correlation between the absence of some "protective" *Lactobacillus* species and BV-associated bacteria, pointing out the development of a specific microbial balance in CIN patients. Overall, our data highlighted an increased inflammatory microenvironment in CIN patients, marked by high levels of pro-inflammatory cytokines (IL1α, IL1β, IL6, IL8, and TNFα), with the highest amounts observed in the CST-IV group. Based on our results, the surgical treatment induced a significant transition between CSTs prevalence, with an evident increase of CST-I and CST-III groups and a drop of CST-IV, among the follow-up population. A significant increase of *L. crispatus* in the whole microbiome profile at follow-up confirmed its protective role in hrHPV clearance and the subsequent patient's recovery. These significant changes in microbiome profile

were exclusively observed in patients that cleared HPV infection, whereas hrHPV-recurrent group did not exhibit relevant changes in CST distribution, with the maintaining of the initial CST-IV prevalence. *G. vaginalis* was constantly detected in the microbiomes of hrHPV-persistent subjects and may favor the establishment of the polymicrobial biofilm typically associated to bacterial vaginosis and dysbiotic status. All recurrent patients were hrHPV positive at follow-up, and these results could evidence the potential to consider the viral persistence after treatment as a sensitive predictive risk factor for lesion recurrence, together with the presence of an early margin involvement. Our evidence highlighted the importance of specific microbial amounts, rather than the simple microbial presence. For example, the only predominance of *L. crispatus* did not assured hrHPV clearance, if accompanied by the concomitant high presence of *G. vaginalis*, *U. parvum* and *C. albicans*, as observed in the cohort of CIN clinical recurrence. Moreover, such patients did not present relevant changes between basal and follow-up microbiome profiles, except for a low decrease of *L. crispatus* and a particular increase of species associated to aerobic vaginitis (e.g. *S. agalactiae*, that require further investigation in larger studies. Overall, in our study population, the surgical removal of CIN2/CIN3 lesions has been demonstrated to induce a significant microbiome remodulation, and, on the other hand, the prevalence of specific microbiome profile was associated with a higher rate of HPV clearance. The underlined crosstalk between HPV infection and the local vaginal microbiome may open the way to novel approaches aimed at remodulating vaginal microbiome. The administration of specific probiotic bacteria or the use of targeted antimicrobials (from the most conventional use of antibiotics to more promising approaches, consisting of bacteriophage therapy), could represent potential tools to enhance the viral clearance and consequently increase the rate of favorable outcomes after lesion removal.

In conclusion, the research conducted during my PhD period pointed out different findings and open the way to further interesting investigation, in particular:

1. The effectiveness of NGS technique has been reported as a strategy to implement the environmental surveillance and control of HAI transmission. The use of 16S rRNA sequencing, flanked by PCR techniques for resistome characterization, and further implemented by using amplicon sequence variants (ASV) and mycobiome 18S analyses, could provide a deeper knowledge of the hospital microbiome, potential leading to the improvement of protocols for the monitoring of hospital surfaces.

2. The environmental hospital microbiome, including its MDR component, has the potential to colonize preterm newborns' nasal cavities, thus confirming the importance of monitoring the hospital environment by using molecular

methodologies.

3. The innovative probiotic-based PCHS sanitation system showed long-lasting *in vitro* antiviral activity and sustainability features, in contrast to conventional chemical disinfection. Its application in the Emergency Room ward of an Italian pediatric hospital, during COVID-19 pandemics, highlighted the effectiveness of its use in control virus spread, and the parallel ability to remodulate environmental microbiome and stably counteract the spread of AMR pathogens. Further PCHS optimization has been currently investigated, with particular focus on the use of bacteriophages, in order to obtain a more rapid and specific action against selected pathogens. In addition, to meet the needing of the One Health approach, will be interesting to enlarge the application of this system in other built environment and also in animal livestocks, to counteract the spread of zoonotic infections.

4. The investigation of human oral microbiome by WGS analyses and qPCR microarrays allowed the definition of a site-specific microbiome map, including the resistome features of the microbes colonizing the healthy oral cavity. Secondly, the analysis of oral microbiome in COVID-19 patients showed a significant correlation between oral dysbiosis and individual susceptibility to SARS-CoV-2 severe infection, with also impact on local inflammation and mucosal responses. The use of probiotic therapies could be useful to rebalance the oral microbiome and contrast viral infection and requires interesting clinical studies in the future.

5. The vaginal microbiome and the inflammatory microenvironment were significantly altered in CIN2/CIN3 women, and a specific microbiome/cytokine profile could be associated to hrHPV clearance or persistence at 6 months from lesions removal. Also in this case, the vaginal microbiome balancing could represent a novel strategy to modifying the progression of hrHPV-related cervical cancer.

In conclusion, the data collected during my PhD support the existence of a deep interconnection between the environmental and human microbiome, and a significant role of the human microbiome in maintaining the health or favoring disease onset. This open the way to novel approaches, aimed to remodel the microbiome, whatever it is the environmental or human one, in order to maintain and improve the health of humans, animals, and the whole ecosystem.

This approach would be also in line with the "One Health" concept, considering the environmental and human microbiomes strongly interconnected. In this perspective, the data already obtained by us in the hospital environment are strongly sug-

gestive that such an approach, through a comprehensive characterization of microbiomes, may be successful in achieving a deeper understanding of the environment-humans microbial network and a consequent better control of the infectious risk.

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