Università degli Studi di Ferrara

UNIVERSITETI POLIS

INTERNATIONAL DOCTORATE in ARCHITECTURE AND URBAN PLANNING

Cycle XXXVII

IDAUP Coordinator Prof. Theo Zaffagnini

Thesis Title

Microbiological safety in public teaching buildings and other confined spaces: critical analysis of architectural, plant engineering and cleaning factors aimed to preserve human health.

Curriculum Architecture / Urban planning (MEDS-03/A Microbiology and Clinical Microbiology)

Candidate
Matteo BISI

(UniFe Matr. N.047883)

(Polis Univ. Reg. N. PL581N100012)

Supervisor DA / POLIS
Prof. Elisabetta CASELLI

Supervisor DA / **POLIS**
Prof. Kritsaq GJINO

External Expert
Prof. Sante MAZZACANE

Year 2021/2025

INDEX

1.INTRODUCTION

1.1. The Microbiome: The Holobiont Theory and One Health approach

According to Whipps et al. (1988), the term *microbiome* not only refers to the community of microbes that make up the microbiota, defined 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) but also refers to their "theater of activity," that is, all types of molecules produced by these microorganisms, including structural elements, metabolites/signal molecules and surrounding environmental conditions (Whipps J. et al., 1988).

Recently, the definition of Whipps et al. has been updated to provide a comprehensive and integrated description of the term (Berg et al., 2020), as shown in **Figure 1**.

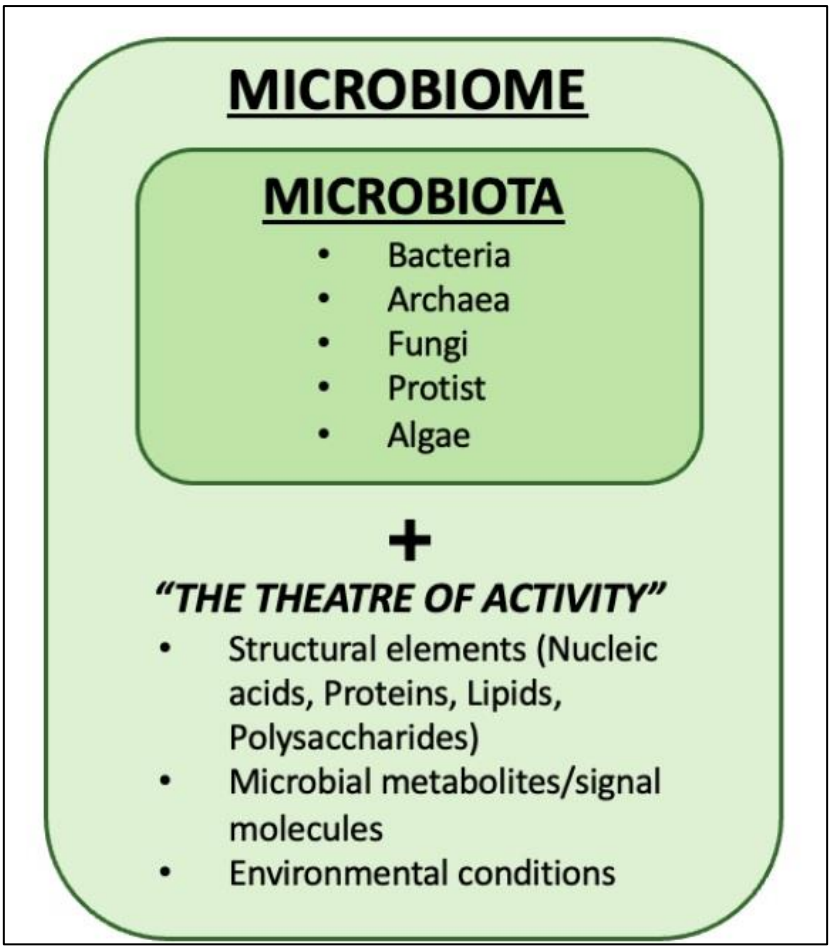


Figure 1. Graphical representation of the terms *microbiome* and *microbiota*: the microbiome includes both the community of microorganisms (*microbiota*) and their "Theatre of activity" (modified from Berg et al., 2020).

The living part of the microbiome is the *microbiota*, including bacteria, archaea, fungi, algae, and small protists—in other words—all the living members of the *microbiome* (Marchesi & Ravel, 2015). Microorganisms, the most ancient organisms in the biosphere, are ubiquitous, vital to the environment, and associated with both positive and negative health effects. In nature, they have not only an essential role in biochemical cycles, such as the carbon cycle, by converting atmospheric carbon dioxide into organic material (Gougoulias et al., 2014), but they also play a vital role in the survival of living macro-organisms, contributing crucial functions for their survival and health. Indeed, microorganisms can establish complex interactions with their host (humans, animals, and plants), with whom they have coevolved (Moran, 2007). Up to 10 trillion living microorganisms are estimated to be present in the human body (Sender et al., 2016), which means a ratio of 1:1 to 10:1 between the number of colonizing microorganisms and that of the total human cells (Fijan, 2014). The human microbiome has thus a profound impact on the functions of the human body, influencing our state of health or disease based on its composition. Accordingly, the XIX century "separation approach," according to which microorganisms can be subdivided into pathogens, symbionts, and neutrals based on their interactions with the host, first used to analyze microbial-host relationship (Lederberg & Mccray, 2001), has been replaced by the so-called "holistic approach", which looks to the host and its associated microbiome as a single entity (the holobiont) (Zilber-Rosenberg & Rosenberg, 2008; Berg et al., 2020). The paradigm shift that has characterized the microbiological research of the last decades is based on this crucial observation: a stable and resilient microbiome, rich in biodiversity (a state called *eubiosis*) is necessary for the holobiont to be in a healthy state, since it favor the health of the host (Trinh et al., 2018; van Bruggen et al., 2019). Conversely, when the microbiome loses its balance (a state called *dysbiosis*) has been linked to illnesses in the host.

To note, dysbiotic microbiomes can be spread from hosts to the environment and vice versa, influencing each other. The deep connections between the environment, plants, animals, and humans have resulted in the development of the "One Health" concept, which refers to "an integrated, unifying approach that aims to sustainably balance and optimize the health of people, animals, and ecosystems. It recognizes that the health of humans, domestic and wild animals, plants, and the wider environment (including ecosystems) are closely linked and interdependent" (One Health High-Level Expert Panel (One Health High-Level Expert Panel - OHHLEP et al., 2022). According to the World Health Organization document of reference, this integrated approach, schematically shown in **Figure 2**, recognizes the close connections between the health of humans, animals, plants, and the environment, highlighting the need to consider these connections when developing strategies directed toward the prevention and control of infection and AMR (Antimicrobial resistance) spread (One Health High-Level Expert Panel (OHHLEP) et al., 2022).

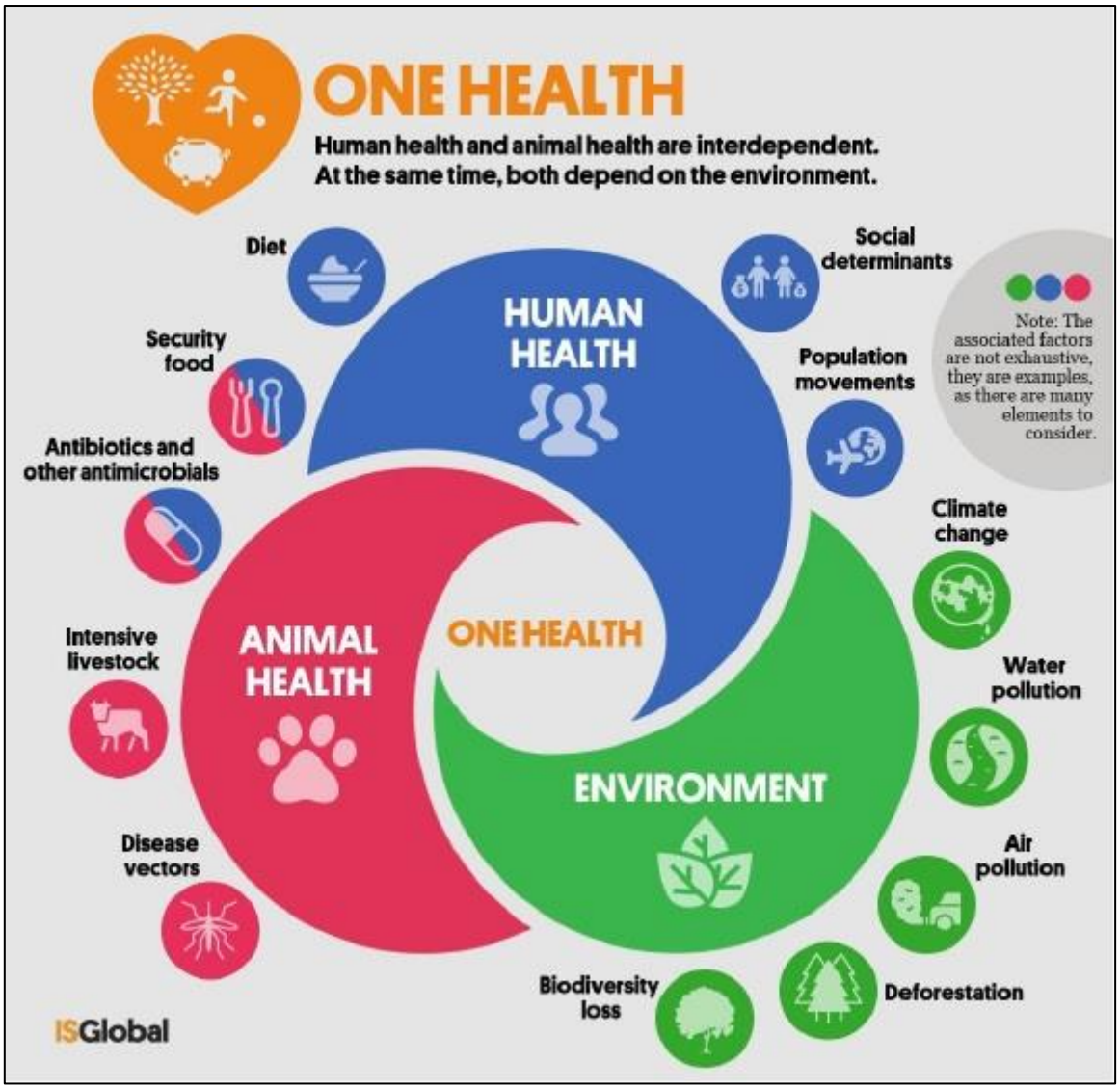


Figure 2. The One Health approach: the health of humans, animals, and our shared environment is strongly interconnected. A collaborative and transdisciplinary approach is needed to counteract global issues (e.g. antibiotic resistance, emerging, zoonotic diseases, environmental pollution) and achieve global health (LabEuropa, 2022).

The increasing accessibility of microbiome data has contributed to a better understanding of the features and functions of the microbiome. Currently, the main obstacles facing microbiome research are gaining a deeper comprehension of the mechanisms associated with microbiome eubiosis and dysbiosis and investigating methods to modify the microbiomes to enhance the health of people, animals, plants, and the ecosystem as a whole. To clarify this last statement, only through continuous interaction with the microbiome our body can perform all the essential functions for life, underscoring how the presence of the microbiome is fundamental to the health of the holobiont.

When the microbiome is healthy and balanced, the state defined *eubiosis*, its composition is typically characterized by high biodiversity. When this balance is altered (due to poor nutrition, an unsuitable lifestyle, the use of antimicrobial drugs, or other factors), the microbiome eventually alters, acquiring

a composition defined *dysbiosis*, which is chacterized by loss of biodiversity and frequent association with a decline in health and with development of several diseases, as shown in **Figure 3**.

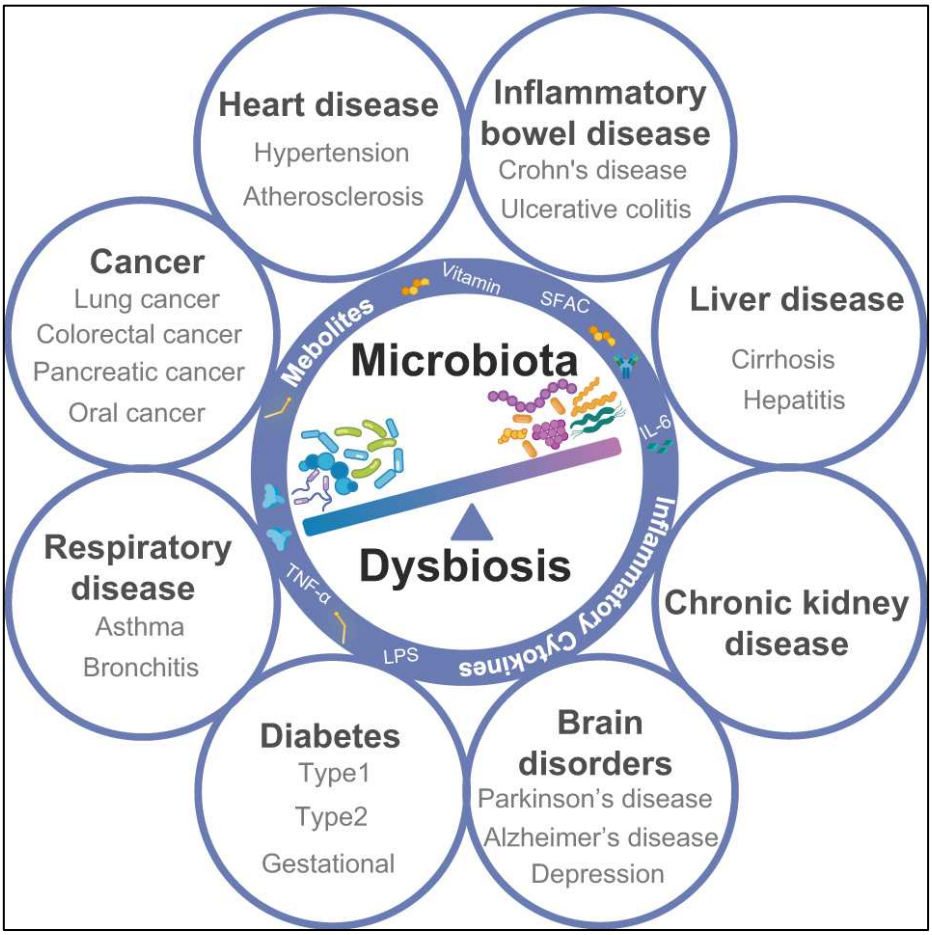


Figure 3. The various effects of a microbiome in dysbiosis.

1.2. The Built Environment (BE) microbiome

Microorganisms can be found in all types of environments on Earth and have even evolved to live in extreme environments; thus, the term *environmental microbiome* broadly describes the ubiquitous distribution of microorganisms across various habitats, including air, soil, aquatic environments, and human-inhabited indoor spaces.

Notably, in recent times, humans have started spending most of their lives in indoor spaces and built environments (BEs), including all the types of community settings (homes, workplaces, schools, public transportation, hospital settings, etc.). Based on the continuous interaction between microbes in the environment and in hosts, the health of human BE occupants has become more and more

affected by the continuous exposure to BE indoor microbiomes, rather than those from the external environment of rural life, potentially influencing the human health (Dai et al.,2017).

A “healthy” BE microbiome is characterized by high biodiversity, being composed by commensal and beneficial microbes spread by healthy humans, plants, and pets; pathogenic microorganisms that may cause human diseases can also be present, but they generally represent a minor part. By contrast, some BEs are essentially populated by microbes spread by humans, generating a mostly anthropic microbiome with less biodiversity. In particularly controlled and restricted BEs, such as in the hospitals, microbes are essentially spread by infected individuals, contributing to increase the quote of potential human pathogens in the BE microbiome. Moreover, such pathogens can be selected by the massive use of disinfectants and antimicrobials, rendering the BE microbiome a “non-healthy” one, potentially associated with the risk to develop diseases for the human occupants. In the hospital BE, this kind of microbiome is in fact responsible for the onset of the so-called healthcare associated infections (HAIs), which represent a global concern for hospitalized subjects.

In the BE, potential pathogens can be found not only on surfaces but also in the air, particularly in indoor spaces with air ventilation systems, causing BE air contamination (**Figure 4**), which can be associated with the occurrence of the so-called “Sick Building Syndrome” (SBS) (Prussin & Marr, 2015), characterized by symptoms like headache, eye, nose, or throat irritation, nausea, fatigue, difficulty concentrating, and other acute distress (EPA, 1991).

Figure 4. BE microbiome: microbial sources.

As mentioned, in the so-called high-traffic BEs (i.e. BEs highly frequented by humans), people represent the major source of microorganisms that can be found within the BE, which consequently develops an essentially anthropogenic microbiome (Zampolli J. et al., 2024). This is true not only for

healthcare structures but also for community environments, such as schools and mass transports. More closed, confined, and controlled BEs have microbiomes with lower biodiversity, compared to unrestricted environments, and with greater level of resistance to antimicrobials, due to the continuous selective pressure exerted by the use of antimicrobial compounds in crowded BEs (**Figure 5**).

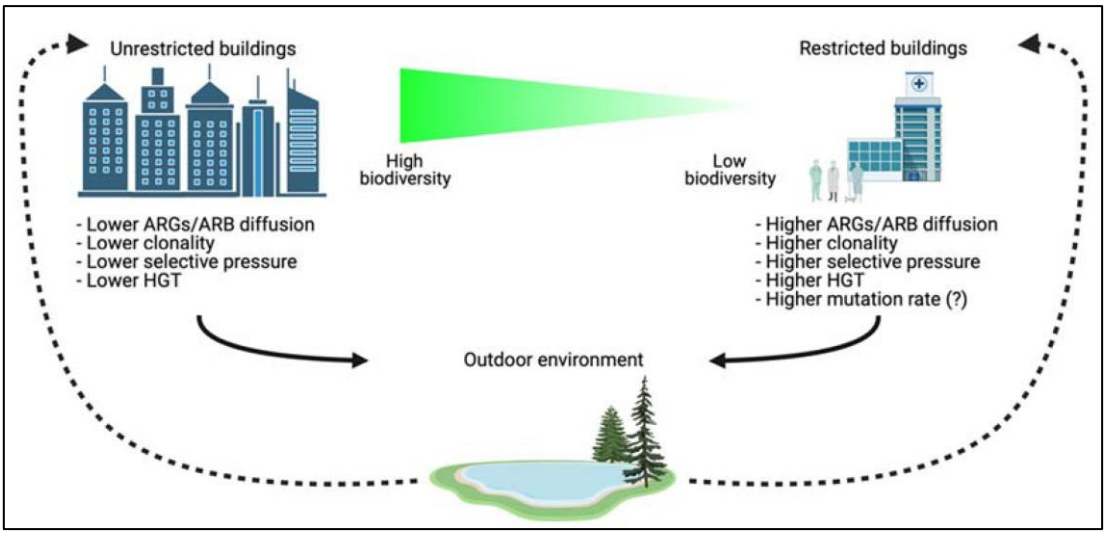


Figure 5. Microbiome of unconfined and confined environments: biodiversity and resistance.

The BE microbiome is also 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), thus different types of indoor spaces exhibit different microbiome composition, abundance, and diversity (Adams et al., 2015; Shin et al., 2015; Bragoszewska & Biedroń, 2018). Moreover, the interactions between BE occupants, the BE itself, and the BE microorganisms are highly dynamic and complex, originating a peculiar ecosystem made up of various organisms that interact with the surrounding environment and among themselves (Dai et al., 2017; Mahnert et al., 2019).

Consistent with this, recent studies characterizing different BE microbiomes showed that they can include several taxonomic groups (Marimuthu et al., 2014) and that higher occupancy levels are associated with higher microbiome abundances (Adams et al., 2016), with lower microbiome biodiversity and higher presence of multi-drug resistant (MDR) species compared to unrestricted environments (Kang et al., 2018; Mahnert et al., 2019; Nowrotek et al., 2019). These features confirm that the higher level of control and hygienization applied in highly restricted BEs exerts a strong selective pressure on the resident microbes, finally selecting the most resistant microbes (MDR strains) (**Figure 6**). In addition to hospitals, similar conditions can be found also in agriculture, animal husbandry (Chokshi et al., 2019), and other highly occupied indoor environments, including domestic and community ones (Jovel et al., 2016; Li et al., 2018; Xu et al., 2018; Lutz et al., 2014).

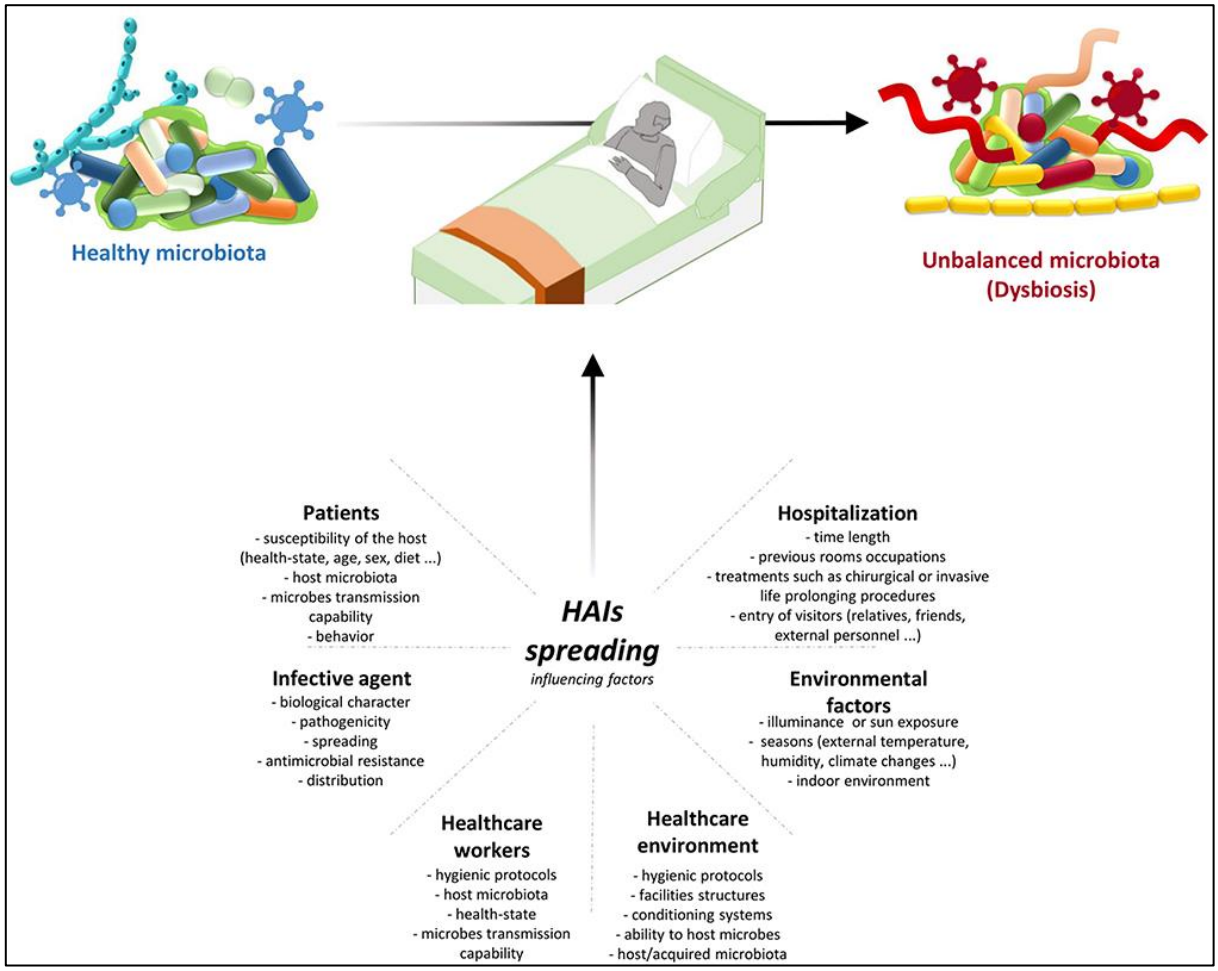


Figure 6. Risks associated with the hospital microbiome.

1.3. The features of the BE microbiome

BEs microbiomes include bacteria, viruses, and fungi (Gilbert & Stephens, 2018; S. M. Smith et al., 1996). Microbial contamination arises from multiple sources within indoor environments, including human occupants, building materials, and the outdoor environment (Caselli, 2017).

As already mentioned, human presence has a major impact on the composition of the BE microbiome, which is usually highly populated with bacteria that colonize human skin, such as Gram-positive Staphylococci, as well as Gram-negative bacteria like members of the Enterobacteriaceae family, fungi, and viruses.

The Home Microbiome Project has illustrated a close relationship between microbes, people, and their homes, showing rapid colonization of the home environment by the family's microbiota (Lax et al., 2014; Li et al., 2021). Various bacterial species inhabit domestic settings such as toilets, kitchens, and refrigerators, potentially becoming direct sources of foodborne diseases (Jeon et al., 2013).

Bacteria detected in these environments include *Proteobacteria*, *Firmicutes*, *Actinobacteria*, and *Bacteroidetes*, with common genera being *Propionibacterium*, *Bacteroides*, and *Staphylococcus* (Jeon et al., 2013). Additionally, *Staphylococcus* and *Micrococcus* genera have been detected in washing machines, with half being potential opportunistic pathogens, highlighting the need for effective cleaning and control strategies (Jacksch et al., 2021). *Staphylococcus* species, typically colonizers of human skin and which can also be found in the upper respiratory tract, are frequently transferred from humans to BE surfaces and air (Xu et al., 2015; Madsen et al., 2018; Cave et al., 2021). These bacteria have been linked to chronic conditions and allergic diseases within domestic environments and urban areas due to their ability to persist in dry environments and on surfaces (Oie & Kamiya, 1996; Dietze et al., 2001). Among *Staphylococcus* species, *Staphylococcus aureus* is notable for causing both community and hospital-associated infections (Aires de Sousa & Lencastre, 2004). Furthermore, its increasing antibiotic resistance, particularly in the form of methicillin-resistant *S. aureus* (MRSA), has made it a major pathogen not only in the nosocomial but also in the community settings (Tong et al., 2015; Cave, Cole, et al., 2021). Similarly, coagulase-negative staphylococci, which include species such as *Staphylococcus epidermidis*, *Staphylococcus haemolyticus*, *Staphylococcus hominis*, and *Staphylococcus saprophyticus*, were initially linked with severe infections in hospital settings. However, these bacteria are now frequently detected in non-healthcare environments as well (Becker et al., 2014), playing a significant role in the horizontal transfer of AMR genes (Davis et al., 2012). Likewise, Enterobacteriaceae, a family of Gram-negative bacteria typically present in the human gut microbiota, are also found in food, water, and home surfaces (Manshadi et al., 2013; Ashbolt, 2015). These bacteria show widespread resistance to beta-lactam and carbapenem antibiotics and are increasingly reported in non-healthcare settings, highlighting a potential public health threat (Meletis, 2016; Kelly et al., 2017). Notably, the recent spread of mobile colistin resistance (*mcr*) genes among MDR *Enterobacteriaceae* has rendered colistin, a last-resort antibiotic, ineffective (Morrill et al., 2015). This has significant implications for human health, as it further limits treatment options for infections caused by these resistant bacteria (Wang et al., 2017). Indoor environments are also home to fungi, and saprotrophic fungi such as *Cladosporium*, *Aspergillus*, and *Penicillium* represent a large portion of the indoor mycobiome (Nevalainen et al., 2015; Flannigan et al., 2016). Its diversity, abundance, and composition are influenced by climate and local environmental variations (Adams et al., 2016; Stephens, 2016). Similar to other contaminant microorganisms, fungi can survive on BE surfaces for days to months (Kramer et al., 2006), thus the release of spores, hyphal fragments, and mycotoxins represents a significant source of indoor pollution.

In universities and high school classrooms, factors such as occupancy density, ventilation, and indoor humidity levels directly influence the diversity and abundance of airborne and surface-bound microbes (D'Accolti et al., 2021). Research has consistently shown that high levels of microbial contamination correlate with increased health symptoms, particularly among children and young adults who are more susceptible to airborne pathogens (Andualem et al., 2019). As a matter of facts, contaminated surfaces in high-traffic community BEs constitute significant reservoirs for pathogens, posing risks to public health. This assertion is substantiated by multiple studies that highlight the prevalence of harmful microorganisms in these environments. The research by Andualem et al. reveals the correlation between indoor bacterial loads in classrooms and adverse health outcomes, noting that older buildings, which often retain moisture, demonstrated higher levels of bacterial contamination, thus worsening indoor air quality and health risks associated with respiratory infections and allergies (Andualem et al., 2019). Additionally, Jiang et al. discuss the presence and health implications of bioaerosols in office environments, establishing a direct link between microbial exposure and health complaints among occupants, thus reinforcing the need for increased scrutiny on indoor microbiological content (Jiang et al., 2023). Moreover, public transport systems, which serve as essential components of urban infrastructure, are similarly affected. The analysis by Leung et al. demonstrates that public transit environments harbor a distinctive microbial ecosystem, revealing geographical specificity in the microbial composition and antibiotic-resistance (Leung et al., 2021). This highlights the danger posed by potential pathogen dissemination in crowded transport spaces, where airborne and surface-associated microbes can proliferate, leading to increasing public health concerns. The integrated assessment of microbial contamination in these settings necessitates detailed monitoring protocols. Viruses, bacteria, and fungi not only thrive in poorly ventilated spaces, but can also be distributed via droplets and surface contact (Adetitun et al., 2024). Consequently, the deployment of advanced surveillance techniques, such as Quantitative Polymerase Chain Reaction (qPCR), may allow for the quantification of pathogenic microorganisms in these environments, as suggested by the findings of Leung et al. (Leung et al., 2021) and reinforced by the necessity for air quality improvement strategies highlighted by Andualem et al. (Andualem et al., 2019). Furthermore, Schütze et al. emphasize that employing sensor systems to monitor indoor air quality can provide timely data, facilitating the implementation of control measures against microbial contamination in real-time (Schütze et al., 2017).

In the context of these findings, it becomes clear that comprehensive monitoring and assessment protocols for indoor microbial contaminants are not only warranted but essential in mitigating the health risks posed by contaminated environments in schools and public transit systems.

1.4. Health implications of poor Indoor Air Quality (IAQ)

Several studies have linked poor indoor air quality (IAQ) and increased exposure to specific indoor microbes with respiratory problems, allergic reactions, and other health issues. Evidences increasingly points to the detrimental effects of indoor microbial communities on human health, particularly concerning respiratory problems and allergic reactions (Adams et al., 2015). Significant association was observed between the of airborne bacteria and respiratory symptoms, emphasizing the health risks posed by microbial contaminants in indoor environments (Meadow et al., 2013). Besides, significant association was reported between the presence of fungal DNA in classroom dust and ocular symptoms among students (Norbäck et al., 2017). Beyond ocular symptoms, indoor microbial exposure has been correlated with respiratory infections, especially in vulnerable populations such as children (Prussin et al., 2015).

Preventing exposure to harmful microbial environments seems thus essential for maintaining healthy learning spaces and ensuring optimal student performance, highlighting the need for effective strategies to mitigate microbial hazards in indoor environments.

Microbial contamination and its impact on IAQ are exacerbated by factors such as ventilation strategies, building design, and human occupancy (Landry et al., 2018). Meadow et al. demonstrated that both human occupancy and ventilation strategies significantly influence airborne bacterial community composition in indoor environments, suggesting that improved ventilation can mitigate harmful airborne pathogens (Meadow et al., 2013). The findings suggest that well-ventilated spaces often reflect the microbial composition of the outdoor environment, reducing the risk associated with indoor microbial exposure. Similarly, Kembel et al. emphasized that building architecture has a pronounced impact on the distribution of indoor microbial communities, indicating that design interventions could potentially create healthier indoor environments (Kembel et al., 2014). Assessing microbial contamination needs comprehensive and multifaceted approaches. Surface sampling is one effective methodology, allowing researchers to identify and locate specific sources of bio-contamination. Surface sampling can conveniently complement air sampling in determining the microbial quality of indoor spaces, showcasing its utility in identifying and addressing contamination sources (Piñar et al., 2013). Of note, indoor air can harbor a complex interplay of dormant bacteria and viruses, further complicating the assessment of microbial risks and suggesting that indoor environments might serve as reservoirs for pathogenic microorganisms (Prussin et al., 2015). In educational settings, the risks of poor IAQ and microbial contamination can have profound implications for student health and performance. Studies have indicated that improving IAQ can lead to enhanced student health, since higher indoor particulate matter levels were associated with respiratory symptoms in children (Ana et al., 2013). This underscores the importance of addressing

microbial contamination through effective ventilation and hygiene practices, especially in spaces frequented by children. An investment in clean indoor air can thus be seen as a necessary step towards fostering better learning environments, where health and performance are not compromised by microbial hazards. To further understand IAQ issues, ongoing research indicates that both chemical and biological pollutants contribute to indoor air challenges, necessitating comprehensive management strategies (Adetitun et al., 2024). The application of innovative technologies, such as next-generation sequencing (NGS) for microbial analysis, has recently allowed to profile the complexities of indoor microbiomes, enabling a more comprehensive approach to managing microbial health risks (Rocha et al., 2012). Such advancements in IAQ research are crucial for developing effective interventions that can maintain safe and healthy indoor environments. The implications of poor IAQ are in fact extensive, significantly impacting health outcomes and overall well-being. The interplay of microbial presence, environmental factors, and human interaction needs continuous monitoring and proactive management to foster healthy indoor environments favoring health and productivity.

1.5. The impact of air exchange on BE microbiome and IAQ.

Mechanical air exchange is essential to improve IAQ by facilitating the dilution and removal of indoor contaminants, including microorganisms. These systems are particularly important in densely occupied environments such as community and mass transport environments, where a high occupancy load can worsen air quality issues and increase the risk of airborne disease transmission. Improved ventilation has been shown to significantly lower airborne microbial concentrations by ensuring that fresh air is continuously circulated. This continuous airflow helps to reduce the overall burden of pathogens, thereby mitigating health risks associated with poor IAQ (Allen et al., 2016). Furthermore, a well-ventilated environment was observed to contribute to improved cognitive function, thereby linking effective ventilation to enhanced productivity (Allen et al., 2016). By contrast, inadequate ventilation has been associated with various health issues, including respiratory infections and chronic illnesses, by promoting the growth of harmful fungal species, which are common indoor allergens and can exacerbate conditions such as asthma (Wang et al., 2017). Notably, the connection between IAQ, humidity, and the prevalence of respiratory infections, was confirmed by evidencing that appropriate ventilation could serve as a preventive measure against such health complications (Wang et al., 2017). The role of mechanical ventilation was particularly highlighted during the COVID-19 pandemic, when it was emphasized that well-maintained ventilation systems are necessary to reduce airborne viral loads in public spaces (Allen et al., 2016). Effective air exchange can mitigate the spread of airborne pathogens, proving essential during periods of increased infectious disease

transmission. Such interventions are crucial for managing existing microbial loads and reducing the likelihood of future contamination events, thus creating healthier environments for all occupants. However, mechanical ventilation systems can inadvertently become reservoirs for microbial growth if not properly maintained. Therefore, regular monitoring and cleaning are crucial to ensure their effectiveness. Maintenance routines should include filter checks and system cleanings to prevent the accumulation of microbial matter, which can then become aerosolized and reintroduced into indoor air (Baldelli et al., 2022). This underscores that the design and upkeep of mechanical air systems are as critical as their operation.

Based on these evidences, mechanical air exchange systems are crucial to maintain healthy indoor environments by effectively managing the dilution and removal of indoor contaminants. Their role extends beyond improving IAQ; these systems play a fundamental part in safeguarding occupant health, preventing the spread of infections, and enhancing overall productivity in community settings. Investing in effective ventilation systems can yield significant long-term health benefits for communities at large. Despite the clear benefits of mechanical air exchange systems, their implementation faces numerous challenges associated with costs, energy consumption, and maintenance issues. The economic burden of these systems often acts as a barrier to their deployment, particularly in educational environments where budgets are strained. The initial costs for the installation of advanced ventilation technologies can be substantial, and many existing infrastructures lack the necessary design features to accommodate such systems, which further exacerbates the financial hurdles (Liu et al., 2022). Interestingly, studies indicate that while the operational expenses of mechanical ventilation systems can be significant, optimizing energy use through strategies such as demand-controlled ventilation (DCV) can lead to energy savings as high as 75%, thereby mitigating the costs in the long run (Blubaugh et al., 2022). Energy consumption is another pivotal consideration, especially since HVAC (heating, ventilation, and air conditioning) systems account for approximately 40% of total building energy use (Liu et al., 2022). This energy demand can escalate further in systems that are not effectively designed to utilize natural ventilation methods (Cîrstolovean et al., 2018). Consequently, the potential for energy savings becomes a critical aspect of any building's ventilation design. For example, improved thermal insulation and efficient system controls that harness natural ventilation principles can significantly reduce energy consumption by approximately 40% (Cîrstolovean et al., 2018). Moreover, the implementation of advanced controls that adapt to occupancy patterns can optimize energy usage without compromising IAQ (Sha et al., 2021). Additionally, variability in user acceptance and understanding of these systems poses another obstacle to their widespread adoption in educational institutions. Research indicates that users often hold misconceptions regarding the benefits and operational aspects of mechanical ventilation

systems, which can lead to resistance when these systems are introduced (Liu et al., 2022). This variability in acceptance highlights the importance of educational initiatives to promote user understanding, as occupants significantly influence the operational efficiency of HVAC systems (Liu et al., 2022). Involving users in the decision-making process and providing training on the expected benefits and proper use of these systems may enhance acceptance and eventual implementation. In conclusion, addressing the challenges of mechanical air exchange systems requires a multifaceted approach that considers initial costs, energy consumption patterns, and user acceptance levels. By integrating advanced energy-saving strategies, optimizing system designs to fit existing infrastructures, and investing in user education, stakeholders can significantly enhance the deployment and effectiveness of these essential technologies in educational settings. Furthermore, A commonly used parameter for assessing indoor air quality is the concentration of CO_2 (Szczurek et al., 2015, Sanchez-Fernandez et al., 2023). This metric is essential in evaluating occupant well-being, especially in crowded spaces like daycares and schools, where monitoring becomes increasingly relevant (Schibuola, L et al., 2020, Settimo, G et al., 2024). When carbon dioxide concentration reaches critical thresholds of 1000 ÷ 1500 ppm(v), it may become a health concern, leading to a decline in cognitive abilities (Satish et al., 2012) and having a narcotic effect; for this reason, particular attention is given to settings such as schools and hospitals, which have been the focus of numerous analyses in the scientific literature (Ballerini et al., 2015, Ballerini et al., 2023, Loreti et al., 2016, Cesari et al. 2020, Krawczyk et al, 2016). Within this framework, low-cost sensors were employed in the international project by Sahme (Chatzidiakou et al., 2023). Recommended indoor CO_2 concentrations typically range between 1000 ÷ 1500 ppm (Dimitroulopoulou et al., 2023; Lowther et al., 2021). In particular, in schools, IAQ is deemed good if CO_2 remains below 1000 ppm and acceptable if it is below 1500 ppm (Lowther et al., 2021). Standards such as EN 16798:2019 categorize indoor air quality by CO_2 concentration levels. For instance, in residential environments, a Category I rating (the highest quality) requires CO_2 levels to stay within 500 ppm above outdoor levels in living rooms and 380 ppm in bedrooms. This aligns closely with Lowther's review (Lowther et al., 2021), considering the global average outdoor CO_2 concentration of 419 ppm, as reported in the NOAA's (National Oceanic and Atmospheric Administration) 2023 annual report (NOAA, 2025).

1.6. Microbial Monitoring in indoor BEs

The characterization of the indoor microbial bioburden can provide important information about the amount and type of the BE microbiome, also allowing to evidence the effectiveness action of the sanitation procedures, and to take adequate countermeasures when needed.

To this purpose, different methods can be used. Conventional microbiological methods are largely used, primarily culture-based methods, allowing the counting of colony-forming units (CFU) of specific microorganisms. These methods are based on the use of RODAC plates (Replicate Organism Detection and Counting plates, containing general or specific agar media), nitrocellulose membranes, dip slides, swabs, sponges, and wipes that can collect microbes from surfaces and air. Though their wide usage, all conventional culture-based techniques are time-consuming, since they depend on the growth of microbes to be tested. Also, these methods can only evidence cultivable microbes and depend on the type of culture media used. To overcome these limitations, providing quick and whole characterization of the analyzed BE microbiome, molecular methods have been introduced as a useful tool for accurate and informative analysis on environmental bioburden (D'Accolti et al., 2019a). These methods are based on DNA technologies and can evidence even traces of microbes. Moreover, quantitative molecular methods can provide quantitation of each microbe composing the BE microbiome, providing not only a yes-no result. Besides, these methods can be used to evidence particular genes harbored by the microbes of the BE microbiome, such as those associated with antimicrobial resistance (AMR) of the microbial population, providing identification and quantitation of several antimicrobial resistance-coding genes (ARGs) (Caselli et al., 2016, 2019; Caselli et al., 2018). Last, the recent DNA sequencing-based technologies has led to the possibility of defining complex microbial populations by NGS (Jovel et al., 2016), providing a potentially useful strategy for environmental microbial surveillance (D'Accolti et al., 2019b). NGS is in fact based on deep sequencing technologies able to characterize the whole microbial community: the whole bacteriome can be profiled by sequencing the gene coding for 16S rRNA (conserved in bacteria), and similarly the whole fungal community can be characterized by 18S rRNA sequencing. Furthermore, a more comprehensive picture can be obtained by using shot-gun techniques, such as Whole Genome Sequencing (WGS), which can simultaneously provide the whole profile of a microbial community, identifying bacteria, virus, fungi, protozoa, etc. A combined approach of these procedures, as schematically shown in **Figure 7**, may provide a complete picture, allowing timely countermeasures to be taken, if necessary (Cason et al., 2022).

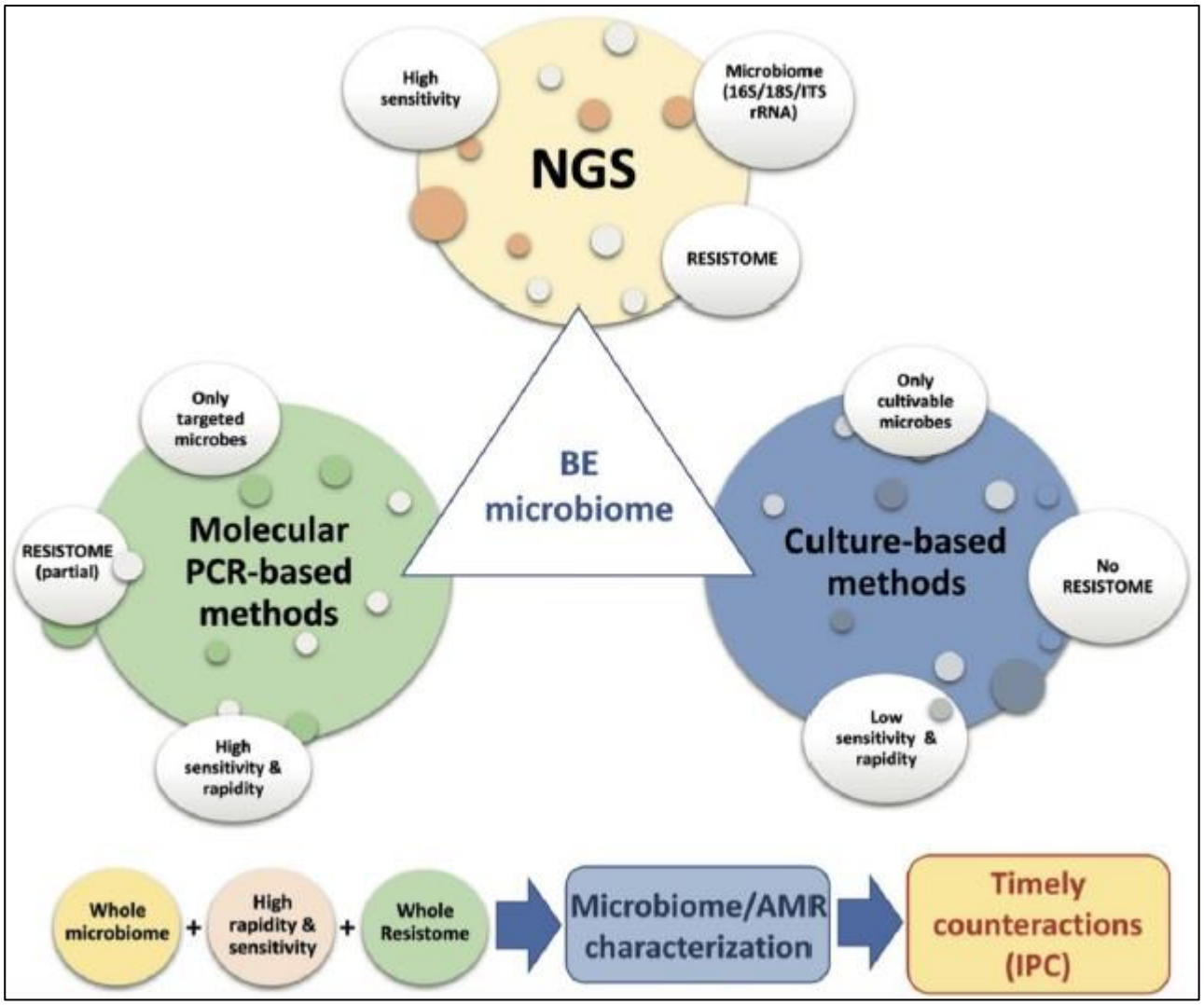


Figure 7. Schematic representation of different molecular and conventional methods useful for characterizing the microbiome of the hospital environment, with a focus on AMR surveillance.

1.7. Sanitation to counteract BE bioburden

To control the BE bioburden, chemical-based detergents and disinfectants have been used so far. However, disinfectants have several significant drawbacks. First, chemical disinfectants can worsen water and soil pollution and have a significant negative impact on the environment (Nabi et al., 2020; Zhang et al., 2020). Second, their effects are partial, as over 50% of surfaces cleaned with chemical products are not sufficiently decontaminated (Carling et al., 2008), and several microbes have been found to persist on the surfaces after treatment (Kramer et al., 2006; Goodman et al., 2008; Lawley et al., 2010; Boyce, 2016). Furthermore, chemical sanitation has only a temporary effect, as it is not effective in avoiding recontamination which occurs rapidly, within 0.5-2 hours after disinfection, depending on the type of disinfectant used (Rutala & Weber, 2014; D'Accolti et al., 2021a). Last, chemical sanitation can enhance the selection of microbes tolerant or resistant to the disinfectant itself (McDonnell & Russell, 1999; Caini S. et al., 2013; Cornejo-Juarez et al., 2015), nullifying the

disinfectant's effect. Most importantly, the resistance towards disinfectants can become resistance against antimicrobial drugs, due to cross-resistance phenomena (B. Chen et al., 2021; Clancy et al., 2020; Kampf, 2018; Lai et al., 2021; Wand et al., 2017; Wu-Chen et al., 2023). One of the first disinfectants reported to induce cross-resistance is chlorhexidine, which has been shown to promote resistance to several antibiotics, including ceftazidime, sulfamethoxazole, imipenem, cefotaxime, tetracycline, and colistin (Wand et al., 2017). Besides, benzalkonium chloride can induce diverse Gram-negative bacteria to become resistant to ampicillin, cefotaxime, and sulfamethoxazole (REF). Similar cross-resistance phenomena were also observed with triclosan, octenidine, sodium hypochlorite, and didecyldimethylammonium chloride (Kampf, 2018).

Chemical sanitation is thus associated with "side effects" which are undesirable and unsafe, both for environmental and human health, given the growing AMR diffusion. To overcome the limitations of the conventional chemical disinfection, several alternative approaches have been proposed for BE hygienization. These include the use of ultraviolet (UV) light or hydrogen peroxide (Weber & Rutala, 2013; Boyce, 2016), automated no-touch technologies, and self-disinfecting surfaces that work by acting as antimicrobial agents such as iron, copper, and silver (Lansdown, 2006; Noyce et al., 2006; Casey et al., 2010). However, despite their effectiveness, such technologies can only be applied in the absence of patients, are not suitable for all types of surfaces and are not cost-effective (Dancer, 2014). Moreover, more recent research has shown that a sanitation strategy meant to completely eradicate microbial contamination in the environment is harmful rather than helpful (Vangay et al., 2015). Pathogenic species can more easily colonize an environment free of natural contamination and lacking in "good" competitor bacteria. This can result in the spread of more virulent and resistant strains of the pathogen. Consistent with this, an alternative approach has been considered, based on rebalancing the microbial populations by reintroducing beneficial microbial species instead of "oversterilizing" the environment. This method has been defined "Bidirectional Hygiene" or "Bygiene" (Al-Ghalith & Knights, 2015) (**Figure 8**) and is based on the observations made in the living organisms, where it is known that microbiota depletion, such as those resulting from prolonged antibiotic therapy, can encourage the colonization by potentially pathogenic microorganisms, leading to a dysbiotic condition that may play a role in the development of disease (Hawrelak & Myers, 2004; Deshpande et al., 2013). Toward this goal, an innovative sanitation approach has been set-up and tested in our laboratory, based on the addition of beneficial microbes (probiotics) to the cleaning product. This system was named PCHS (Probiotic Cleaning Hygiene System) and is composed of a fully eco-sustainable detergent containing three species of the sporogenic probiotics belonging to the genus *Bacillus*.

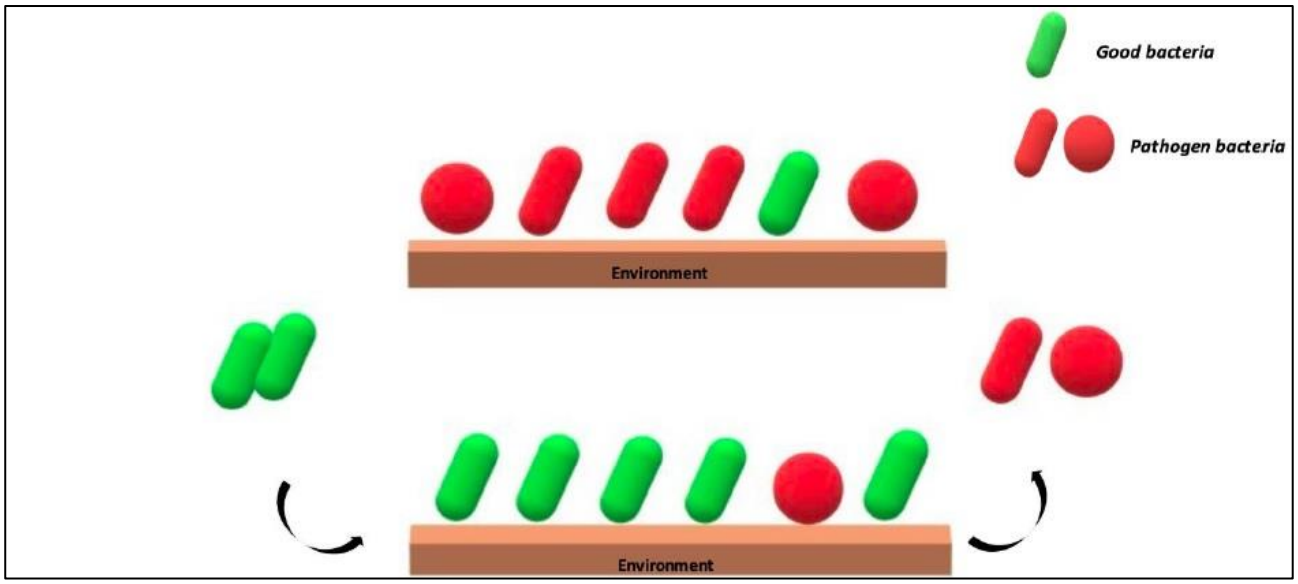


Figure 8. Schematic representation of the "Bygiene" principle. The introduction of good bacteria (green) in the environment counteracts the colonization of potential pathogens (red).

1.8. Probiotic-based sanitation as a strategy to control BE bioburden

Probiotic-based sanitation (PBS) systems are defined as cleaning techniques based on the addition of beneficial probiotics to the cleaning products. In 2010 we set-up a particular PBS, named Probiotic Cleaning Hygiene System (PCHS), based on the use of probiotic bacteria belonging to the *Bacillus* genus, to compete with harmful pathogens and promote a more stable microbial balance in indoor environments. Notably, PCHS represents a significant shift from conventional cleaning methodologies, which predominantly employ chemical disinfectants that can contribute to pathogen resistance and environmental harm (Caselli, 2017). During over 15 years of research, PCHS was shown to effectively lower pathogens 80% more than disinfectants in sanitary settings, significantly outperforming conventional techniques that often fail to prevent surface recontamination (Caselli et al., 2018). Furthermore, in contrast with chemical disinfectants, PCHS did not induce selection of AMR but rather promoted a 99.9% decrease of the pre-existing ARGs, thus positively impacting on the AMR spread (D'Accolti et al., 2023; D'Accolti et al., 2021). In addition to reducing pathogens, PCHS also induced remodulation of the indoor microbiome, fostering a microbiological environment that enhances resilience against future contaminations. PCHS, in fact, not only significantly decreased pathogens but also redefined the resistome of the microbial community persisting on hospital surfaces, enabling more favorable microbial communities (Caselli et al., 2016; Caselli et al., 2019). This shift towards a healthier microbiome is crucial for healthcare facilities, where the risk of healthcare-associated infections (HAIs) is a persistent concern (Caselli et al., 2018; Caselli et al., 2019). Furthermore, the sustainable nature of probiotic cleaning offers an interesting alternative for

institutions aiming to implement eco-friendly practices while addressing microbial contamination, an imperative that has gained significant traction in recent years (Caselli et al., 2018; Caselli et al., 2019). Compelling evidence suggests that the efficacy of PCHS is mostly due to the competitive exclusion mechanism exerted by the *Bacillus* probiotics, that can gradually displace and replace pathogens. Thank to this mechanism, PCHS was capable to displace pathogens, including those with AMR, thereby enhancing hygiene standards in healthcare settings (D'Accolti et al., 2023; D'Accolti et al., 2022). Comparative studies have shown PCHS's superior effectiveness in various trials across multiple hospital environments, reinforcing its practical application and efficacy (Caselli et al., 2018; Caselli et al., 2019). In conjunction with the growing body of evidence advocating for the adoption of probiotics within environmental sanitation, these findings emphasize the need for healthcare institutions to transition towards more sustainable and effective cleaning protocols. In this respect, the Probiotic Cleaning Hygiene System exemplifies an innovative and effective approach to sanitation that not only reduces the presence of harmful pathogens by up to 90% but also contributes to a healthier indoor microbiome and mitigates the emergence of antimicrobial resistance. Given its potential environmental benefits and alignment with sustainable practices, the adoption of such probiotic-based cleaning methods may represent a useful tool to control microbial contamination challenges. Indeed, the integration of probiotic cleaning systems with mechanical air exchange may improve the efforts to manage indoor microbial contamination effectively. In fact, probiotic cleaning can substantially reduce surface contamination while mechanical ventilation systems can ensure the continuous removal of airborne pathogens (Caselli et al., 2018). Research indicates that such synergistic applications may lead to a significant enhancement in indoor environmental health.

1.9. PCHS features

Probiotics are defined by FAO/WHO guidelines as "live microorganisms that confer a health benefit to the host when administered in adequate amounts" (FAO and WHO, 2002). Probiotics have been shown to be effective in preventing various infections, including diarrhea (Squellati, 2018), necrotizing enterocolitis (Patel & Underwood, 2018), upper respiratory infections (Banupriya et al., 2015), and infections in surgical patients (Rayes et al., 2002, 2012; Sommacal et al., 2015). To develop an effective method for environmental sanitation, our research group, in collaboration with Copma S.c.p.a., a leading company in sanitation systems, has developed the already mentioned Probiotic Cleaning Hygiene System (PCHS), which employs three specific species belonging to the non-pathogenic probiotic Bacillus genus, namely *B. subtilis*, *B. pumilus*, and *B. megaterium*. *Bacillus* bacteria are Gram-positive, spore-forming rods commonly found in the environment, such as soil and water, and in the human and animal gut. Except for the two species *Bacillus anthracis* and *Bacillus*

cereus, all the remaining *Bacillus* species are considered safe for humans (EFSA, 2022) and have been widely applied in food production, aquaculture, agriculture, and human and animal therapy. The ability of *Bacillus* to persist in a sporogenic form makes them suitable for inclusion in concentrated detergents, maintaining their viability and capacity to germinate when diluted and applied to surfaces. In vitro studies have demonstrated that PCHS-*Bacillus* can nearly completely inhibit the growth of Gram-positive and Gram-negative bacteria, as well as of fungi (Caselli et al., 2019). This inhibition is primarily due to competitive antagonism (Gause's law) and further to the production of antibacterial compounds known as bacteriocins (Caselli et al., 2019). In addition, PCHS-*Bacillus* were shown to produce various enzymes that can degrade and remove proteins, lipids, and organic material in general (D'Accolti et al., 2021). These features allow a gradual reduction of undesirable microorganisms on PCHS-treated surfaces, with a simultaneous increase in bacteria of the *Bacillus* genus, which maintain the balance achieved over time, leading to a stable modulation of the microbiota on treated surfaces. Indeed, several studies in situ have shown that PCHS leads to a stable remodulation of the hospital microbiome and a significant reduction in AMR within the hospital environment. These studies, performed in various public and private healthcare settings, compared the results of PCHS to conventional chlorine-based cleaning methods (Vandini, Temmerman, et al., 2014) or analyzed outcomes in the same wards before and after the PCHS intervention (Caselli et al., 2016; Caselli et al., 2018). PCHS was observed to reduce pathogen contamination by up to 90% more effectively than chemical disinfectants, without promoting the selection of antimicrobial-resistant pathogens, evaluated using Real-Time quantitative PCR microarray (qPCR microarray) targeting 84 resistance genes (Caselli et al., 2016; Vandini et al., 2014; Caselli et al., 2018). The microbiological surveillance in all treated hospitals ensured both the genetic stability of PCHS-derived *Bacillus* species and the absence of pathogenicity in hospitalized patients (Caselli et al., 2016; Caselli et al., 2018). Moreover, PCHS was demonstrated to inactivate enveloped viruses, including SARS-CoV-2, Herpesviruses, Influenza viruses, and Vaccina viruses (Caselli, 2017). Since conventional disinfectants often end up selecting for resistant microbes, the adoption of PCHS may represent a sustainable and effective alternative in fighting HAIs sustained by MDR microbes in clinical settings (D'Accolti et al., 2023). Hence, further supporting this aspect, PCHS implementation was shown to halve (-52%) the number of HAIs in a multicenter study (Caselli et al., 2017).

This benefit extends beyond hospitals, showing similar results in non-sanitary environments, such as schools and public transport environments. The main features of the PCHS system are reported in **Figure 9**.

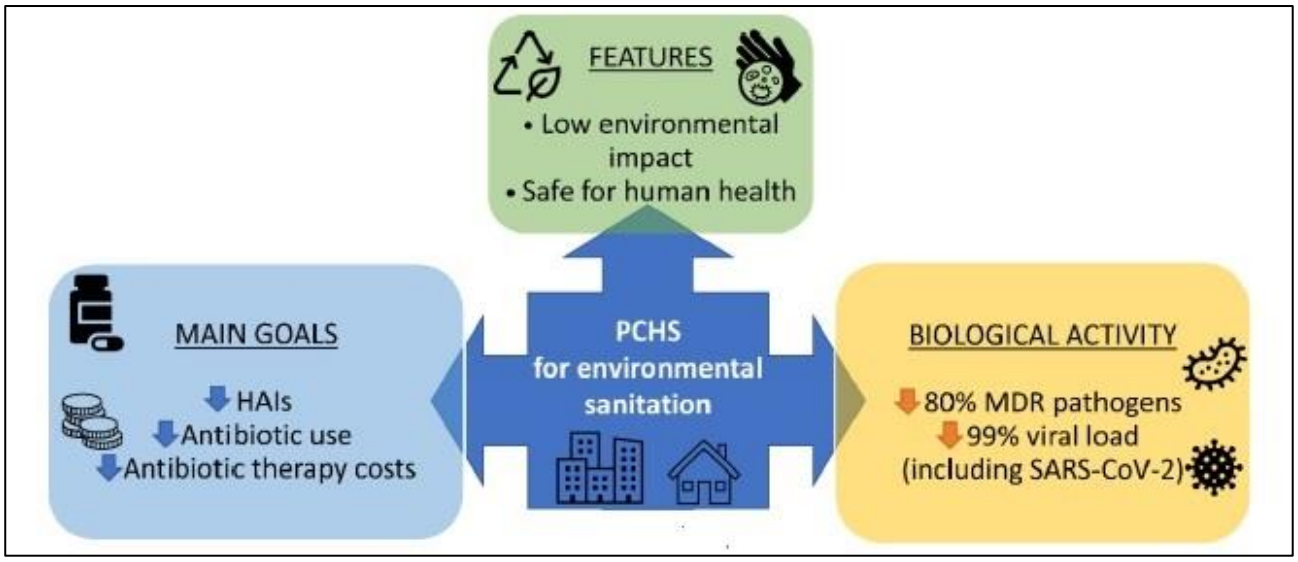


Figure 9. Schematic representation of PCHS features, main goals and biological activity.

The usage of PCHS in these settings could lead to lower pathogen transmission rates, enhancing overall community health and safety (Caselli et al., 2016). The flexibility of PCHS allows them to be integrated into various environments, promoting hygiene without the collateral damage associated with broad-spectrum chemical disinfectants (Caselli, 2017; D'Accolti et al., 2021; Caselli et al., 2016). Furthermore, evidence suggests that the impact of PCHS is not only limited to pathogen reduction but extends to the broad remodulation of microbial communities, contributing to enhanced overall sanitation efforts in both clinical and non-clinical settings (Caselli et al., 2018; D'Accolti et al., 2023). The demonstrated efficacy of probiotic cleaning, coupled with their reduced risk of driving antimicrobial resistance, positions them as advanced sanitation tools across varied environments. With ongoing research supporting their applications, the potential for transformative public health outcomes from widespread adoption remains a compelling proposition (Caselli, 2017; Caselli et al., 2018; D'Accolti et al., 2021; Caselli et al., 2016). Therefore, as institutions seek to navigate the challenges posed by microbial contamination, the proactive incorporation of probiotic cleaning systems could serve as a strategic intervention.

1.10. Safety of *Bacillus* probiotics

As mentioned, all the *Bacillus* species, with the exception of two species, are classified as Generally Regarded As Safe (GRAS) by the Food and Drug Administration (FDA, 1999) and by the European Food Safety Authority (EFSA) (EFSA, 2019), and their safety is extensively reported in the literature (Hong et al., 2008). *B. subtilis*, for example, is considered a Class 1 agent (no risk) in the National Institute of Health (NIH-US) Guidelines for Research Involving Recombinant DNA Molecules (DHHS, 1986) and, additionally, it is not toxigenic (no toxins production), indeed it has been granted

"Qualified Presumption of Safety" status by the European Food Safety Authority (Collins et al., 2011). According to the recent EFSA document, the requirements to be included in the QPS group include lack of pathogenicity and absence of acquired resistance gene (EFSA, 2005). All the species included in the *B. subtilis* group, as well as *Bacillus velezensis* and other *Bacillus* species, are considered to belong to the group that agreed with QPS (EFSA, 2022), being further considered safe as they have never been linked to human infections or diseases. Non-pathogenic bacteria of the *Bacillus* genus have a well-established history of safe use across various fields, including biotechnology, medicine, and agriculture. *Bacillus* species play a key role in the industrial production of bio-products such as proteins (Zweers et al., 2008), enzymes (Contesini et al., 2018), and biopolymers (Jin et al., 2016). Among these, *Bacillus subtilis* stands out as a prolific "cell factory" for producing industrial enzymes and biopharmaceutical proteins (Westers et al., 2004), thanks to its excellent fermentation capabilities and its status as one of the most extensively studied Gram-positive bacteria. These microorganisms have been used in food preparation, particularly for Asian foods, such as a particular strain of *B. subtilis* (*Bacillus subtilis* var. *Natto*), which is used in the production of Natto, a traditional Japanese food made by fermenting soybeans. In agriculture, *Bacillus subtilis* QST 713 is commonly used as a bio-fungicide (e.g., Serenade by Bayer) (Leyva Salas et al., 2017), and can be applied to soil in horticulture and agricultural fields (Trabelsi & Mhamdi, 2013). Similarly, *Bacillus pumilus* spores exhibit fungicidal properties after germination, with *B. pumilus* GB34 being used to prevent infections caused by *Rhizoctonia* and *Fusarium* in soybeans (EPA, 1991). Similarly, *Bacillus megaterium* is used for enzyme production (Vary et al., 2007). In the medical field, *Bacillus*-based spore products have been widely used as immunostimulatory agents to treat diseases, particularly in the gastrointestinal and urinary tracts (Ciprandi et al., 1986) (Mazza, 1994). For instance, Enterogermina® (Italy), containing *Bacillus clausii* spores, and Biosporin® (Ukraine), a mix of *B. subtilis* 2335 and *B. licheniformis* 2336, are used to treat and prevent intestinal disorders often resulting from prolonged antibiotic use (Mazza, 1994). *Bacillus* species have also been suggested as potential food additives for poultry. Multiple studies have shown that supplementing poultry diets with *Bacillus subtilis* PB6 enhances animal health, boosts body weight and egg production, and significantly reduces the presence of the pathogen *Clostridium perfringens* in the intestinal microbiota (Teo & Tan, 2006; Jayaraman et al., 2013). Additionally, *Bacillus subtilis* species are also used in aquaculture, where they have been found to inhibit the growth of pathogenic *Vibrio* species in marine crustaceans and parrot fish (Liu et al., 2018; Vaseeharan & Ramasamy, 2003). The main uses are summarized in **Figure 10**.

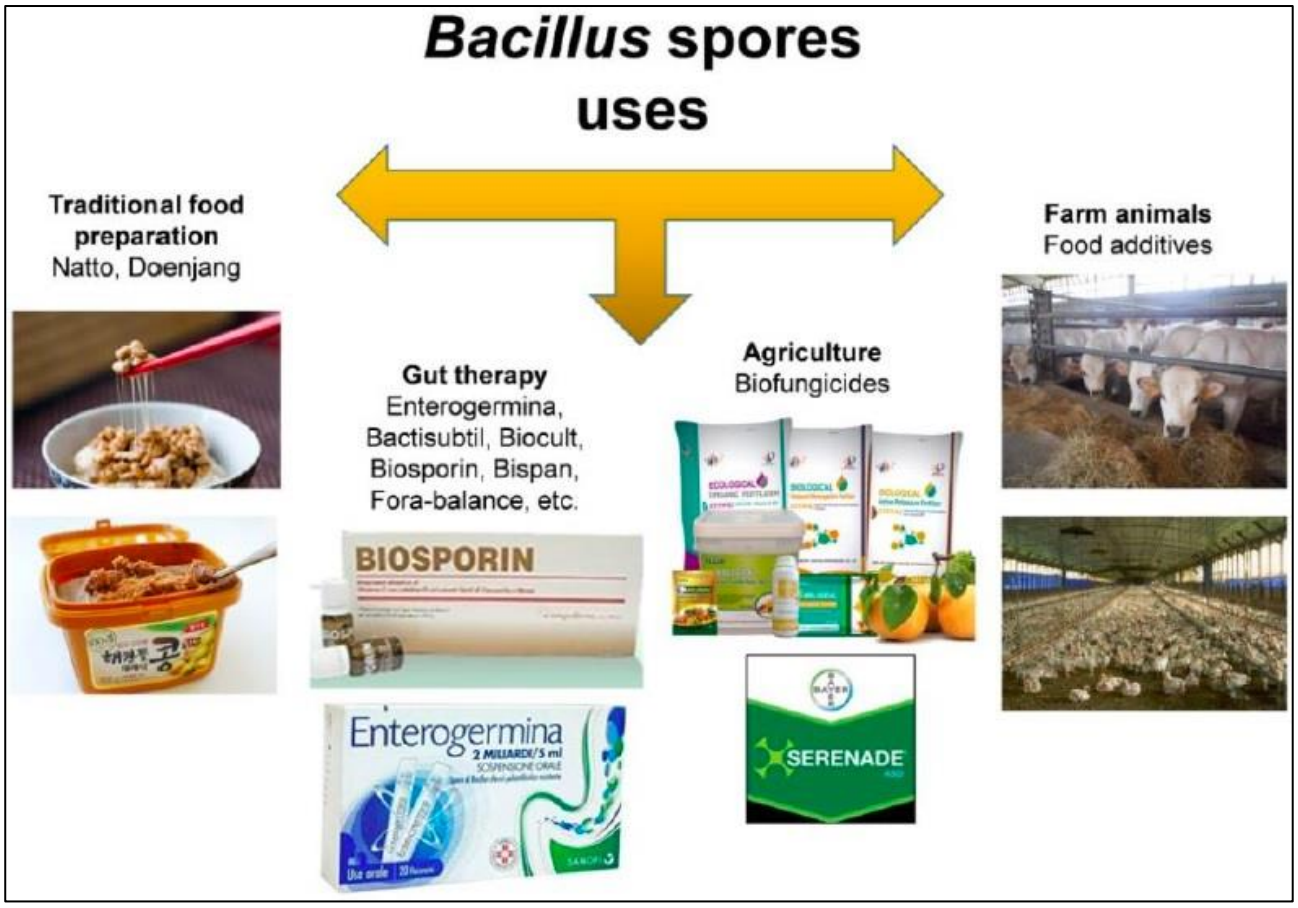


Figure 10. Main uses of *Bacillus* spores.

All these assumptions form the basis of the rationale for using *Bacillus* probiotics as sanitizing agents in the PCHS system. Indeed, PCHS-*Bacillus* have been used safely for decades in many applications aimed to preserve human, animal, and plant health (Caselli et al., 2019; Caselli, 2017), and were proven safe also for use as sanitizers in the hospital environment (Caselli et al., 2016).

Nevertheless, being applied in sanitary environments where potentially susceptible and fragile subjects are hosted, PCHS safety was carefully evaluated. First, a microbiological surveillance was implemented in each enrolled hospital to detect any infection caused by *Bacillus* probiotics in hospitalized patients, or even the presence of *Bacillus* probiotics in blood or urine samples from patients with HAIs. The results, including over 20.000 patients, showed the total absence of infectious or invasive risk in *Bacillus* probiotics (Caselli et al., 2016). Moreover, being *Bacillus* probiotics in continuous contact with surrounding pathogens on treated surfaces, their genetic stability was evaluated, to assess any genetic change over time. The results, obtained in around 300 samples from six different hospitals during almost two years, showed the complete absence of genetic alterations in all the *Bacillus* isolated from treated surfaces, highlighting their high genetic stability even after prolonged contact with pathogens (Bini et al., 2025). Moreover, the analysis performed by WGS

provided the complete genome profile of the species included in PCHS products, showing the absence of genes coding antibiotic resistance, virulence factors, and gene exchange ability.

1.11. COPMA Scpa and PCHS: a collaborative innovation

Copma S.c.p.a. is a Ferrara-based company, leader in cleaning and sanitizing systems, which patented and implemented the PCHS system set-up and optimized by our studies. This collaboration, lasting around 15 years, was based on the strict interaction between our university research group, developing and optimizing the PCHS system, and the productive company, aimed at exploiting the research results, applying the system *in situ* in sanitary and non-sanitary settings, and spreading its usage in the community. Specifically, the company is primarily dedicated to the production of the probiotic detergent, to sign contracts with facilities interested in its sanitization services and to train its own personnel for PCHS application. In detail, the PCHS system consists of six main components (ANMDO, 2014):

1. Process governance: The Always Connected Copma (ACC) portal is the interactive tool through which the entire service delivery process is constantly monitored; planning, management, and reporting of activities and results that are updated at each stage of the process, consistent with the principles of transparency.

2. Biocompetition: The cleaning solution based on probiotic bacteria of the *Bacillus* genus, can promote the dissolution of biofilm by facilitating its mechanical removal on the one hand and developing biological competition to reduce the resistance of potentially pathogenic agents on the other.

3. Materials and equipment: The cleaning equipment and materials are designed to make basic cleaning easier and more effective; thus, the application of the product is made by using prepreg cloths and nebulization. Operationally, the probiotic solution is diluted by the cleaning staff before being applied.

4. Training system: In healthcare, competence, and motivation are necessary, and for every employee, the first requirement must be the Culture of Hygiene. Awareness of one's responsibilities and the consequences of a correctly performed cleaning operation is crucial for the company. Hospitalized patients are the first recipients of hygiene services and require facilities with high levels of hygiene. For this reason, employees are trained through targeted training courses.

5. Environmental impact: Environmental policies are a driver for innovation. Environmental performance is designed and certified in accordance with the Minimum Environmental Criteria and in line with the Green Economy Act of 28 December 2015. The model developed with the PCHS system not only reduces the environmental impact with a significant decrease in consumption (-30/40% Kg of material waste, -4/12% Kg of products/detergents, -40/50% Kg of hazardous chemical substances, -15/28% of water and energy resources) but also guarantees the adoption of solutions aimed at zeroing the climate-altering effects (CO_2 emissions), through compensatory measures (planting trees, purchasing green energy, international greenhouse gas reduction projects, etc.). All this is guaranteed by the use of the registered trademark "Copma Zero CO_2", acquired by the PCHS system, which represents an eco-sustainable methodology.

6. Controls and certifications: As hygiene is the goal of the entire sanitization process and an integral part of infectious risk reduction strategies, it must be monitored to keep potential pathogens under control. Therefore, quality control is carried out against the certification systems indicated in the Operational Protocols. The service is verified at both process and result levels on the basis of self-control procedures and compliance with the following standards:

• UNI EN ISO 9001, to evaluate the customer satisfaction;

• UNI EN ISO 45001, to assess health and safety at work;

• Social Accountability System SA 8000, for the labor protection for company and workers;

• UNI EN ISO 1400, to evaluate the environmental management system;

• ANMDO-CERMET Standards, for the evaluation of good cleaning and sanitation procedures in healthcare facilities;

• PCHS Standard Compliance Indicators, to assess microbiological parameters defined by the University of Ferrara for the PCHS system (Vandini, Antonioli, et al., 2014);

• Carbon Footprint (CFP) ISO 14037, to quantify CO_2 emissions;

• UNI EN ISO 16636, for the management and control of pests (pest management) and the skills that must be possessed by professional service providers in order to protect public health, property, and the environment;

• EU Ecolabel certificate, for indoor cleaning services.

All the six main features of the PCHS system are summarized in **Figure 11**.

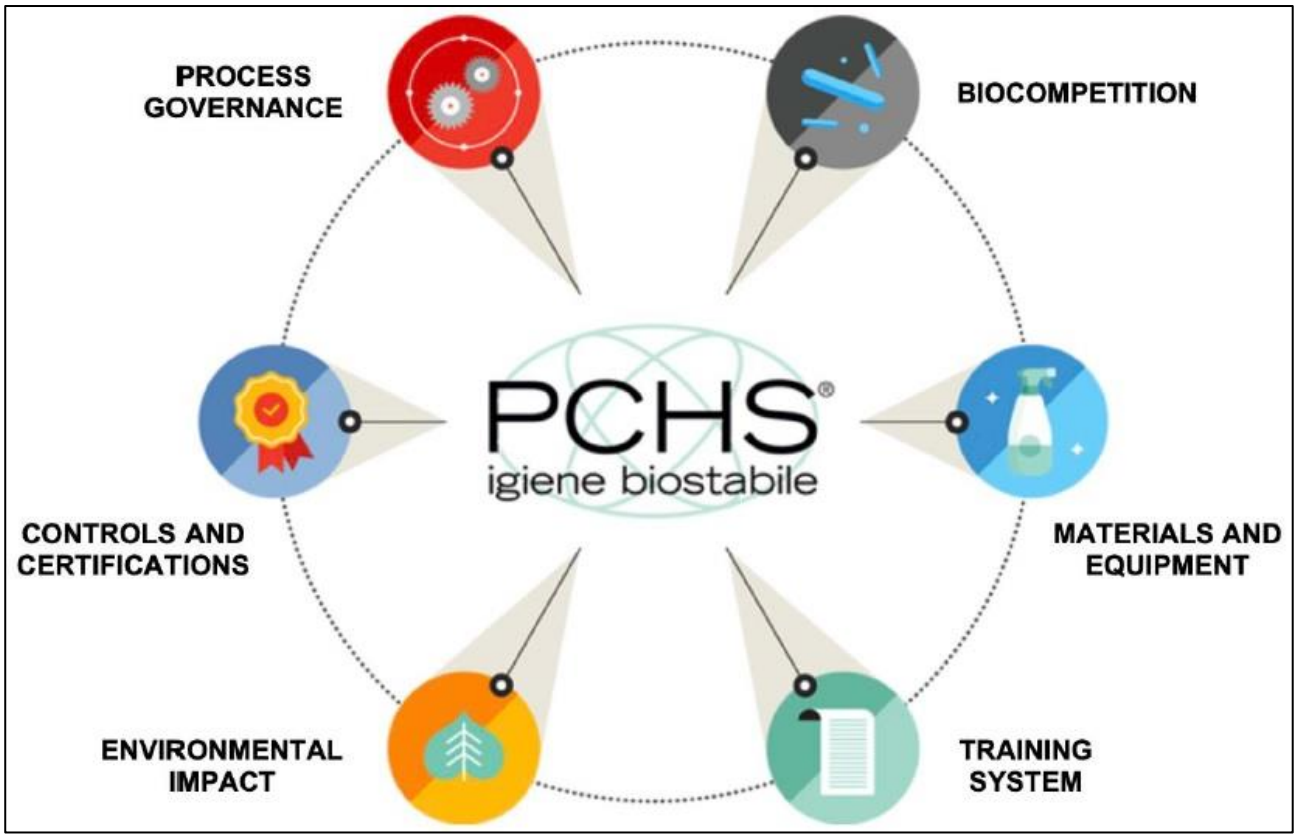


Figure 11. PCHS system and its six main components (https://pchs.it).

For Copma S.c.p.a., innovation is essential because it drives the development of safer, more efficient, and sustainable solutions that respond to evolving problems, such as AMR. In this light, the PCHS system is accompanied by a constant microbiological monitoring performed by our group, to assure that the system is applied correctly and that the results are in line with those obtained during the research and optimization phases.

2. AIM OF THE RESEARCH

The project of my PhD course was focused on developing and optimizing sustainable interventions addressed to infection control through a stable remodulation of the BE microbiome.

Conventional chemical disinfectants, used so far to control the BE bioburden, have a high environmental impact, can allow rapid recontamination of treated environments, and favor the persistence of resistant pathogens, contributing to AMR spread and increase. To overcome these limitations, we previously developed a Probiotic Cleaning Hygiene System (PCHS) able to stably decrease human pathogens in the sanitary environment 80% more than disinfectants, in parallel reducing existing AMR (up to -99.9%) and HAI incidence (-52%) compared to what observed with chemical sanitation.

Since PCHS studies performed so far were exclusively conducted in sanitary settings, during my PhD my research project aimed to assess its applicability and effect in non-sanitary environments, with the purpose to expand its potential usage particularly in high-traffic BEs. In parallel, other potential sustainable interventions were analyzed, aimed to improve the indoor microbial and air quality, namely the introduction of green plants and mechanical air ventilation systems.

To this aim, different studies have been performed during my PhD course in community environments characterized by high human density:

1) Two studies about PCHS applicability and effect on indoor microbial bioburden in the mass transportation environment; both studies were performed in collaboration with Azienda Trasporti Milano (ATM) and included the introduction of PCHS in two subway lines located in Milan (Italy) in pandemic and non-pandemic periods;
2) One study about the effect of combined sustainable interventions on indoor microbial and air quality in the school environment; the study was performed in collaboration with the high school Liceo Classico Ariosto (Ferrara, Italy), and included the introduction of green plants, ventilation systems, and PCHS.

In both kinds of study, a pre-post and case-control study design was applied, where PCHS and the other interventions were compared to previous and/or simultaneous controls.

All studies included the analyses of surface and air microbiomes. The samples were analyzed by both microbiological and molecular methods, in order to profile the composition of the BE microbiome and its AMR features. Moreover, in the school study, environmental parameters including temperature, humidity and CO_2 concentration were included in the analysis.

Overall, the results showed that PCHS can be considered a useful tool to control the BE bioburden without exacerbating the AMR spread, since it decreased the presence of pathogens 80-99% more

than chemical disinfectants, and similarly abated their AMR up to 99.9% compared to controls. Superimposable results were observed in mass transport and school environments.

Furthermore, significant results were also obtained by introducing plants and ventilation systems in the school environment, supporting the use of combined green interventions to ameliorate the BE microbiome and provide healthier community environments.

3. MATERIAL AND METHODS

3.1. Shaping the BE microbiome of on subway trains: first study (pandemic period)

3.1.1. Study design

The study aimed to compare the effects of PCHS versus chemical sanitation on the subway microbiome, focusing specifically on human pathogens and related AMR. Conducted in collaboration with the Azienda Trasporti Milanesi (ATM, Milan, Italy), after approval by the company's technical scientific committee, it involved two driverless underground trains of the line M5 with similar characteristics (**Figure 12**).

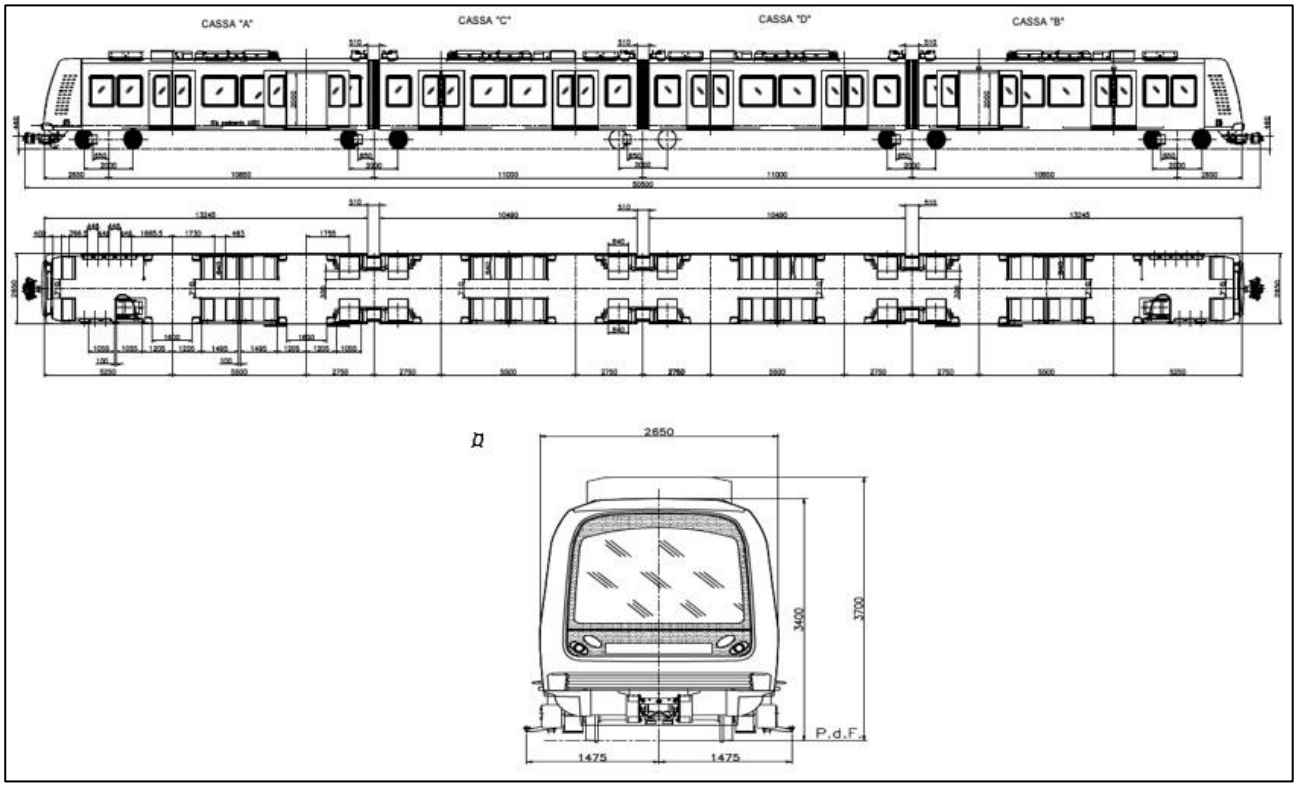


Figure 12. Plan, elevations and characteristics of the subway line M5 being tested

Each train had four compartments, 96 seats, 438 standing places, a length of 50.5 m, and a surface area of 73 m^2. At the time of the study, the ATM cleaning protocol, due to current Italian COVID-19 regulations, consisted in chemical disinfections including ethanol, chlorine, and ammonium salt-based products. To compare the effectiveness of PCHS with chemical disinfection, one train (n°5508)

continued to receive the routine chemical disinfection (control), while the other (n°5511) underwent the PCHS sanitation (Probiotic Cleaning Hygiene System; PCHS, Copma S.c.p.a., Ferrara, Italy). Due to national guidelines requiring chemical disinfectants, PCHS could not completely replace the chemical disinfection. Thus, after compatibility in vitro tests, PCHS was used in substitution of chlorine disinfection, whereas ethanol and ammonium disinfectants remained in use. PCHS was applied using prepreg cloths and nebulization, following the same schedule as the chemical disinfectants, and was applied 30 minutes after alcohol/ammonium disinfection, to preserve the viability of the PCHS probiotics. The study lasted 12 weeks, from September to December 2021. Throughout the study, data on train bioburden and passenger flows were collected to analyze the relationships between contamination levels and the type of sanitation used.

3.1.2. Compatibility in vitro tests

Before PCHS implementation on subway trains, the compatibility between PCHS and each of the used disinfectants was evaluated in vitro. Specifically, we evaluated the viability of PCHS-derived *Bacillus* (referred to as PCHS-*Bacillus*) after exposure to chlorine, ethanol, and ammonium-based disinfectants on hard, non-porous surfaces under controlled laboratory conditions. Two types of assays were conducted to assess:

- the viability of PCHS-*Bacillus* on surfaces subsequently treated with disinfectants
- the viability of PCHS-*Bacillus* on surfaces that had been previously treated with disinfectants.

The latter assay included tests with both PCHS-*Bacillus* spores and germinated vegetative forms. In each assay, sterile water and a sporicidal disinfectant (Viroxid Spray, IDS SpA, Savona, Italy) were used as negative and positive controls, respectively. In the first assay, the PCHS detergent, which contained 10^7 *Bacillus* spores/mL, was diluted 1:100 in sterile water. Then, 10 µL of this diluted detergent (equivalent to 10^3 spores) was applied to a 24 cm^2 surface and left to dry. Chemical disinfectants were prepared according to the manufacturer's instructions and were uniformly applied to the PCHS-treated surfaces using nebulization. The disinfectants were left to act for the durations specified by the manufacturer: 1 minute for chlorine and ethanol-based products, and 15 minutes for ammonium-based products. At the end of the exposure period, residual PCHS-*Bacillus* were collected on 24 cm^2 contact plates (55 mm diameter Replicate Organism Detection and Counting, RODAC) containing tryptic soy agar (TSA, Scharlab, Milan, Italy), a nonselective general medium. The RODAC plates were incubated for 24 hours at 37 °C, after which the number of *Bacillus* CFU was enumerated. In the second assay, disinfectants were uniformly applied to surfaces via nebulization and allowed to dry. After 0.5, 1, 2, 3, 4, and 24 hours from disinfectant application, 10 µL of diluted PCHS detergent (containing 10^3 spores) was spread on a 24 cm^2 area of the pre-disinfected surface.

Following a contact time of 1 minute for chlorine and ethanol-based disinfectants or 15 minutes for ammonium-based disinfectants, the amount of PCHS-*Bacillus* was assessed using TSA RODAC sampling, with CFU counted after 24 hours of incubation at 37 °C. This assay was also performed with germinated spores of PCHS-*Bacillus*, which were first allowed to grow overnight in tryptic soy broth (TSB, Biolife, Monza, Italy) at 37 °C. The bacterial suspension was then diluted to a final concentration of 10^5 CFU/mL, and 10 μL (equivalent to 10^3 bacteria) was applied to the pre-disinfected surfaces. Following 1–15 min of contact time, *Bacillus* CFU were enumerated after TSA RODAC collection and 24 h incubation.

3.1.3. Sanitation protocols on subway trains

Both chemical and PCHS sanitation were carried out by the same cleaning company (Fulgens Italia S.R.L, Italy), leader in environmental sanitation and trained for the use of both disinfectants and PCHS. The chemical disinfection included:

1. Daily cleaning of seats, handrails, doors, and floors with alcohol-based disinfectant (KEM Alcohol Duo, 78% ethanol; Kemika SpA, Alessandria, Italy) and chlorine-based disinfectant (Biospot, 200 ppm active chlorine; Kemika SpA, Alessandria, Italy);
2. Weekly treatment with a quaternary ammonium salt-based product (Hygiene Spray Professional, containing benzalkonium chloride and Ophenylphenol; GEN-ART srl, Lanuvio, Rome, Italy) applied via nebulization;
3. Bimonthly comprehensive disinfection using both alcohol- and chlorine-based products. PCHS sanitation was performed using both prepreg cloths and nebulization, following the same method as for chemical disinfectants, and adhered to the existing cleaning schedule and surface types in the two selected trains.

PCHS sanitation replaced chlorine-based disinfection, while ethanol and ammonium disinfectants continued to be used, based on current national COVID-19 guidelines. According to compatibility assay results, PCHS was applied 30 minutes after the use of alcohol- or ammonium-based disinfectants. All sanitation procedures were performed in the absence of people, at the end of the daily train run.

3.1.4. Monitoring microbial contamination: environmental sampling

Six sampling campaigns were performed on both enrolled trains at the following intervals: T0 (before PCHS implementation) and T1, T2, T3, T4, and T5, which occurred 2, 4, 6, 9, and 12 weeks after PCHS was introduced on the PCHS-train. Sampling was performed biweekly, except for the final

timepoint (T5), which was delayed by an additional week due to the unavailability of ATM personnel to assist sampling technicians during the early December holiday period. Sampling took place late at night, after the train's last run and before the cleaning protocols were applied. At each time point, 12 points across the train were sampled, covering different areas: floors (3 samples), seats (3 samples), handrails (2 samples), doors (2 samples), and air filters (2 samples). Air filters were not available for sampling at T3 and T5. Each point was sampled simultaneously using two different methods according to subsequent microbiological or molecular analyses. All samples were immediately refrigerated at 4 °C and transported to the laboratory within 24 h. For microbiological analyses, samples were taken in duplicate using RODAC (Replicate Organism Detection and Counting) plates (**Figure 13**). The media used included:

- Tryptic Soy agar, for CFU count of total microorganisms including *Bacillus* spp. (Scharlab, Milan, Italy);
- Baird Parker agar, for CFU count of *Staphylococcus* spp. (Scharlab, Milan, Italy).
- Cetrimide agar, for CFU count of *Pseudomonas* spp. (Scharlab, Milan, Italy).
- MacConkey agar, for CFU count of *Enterobacteriaceae* (Scharlab, Milan, Italy).
- *Clostridium difficile* selective agar, for CFU count of *Clostridium* difficile (Lickson, Palermo, Italy).
- Sabouraud dextrose agar, for CFU count of fungi (Liofilchem, Millipore, Milan, Italy).

For molecular analyses, the same 12 points were sampled in duplicate using sterile rayon swabs rubbed over a 100 cm^2 surface (**Figure 14**). The swabs were then placed in either 5 mL of TSB broth (Biolife, Monza, Italy) or 0.4 mL of sterile PBS depending on the type of analysis to be performed. All samples were immediately refrigerated and transported to the laboratory for processing within 12 hours.

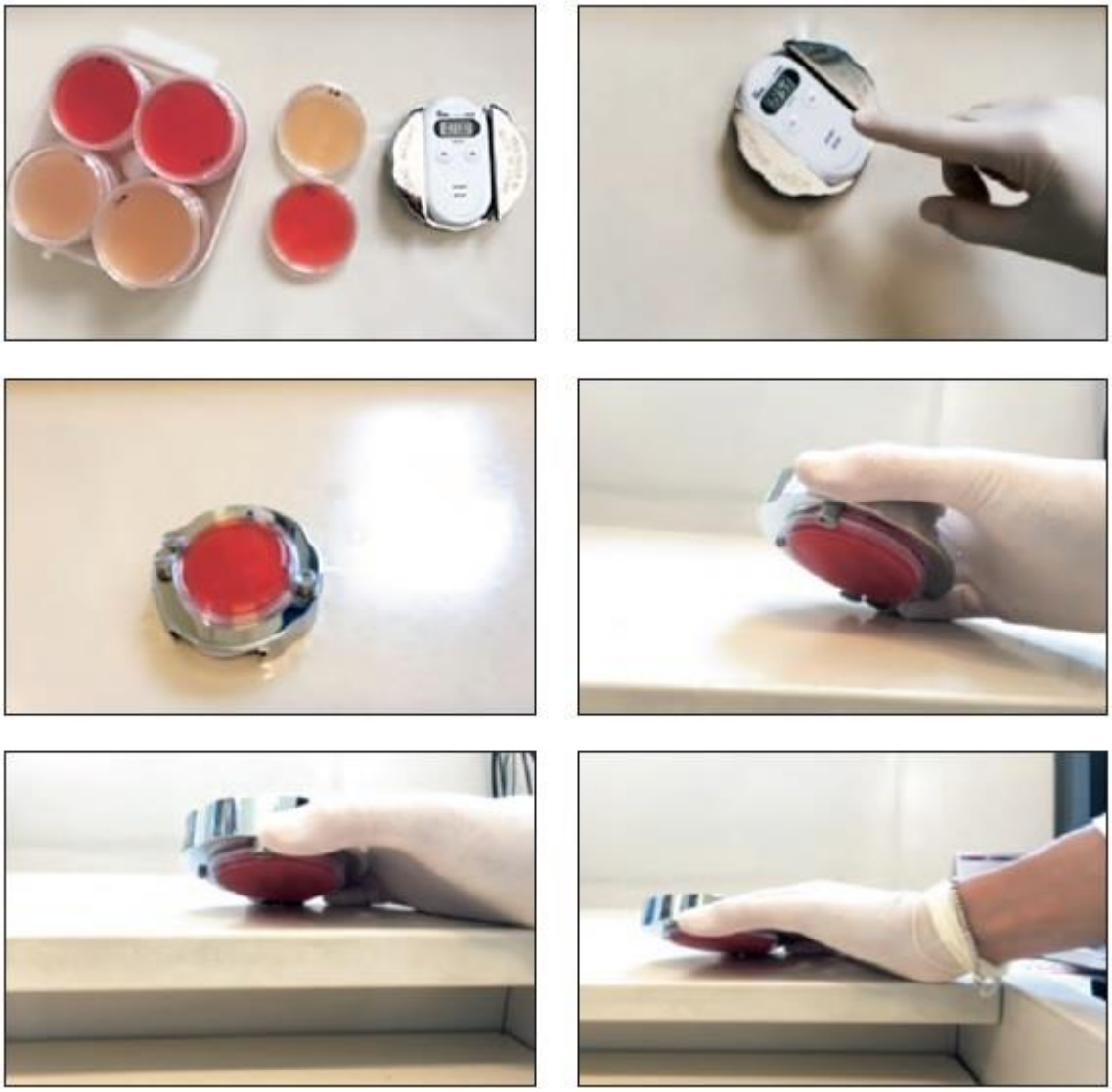


Figure 13. RODAC contact plate procedure.

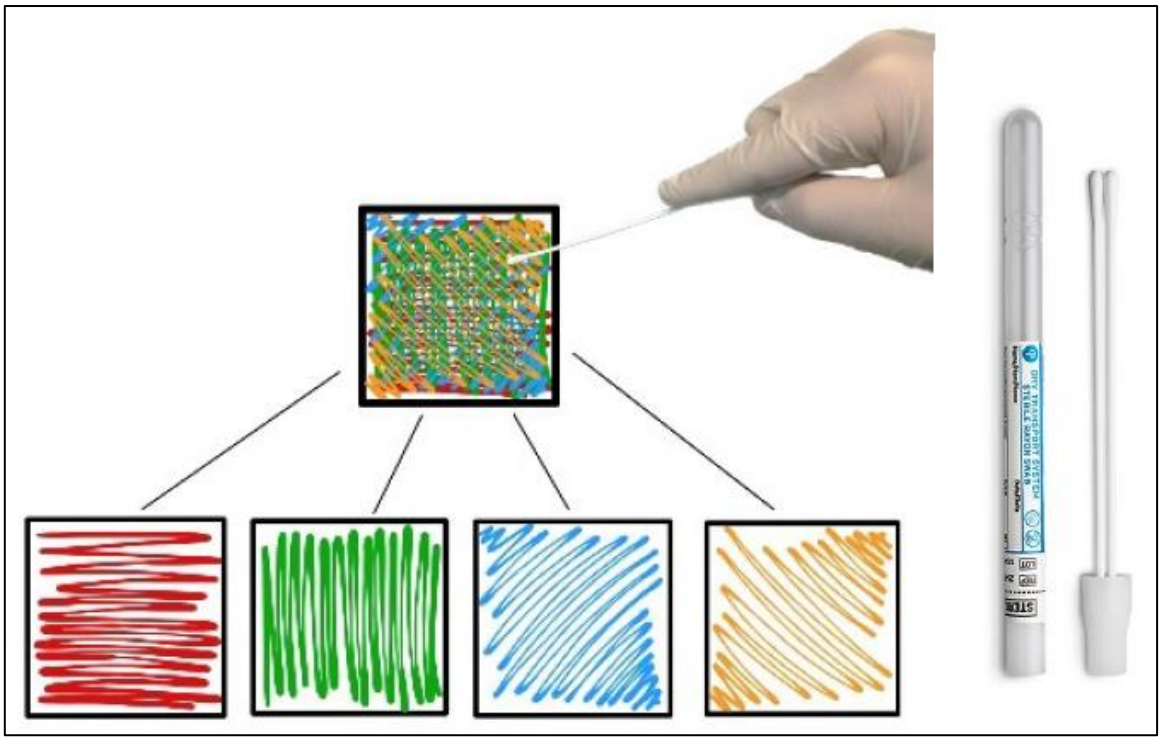


Figure 14. Sampling method by rubbing sterile swab on surfaces.

3.1.5. Microbiological analyses

The RODAC plates obtained from sampling train surfaces and air filters were incubated under specific time and temperature conditions depending on the microorganism type. Briefly, TSA plates were incubated at 37°C for 24 hours, selective bacteria media were incubated at 37°C for 48 hours, and fungal After incubation, CFU grown on the plates were counted. At each time point, a total of 240 samples from surfaces and 48 samples from air filters were collected from both trains, except at T3 and T5, when air filters were unavailable, resulting in 240 samples collected. Overall, 1,632 samples were collected and analyzed throughout the study.

3.1.6. Molecular analyses

Overall, 136 TSB swabs and 136 PBS swabs were collected from each train (120 from surfaces and 16 from air filters), for a total of 272 samples collected and analyzed.

The swab samples collected in TSB broth were incubated at 37 °C for 24 h to obtain a controlled microbial amplification. The microbial suspension was then collected by centrifugation (12,000 xg for 5 minutes), and microbial DNA was extracted from the resulting pellet using the commercial kit (Exgene Cell SV mini kit, GeneAll, Seoul, South Korea). Extracted DNA was kept at -20°C until use. An amount of 1 µg of DNA was analyzed by real-time quantitative PCR (qPCR) microarray, using the Microbial DNA qPCR Array for Antibiotic Resistance Genes (Qiagen, Germany). Prior to qPCR

array analysis, the amplifiability of the extracted bacterial DNA was verified by using a universal PanB PCR. In fact, since the PanB PCR is designed in a conserved region present in all bacterial species, it allows the detection and confirmation of the presence of all bacteria. The qPCR microarray allows for the simultaneous detection and quantification of 84 antibiotic resistance genes belonging to the aminoglycoside, β-lactam, macrolide, fluoroquinolone, tetracycline, vancomycin, and other multiple resistance mechanisms classes. The plate for this type of analysis consists of 96 wells, each containing primers and TaqMan probes specific for each detected gene and for reaction controls. Specifically, this technique permitted the detection of:

- 84 AMR genes (A01-G11, H01);
- *S. aureus* identification (G12);
- *S. aureus* leucocidin (H02);
- IgG binding protein A of *S. aureus* (H03);
- Controls for the presence of bacterial DNA (Pan B-1, Pan B-3) (H04-H09).

The swabs samples collected in PBS were instead frozen at −80 °C until use. At the moment of analysis, the total nucleic acids (TNA9 were extracted by samples using a 52 Maxwell CSC platform equipped with the HT Viral TNA Kit (Promega, Milan, Italy). The manufacturer's TNA extraction protocol was adjusted to enhance the lysis of Gram-positive bacteria by adding an enzymatic lysis step involving lysozyme digestion. An amount of 100 ng of TNA was used to evidence and quantify the SARS-CoV-2 RNA genome by qPCR after retrotranscription. The presence of the SARS-CoV-2 RNA genome in both enrolled trains was assessed by using the SARS-CoV-2 ddPCR Kit (Bio-Rad Laboratories, Milan, Italy) (Soffritti et al., 2021). 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.

In parallel, an amount of 1 μg of TNA was analyzed by 16S rRNA NGS, in collaboration with the University of Trieste. 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 the primers 53 B338F_P1-adaptor and U534R_A_barcode, targeting the V3 region of the 16S rRNA gene, with a different barcode for each sample linked to the reverse primer. Negative control, including no DNA template, was also included in the samples analysis. PCR reactions were performed using Kapa HiFi Hotstart ready mix 2X (Kapa Biosystems, Massachusetts, MA, USA) and BSA 400 ng/μL. PCR amplification products from each sample (100 ng) were combined to create a pooled library with a

final concentration of 100 pM, following the manufacturer's instructions. Template preparation was carried out using the Ion OneTouch™ 2 System and the Ion PGM Hi-Q View OT2 200 kit (Life Technologies, Grand Island, New York, NY, USA). Sequencing was conducted using the Ion PGM™ System and the Ion PGM Hi-Q View sequencing kit (Life Technologies, New York, NY, USA). Sequence data were processed with the Quantitative Insights Into Microbial Ecology (QIIME 2 2020.2) software, which 54 facilitates the analysis of high-throughput community sequencing data (Bolyen et al., 2019). High-quality sequences (Q > 25) were demultiplexed and filtered using default parameters, with the exception of sequence length (150 bp). Sequences containing ambiguous bases or with homopolymer lengths greater than 8 were excluded. Operational Taxonomic Units (OTUs) were clustered, and taxonomy assignment at the genus level was performed using the SILVA V.132 reference database (Quast et al., 2012), with a 97% similarity threshold.

3.2. Shaping the BE microbiome of on subway trains: second study (non-pandemic period)

3.2.1. Study design

The second subway study was also conducted in collaboration with ATM and involved two underground trains belonging to the new M4 metro line, identified by the serial numbers 4404 and 4435. Both trains had similar characteristics: each had four compartments, offering a total of 96 seats and 438 standing places. Each train was 50.5 m long, with a total floor area of 73 m².

In this study, PCHS totally replaced chemical sanitation, as it was not anymore mandatory the use of disinfection for mas transports, after the end of the pandemic COVID-19 regulations. The study was approved by the Company's Technical Scientific Committee. The experimental protocol, as already employed on the two train of the line M5 (as described in the paragraph **3.1.5**. **Microbiological analyses**) was structured according to a pre-post model and divided into two main phases:

- Pre-PCHS phase (T0): sanitation carried out with chemical products such as alcohol, quaternary ammonium salts and conventional chlorine-based disinfectants, following the procedures established by ATM.

- PCHS phase (T1–T3): sanitation carried out using PCHS, applied using pre-soaked cloths for daily cleaning of surfaces and weekly manual misting using sprayers on air vents, ceilings, seats and less accessible areas

To evaluate the effectiveness of the two sanitization methods, four sampling campaigns were conducted for each of the trains, before (T0) and after the introduction of the PCHS system respectively: 2 weeks (T1), 7 weeks (T2) and 4 months (T3) from the beginning of the treatment, as illustrated in **Figure 15**.

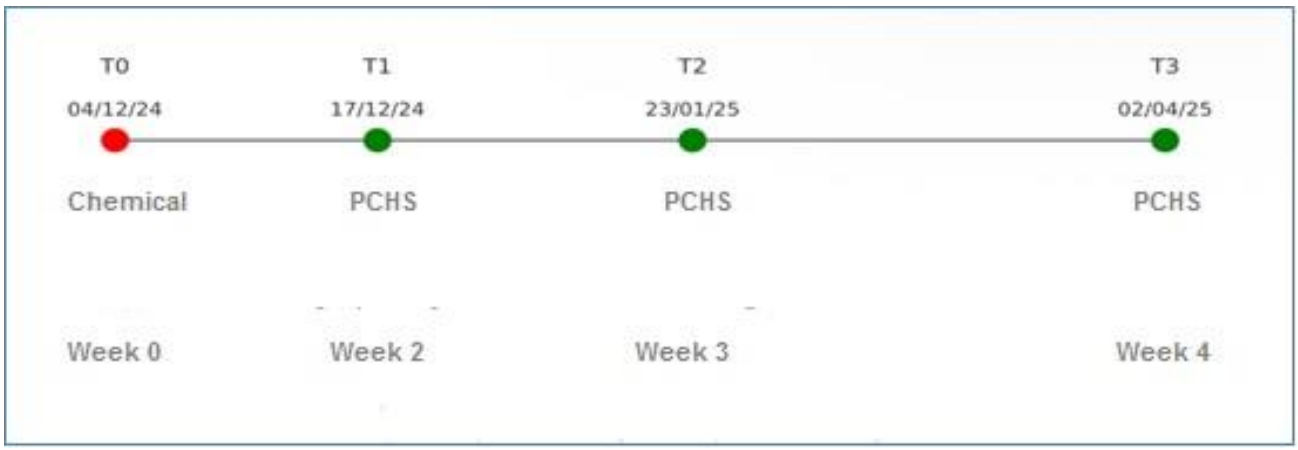


Figure 15. Timeline of sampling campaigns and type of sanitization used.

3.2.2. Monitoring microbial contamination: environmental sampling

Samplings was carried out on both trains at four time points: T0, before the implementation of the PCHS system, and T1, T2, and T3, respectively, 2 weeks, 7 weeks, and 4 months after the introduction of the PCHS. The sampling had been conducted between 11:00 a.m. and 2:00 p.m. at the end of the train's services and before the scheduled train sanitation procedures began. Consistent with previous studies, samples were taken from floors, seats and air filters.

It must be remarked that the air filters were not available at T1. Adopting the same way of sampling employed on the M5 subway line, both molecular and microbiological, each point was sampled simultaneously using two methods: swabs for molecular analyses and RODAC plates for the detection and quantification of bacterial and fungal CFU.

3.2.3. Microbiological analyses

The microbiological analysis has been conducted as already described. Briefly, the RODAC plates contained the following five specific culture media:

- Trypton Soy Agar (TSA) medium (Scharlab, Milan, Italy), a general non-selective medium intended for the growth of total microorganisms present on the sampled surfaces.
- Baird Parker agar (BPA) medium (Scharlab, Milan, Italy), specific for the growth of bacteria of the *Staphylococcus* spp. and *Bacillu*s spp..

- MacConkey agar (MCA) medium (Scharlab, Milan, Italy), specific for the growth of *Enterobacteriaceae* spp..
- Cetrimide agar (CA) (Scharlab, Milan, Italy), specific for the growth of *Pseudomonas* spp.;
- Sabouraud dextrose agar (SDA) medium (Liofilchem, Millipore, Milan, Italy), specific for the growth of fungi, including *Candida* spp and *Aspergillus* spp..

A total of 440 samples were collected from the sampled surfaces.

Following sampling, the plates were placed in a refrigerated container (2-10°C) and transported to the Microbiology laboratory within 8 hours for further analysis. The plates were then placed in an incubator with variable thermal parameters depending on the type of microorganism, as follows:

- TSA RODAC plates: 24 h at 37 °C
- RODAC plates with selective media (BPA, MCA, CA): 48 h at 37 °C
- SDA RODAC plates: 72 h at 25°C

At the end of the incubation times, microbial growth was analyzed by CFU counting.

3.2.4. Molecular analyses

For molecular analyses, the same points were sampled in duplicate using sterile rayon swabs, rubbed on a 100 cm² surface delimited by a special sterile plastic mask, as illustrated in paragraph **3.1.6**. The swabs were then put in 5 mL of TSB, immediately refrigerated and transported to the Microbiology Section within a maximum of 8 hours. Collected samples were incubated for 24 hours at 37°C with shaking, to promote controlled growth of the microbial population collected during sampling. At the end of the incubation, the bacterial cells were collected by centrifugation at 12,000xg for 5 minutes at 4°C and subsequently stored at -20°C until DNA extraction and analysis by the same qPCR microarray (Qiagen, Antibiotic Resistance Genes) previously described.

3.3. Shaping the BE microbiome in the school environment: a combined sustainable intervention including PCHS

3.3.1. Study design

A pre–post case–control study was conducted at the High School “Liceo Ludovico Ariosto” in Ferrara, from July 2023 to May 2024. The investigation represented a part of a Path for Transversal Skills and Orientation (PTSO) project, approved by the school Principal. Two classrooms, each with a floor area of 48.7 m^2 and comparable physical characteristics, were enrolled. One classroom was allocated to an experimental condition (test room, TR) in which specific interventions were

implemented, while the other classroom functioned as a control (control room, CR) and did not receive any intervention. The principal features of the classrooms are summarized in **Table 1**. Both rooms featured large outward-facing windows to provide natural light and ventilation. Additional ceiling windows located at a height of 5.15 m and windows facing the main corridor were arranged opposite the outward-facing windows to promote horizontal airflow within the rooms. Every classroom had a total of 17 student desks and one teacher desk.

Features	Measures
Length	8.47 m
Width	5.75 m
Ceiling height	3.23 m
Maximum height	5.15 m
Floor area	48.7 m^2
Volume	170 m^3

Table 1. Main features of the enrolled classrooms.

3.3.2. Interventions applied in the enrolled classroom

Three successive interventions were carried out in the test room (TR) to enhance indoor environmental quality:

- Introduction of 140 live green plants (T1).
- Installation of a mechanical ventilation system (T2).
- Substitution of the conventional chemical-based sanitation with PCHS (T3).

The plant species selected for T1 were *Epipremnum aureum* and *Tillandsia kammii Rauh*, chosen for their robustness, suitability for greening walls, and ease of handling. Students were engaged in plant care and potential propagation as part of the Path for Transversal Skills and Orientation (PTSO) project. All plant material introduced in the TR was supplied by the Botanical Garden of the University of Ferrara and remained in place throughout the study.

The ventilation upgrade in T2 employed the vertically oriented VEX 380S unit (Aldes, Modena, Italy), delivering a maximum airflow of 1000 m^3/h and incorporating a fan coil unit for heating. The probiotic-based sanitation approach implemented in T3 replaced the prior chemical-based protocol with beneficial microbial formulations designed to mitigate conventional sanitation-related concerns. As in the studies carried out in the subway environment, PCHS was implemented in the TR during the T3 period, and replaced the daily sanitation performed by alcohol-based products. Both conventional and PCHS sanitations were carried out daily at the end of morning lessons by adequately

trained school operators, according to standard procedures. The study design and timing are schematically summarized in **Figure 16**.

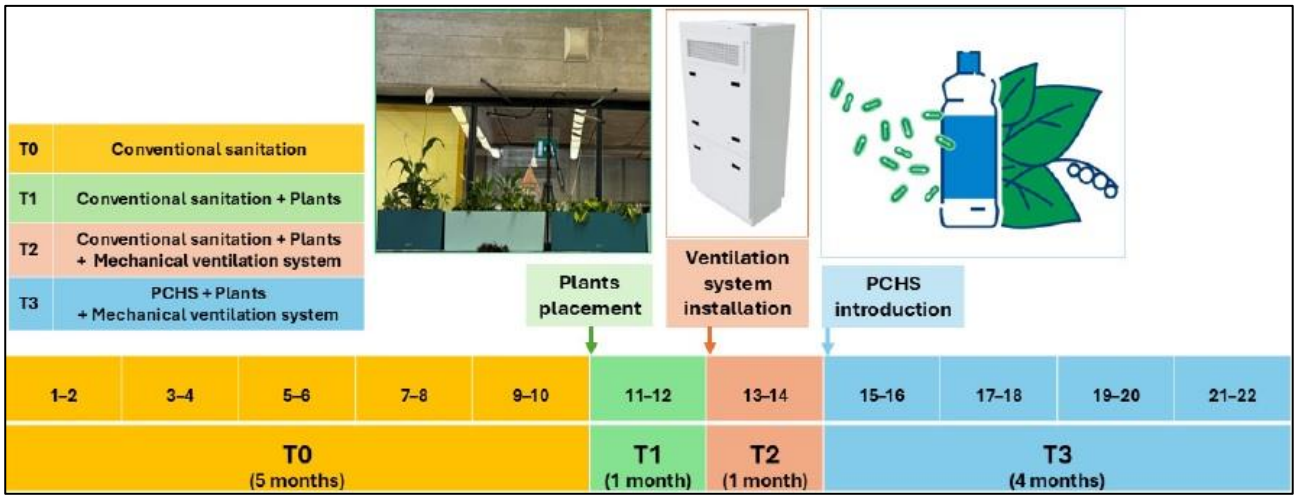


Figure 16. Timeline of sampling campaigns, plants placement, and type of sanitization used.

3.3.3. Environmental monitoring

The TR environment was monitored *in continuum* over the entire study period. Environmental parameters assessed included temperature (°C), relative humidity (RH), and carbon dioxide (CO_2) concentrations. Measurements were obtained with Testo 160 IAQ Wi-Fi Data Loggers (Testo S.p.A., Milan, Italy), which provided continuous readings via two sensors deployed in the enrolled classroom.

Microbial monitoring occurred bimonthly across all study phases, yielding a total of 22 sampling campaigns. Of these, 10 occurred during phase T0, 2 during T1, 2 during T2, and 8 during T3. At each sampling event, both surface and air samples were collected before and after students' lessons to assess the influence of student presence on microbial contamination. Surface sampling targeted the floor, the teacher's desk, and two student tables (**Figure 17**). All sampling points were conducted using two complementary methods, corresponding to the planned analytical workflows described subsequently.

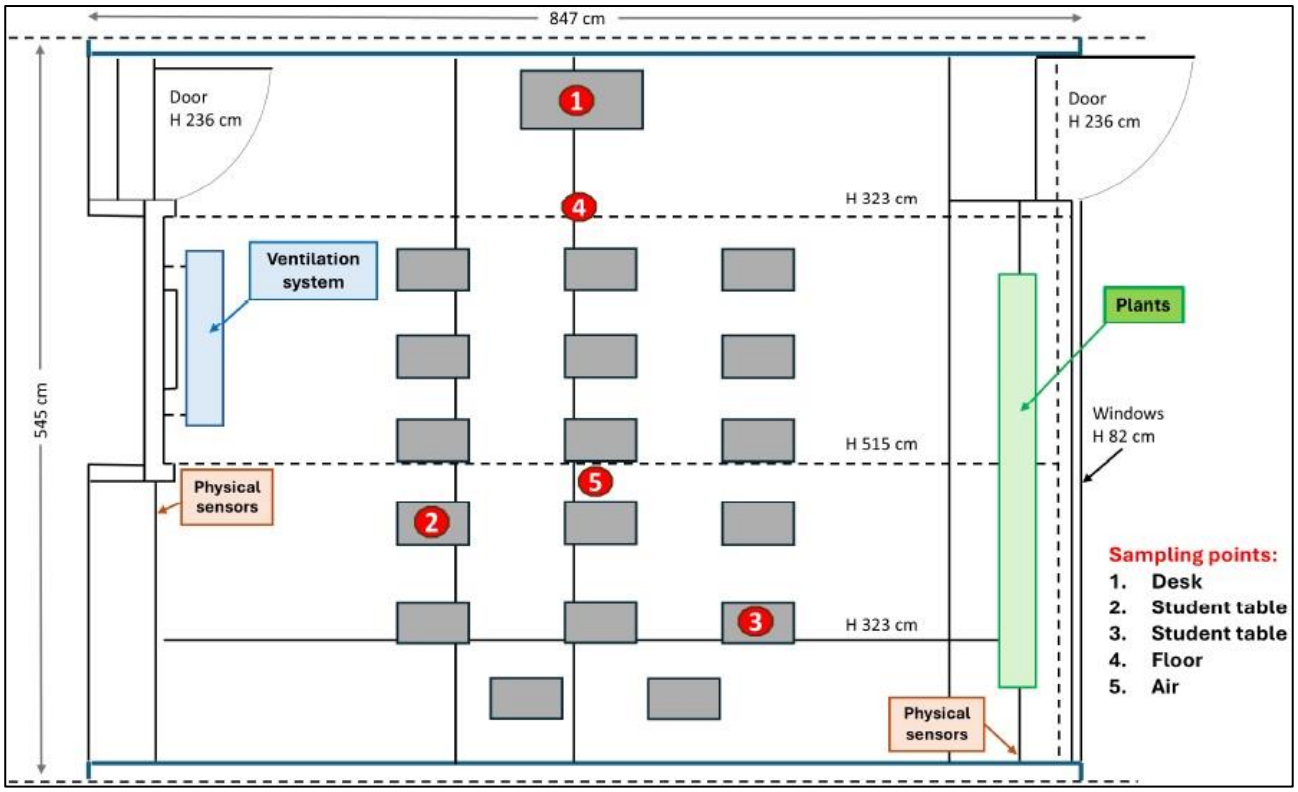


Figure 17. Floor plan of the enrolled classrooms, including sampling point locations.

3.3.4. Microbiological analyses

For microbiological analyses, samples were collected in duplicate by using RODAC contact plates 55 mm diameter (corresponding to a 24 cm^2 surface) containing the following general and specific culture media:

- Plate Count agar (PCA, Biolife, Monza, Italy) for the total bacterial count.
- Baird Parker Agar (BP, Sharlab, Milan, Italy) for *Staphylococcus* and *Bacillus* spp..
- Mac Conkey Agar (MCA, Sharlab, Milan, Italy) for *Enterobacteriaceae*.
- Cetrimide agar (CA, Sharlab, Milan, Italy) for *Pseudomonas* spp..
- Dichloran Glycerol Agar (DG18, Sharlab, Milan, Italy) for the total fungi, including *Candida* and *Aspergillus* spp..

Air sampling was performed at the center of the classrooms via the Surface Air System (SAS) instrument (VWR International, Milan, Italy), collecting 1 m^3 of air on the same media used for surface sampling (**Figure 18**).

Figure 18. insertion of a RODAC plate on a SAS air sampler

Microbiological analyses were carried out on the collected RODAC plates by appropriate incubation and CFU count. Briefly, bacteria were incubated at 37 ˚C for 24 h or 48 h on general or selective media, respectively. Fungi were incubated at 25 ˚C for 72 h. At the end of incubation, microbial growth was determined by CFU enumeration. Overall, a total of 2688 microbiological samples were collected from surfaces and the air, including 1280 samples collected during T0, 256 during both T1 and T2, and 896 during T3.

3.3.5. Molecular analyses

For molecular analyses, duplicate samples were collected from the same surface points by sterile rayon swabs rubbed on a 100 cm^2 area and then placed in 5 mL of sterile TSB (Biolife, Monza, Italy). All collected samples were immediately refrigerated at 2-10 °C and transported to the laboratory within 12 hours. Molecular analyses were performed on the swabs collected in TSB broth. Samples were incubated at 37°C for 24 h, and microbes were collected by centrifugation (12,000× g for 5 min at 4 ◦C). Total DNA was extracted from the pelletized microbes by a commercial kit (Exgene Cell SV mini kit, Gene All, Seoul, South Korea), following the manufacturer's instructions. Extracted DNA was quantified by spectrophotometric reading using a nanodrop at 260/280 nm wavelength, and DNA amplifiability was checked by qualitative PCR amplifying a conserved region in the bacteria (*panB*) (Caselli et al., 2018, Caselli et al. 2016). One µg of extracted DNA was then analyzed using the Microbial DNA qPCR Array for Antibiotic Resistance Genes (Qiagen, Hilden, Germany), as previously described.

3.3.6. Assessment of indoor air quality

The main purpose of this phase was to evaluate the indoor air quality (IAQ) and CO_2 concentration in the enrolled classroom (**Figure 19 a,b**), during natural ventilation (first phase) and mechanical ventilation (second phase), by using a dual-flow heat recovery unit with post-heating support (MVHR, mechanical ventilation with heat recovery). Data were recorded over an entire school year period, specifically focusing on temperature, humidity, and CO_2 concentration levels within the classroom, as well as in the adjacent corridor and outdoors. Measurements were collected over nine months during the season from March 2023 to June 2024. The first period, from March to June 2023, was utilized to check the sensors installed in the classroom and external environment, as well as to verify the data collection system. The two months of data—from October to December 2023—correspond to conditions without mechanical ventilation, while the remaining months correspond to periods with active mechanical ventilation (from January 2024 to June 2024). During the latter phase, the power consumption of the installed ventilation system was recorded using an accessible power meter, including the electric power required by the post-heating battery. After the installation of the mechanical ventilation system, additional sensors were installed near the air return inlet to monitor air quality in terms of CO_2, temperature, and humidity levels at the ventilation unit. These parameters were tracked between February 2024 and November 2024. The comprehensive dataset on environmental conditions and IAQ thus provides a thorough overview of indoor air quality within the public building, both before and after MVHR installation. For the first dataset (October 2023—June 2024), the sensors used were two factory-calibrated Testo 160 IAQ CO_2 meters, one installed in the classroom and another placed outside, with their main features listed in **Table 2**. The characteristics of the power meter used are specified in its datasheet, indicating a measurement uncertainty of ± 5% of the recorded value. **Table 2** also includes the accuracy and the resolution of the various measured quantities and the measurement ranges.

Variable	Range	Accuracy	Resolution
Temperature	0–50 °C	±0.1 K	0.1 K
Humidity	0–100%	±2% (range 20–80% at 25 °C)	0.1%
CO_2	0–5000 ppm(v)	±(50 ppm(v) + 3% m.v.)	1 ppm(v)

Table 2. Measurements range, accuracy, and resolution for Testo 160 IAQ sensor. "m.v." refers to the measured value.

The Testo sensors used were placed in the classroom and outdoors/in the adjoining corridor at a height of 1.70 m (Sensor 1, Figure 1b). In addition to measuring CO_2 concentration in ppm(v) (parts per

million by volume), they also allow for the monitoring of temperature and humidity in the surrounding environment, as shown in **Table 3**.

Variable	Range	Accuracy
Temperature	4–40 °C	0.5 K
Humidity	Up to 85%	3%
Pressure		0.6 hPa
Radon	0–20,000 Bq/m^3	±10% m.v.
Particulate ($PM_{2.5}$)	0–500 µg/m^3	±5 (µg/m^3 +15% m.v.), range 0–150 µg/m^3
VOCs	0–10,000 ppb	-
CO_2	400–5000 ppm	±(50 ppm(v) + 5% m.v.)

Table 3. Measurements ranges and accuracy for AIRTHINGS sensor.

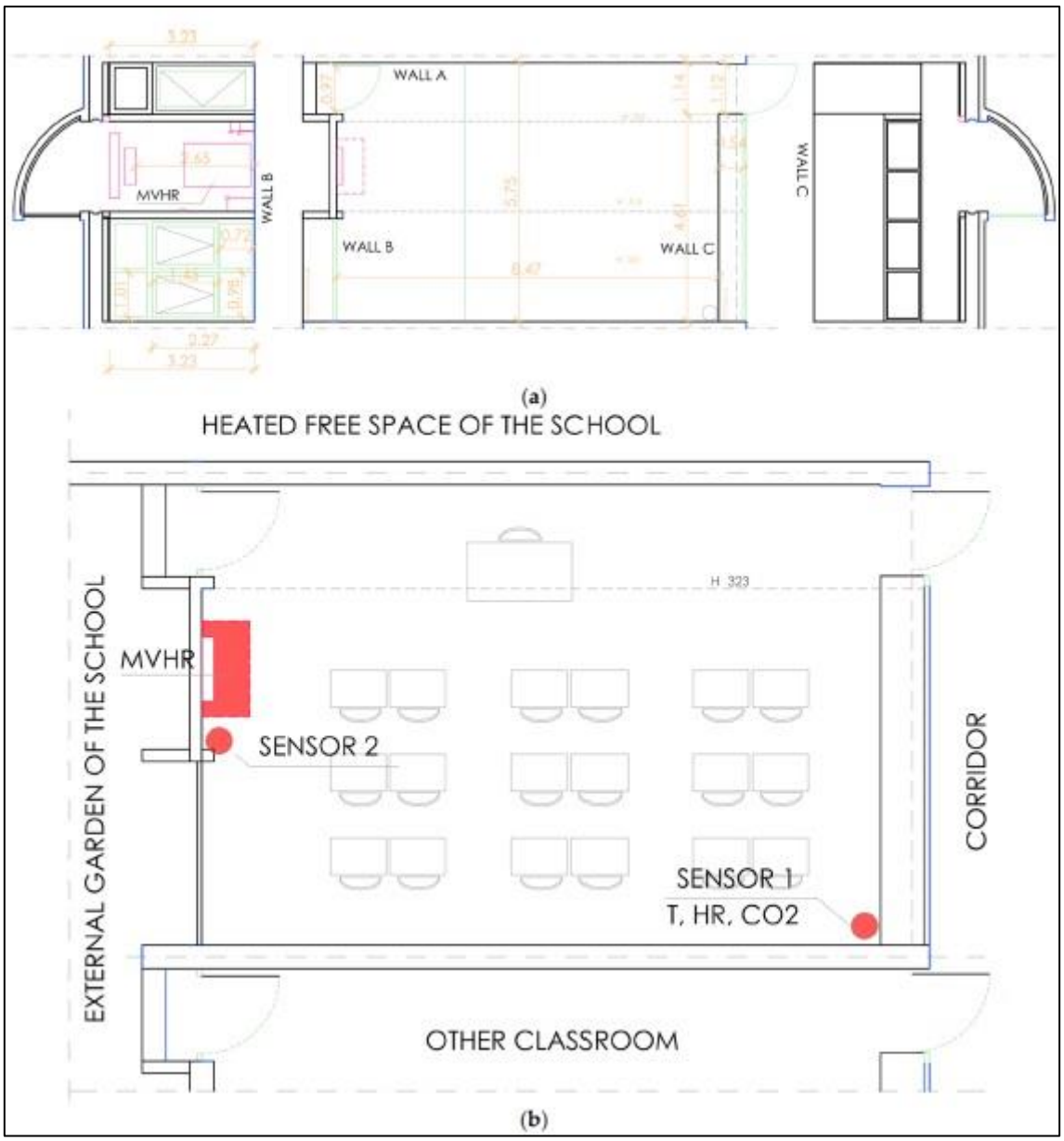


Figure 19. Layout and section of analyzed classroom (**a**); detail of MVHR and sensor position (**b**)

The additional data on CO_2, temperature, humidity, and the general IAQ at the entrance of the heat recovery unit were recorded (Sensor 2, Figure 19b). These measurements were collected starting from February 2024 utilizing a View Plus AIRTHINGS sensor; the measurement ranges and uncertainties are reported in Table 2 for each variable. For these collected data, there are different time granularities, and the time between a record and another is in the range of 2-6 minutes.

3.4. Statistical analysis

Statistical analyses for all the studies reported (subway line trains and classrooms) were performed using the GraphPad Prism 5.03 software (Graph-Pad Software, San Diego, CA, USA). The parametric Student's t-test was used assuming as statistically significant a *p*-value of at least <0.05. To analyze the resistome data, the Bonferroni correction for multiple comparisons was applied to the value detected in the Student's t-test, assuming a corrected pc value of 0.05 as statistically significant.

4.RESULTS

4.1. Shaping the indoor microbiome of the high-traffic subway BE: a case-control study during the pandemic period

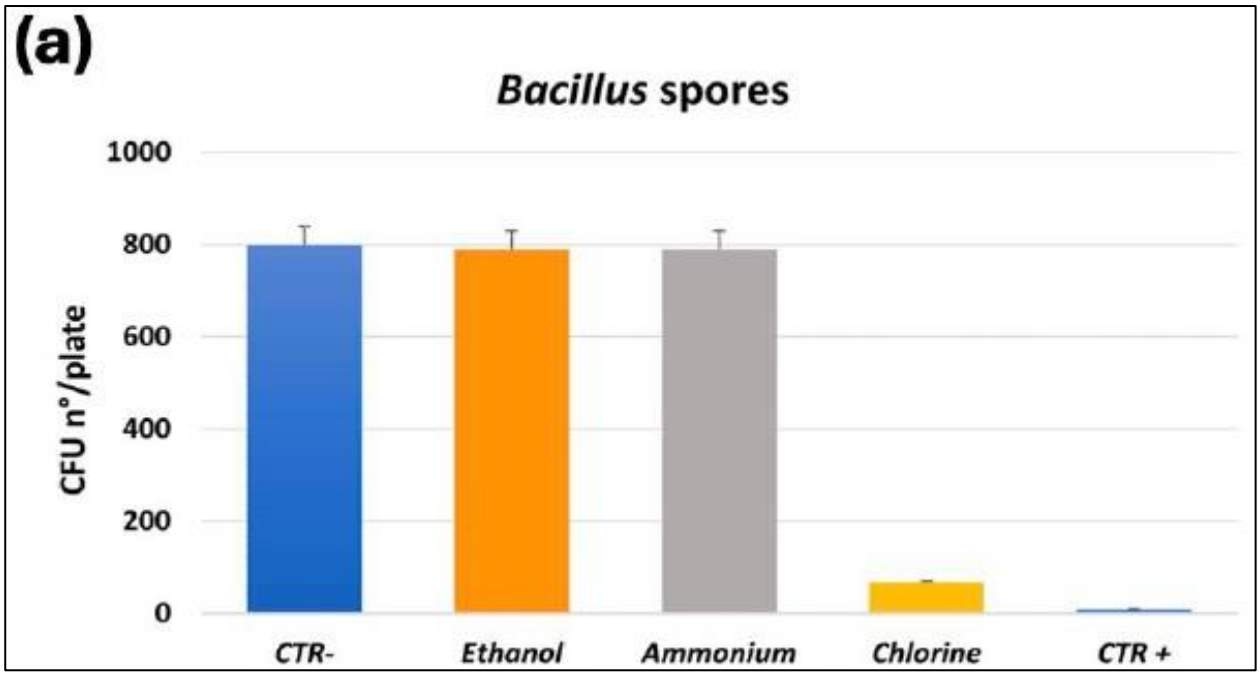
4.1.1. Study design and preliminary assays

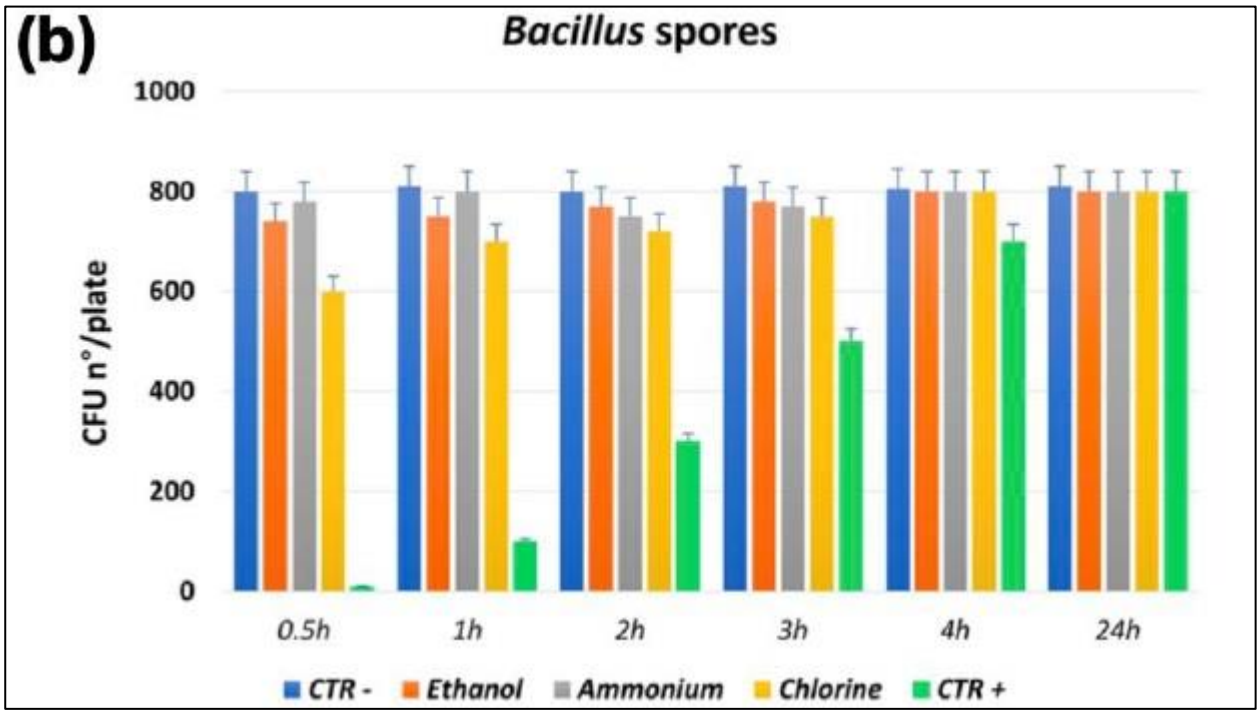
The study aimed to compare the effects of PCHS sanitation to those obtained by conventional chemical sanitation on the subway microbiome.

To achieve this, two driverless trains of the line M5 operated by the Milan Transport Company (Azienda Trasporti Milanesi, ATM; Milan, Italy), with nearly identical characteristics, were selected for a 12-week study conducted during the COVID-19 pandemic (September–December 2021).

Due to anti-COVID-19 regulations for public transport sanitation, which mandated the use of chemical disinfectants, a full replacement of chemical disinfectants with PCHS was not feasible at the time of the study. The mandatory use of chemical disinfectants was outlined in the "Technical Specifications and Extraordinary Hygiene Measures for the COVID-19 Pandemic," established by ATM in accordance with the Italian Ministry of Health guidelines (ISS, 2020). As part of this protocol, ATM sanitation measures for trains involved daily applications of alcohol and chlorine, as well as a weekly treatment with ammonium-based products.

Since the PCHS sanitation relies on competitive exclusion by the live probiotics in the cleanser, preliminary compatibility tests were conducted to evaluate the viability of PCHS-*Bacillus* when exposed to the chemical disinfectants required by the ATM protocol. Two types of assays were performed to independently evaluate the viability of PCHS-*Bacillus* on surfaces treated either before or after the application of each disinfectant. The results showed, as expected, that the chlorine-based product was effective in inactivating > 90% of *Bacillus* spores on surfaces, whereas no significant spore reduction (< 10%) was observed when alcohol- or ammonium-based products were applied after PCHS use (**Figure 20a**). Likewise, no significant reduction in *Bacillus* spores (< 10%) was observed when PCHS was applied 1 h after previous disinfection with chlorine-, ethanol-, or ammonium-based products (**Figure 20b**), whereas a significant reduction (> 90%) was observed when *Bacillus* vegetative forms, instead of spores, were spread on surfaces within 1 h from disinfectant application (**Figure 20c**). At 30 min after application, disinfectants were inactive on spores except for chlorine (causing a 25% reduction of spore titer), whereas all disinfectants were still active against the vegetative form of PCHS-*Bacillus* (**Figures 20b, c**).


(a)
Bacillus spores
1000
800
600
400
200
0
CFU n°/plate
CTR-
Ethanol
Ammonium
Chlorine
CTR +


(b)
Bacillus spores
1000
800
600
400
200
0
CFU n°/plate
0.5h
1h
2h
3h
4h
24h
CTR -
Ethanol
Ammonium
Chlorine
CTR +

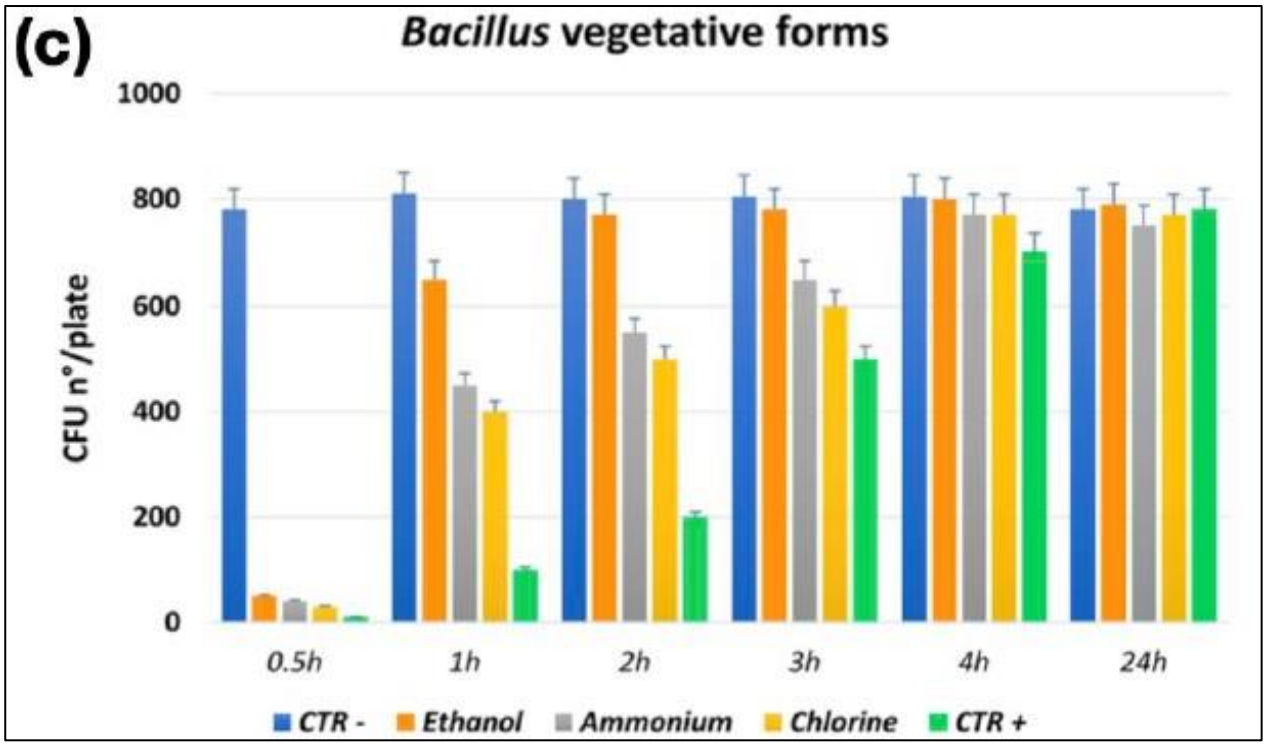


Figure 20. Compatibility tests between PCHS-*Bacillus* and disinfectants. PCHS-*Bacillus* viability in the presence of the indicated disinfectants was assessed *in vitro* on hard nonporous surfaces. **(a)** PCHS-*Bacillus* spores spread on surfaces and subsequently treated with the indicated disinfectants. **(b)** PCHS-*Bacillus* spores spread on surfaces previously treated with the indicated disinfectants. **(c)** PCHS-*Bacillus* growing bacteria seeded on surfaces previously treated with the indicated disinfectants. All results are expressed as mean CFU ± S.D. of duplicate samples in two independent experiments, measured after 24h of incubation on TSA plates.

Thus, based on the results of the compatibility assays and in consideration of the PCHS-*Bacillus* germination time, the experimental sanitation protocol applied included the use of PCHS as a substitute for chlorine-based disinfection, and it was applied 30 min after disinfection with the alcohol- or ammonium-based disinfectants (D'Accolti et al., 2023).

Consistent with this protocol, the control train (n°5508) continued to undergo routine chemical disinfection (chlorine, ethanol, and ammonium) throughout the study, whereas in the PCHS-treated train (n°5511), PCHS sanitation replaced chlorine disinfection, maintaining ethanol and ammonium-based disinfection, as detailed in **Figure 21**. In detail, the control train underwent daily disinfection with ethanol and chlorine, along with weekly ammonium nebulization, while the PCHS-treated train received daily sanitation with ethanol and PCHS, and weekly ammonium/PCHS nebulization. PCHS was applied 30 minutes after ethanol or ammonium disinfectants.

All sanitation procedures were carried out simultaneously on both trains in the absence of passengers. Six sampling campaigns were conducted to assess microbial contamination on surfaces and in the air filters of both trains, starting before PCHS implementation (T0), and then at 2 (T1), 4 (T2), 6 (T3), 9

(T4), and 12 (T5) weeks, as shown in **Figure 21**. At each time point, 12 points were sampled in duplicate from surfaces (floors, seats, handrails, and doors) and air filters (though air filters were not sampled at T3 and T5). Samples were collected using both RODAC contact plates and sterile swabs for microbiological and molecular analysis, as described in Materials and methods.

Passenger flow data were also monitored throughout the study, revealing no significant differences between the control and PCHS-treated trains (**Table 4**), although slightly higher passenger flows were recorded in the PCHS train during all monitored periods (D'Accolti et al., 2023).

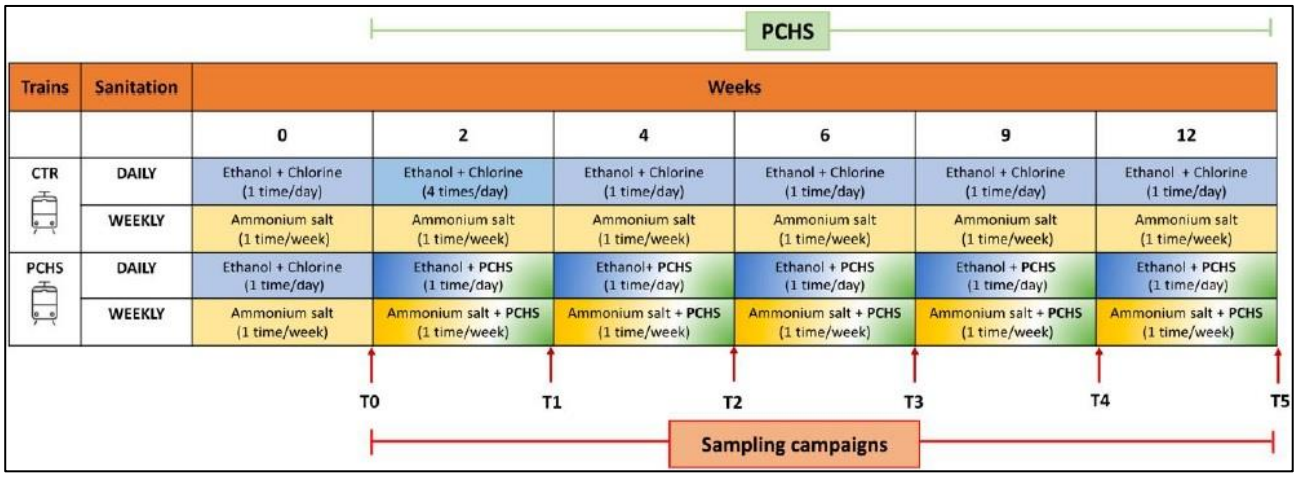


Figure 21. Schematic overview of the study design. Two trains were enrolled: the control train (CTR), which continued to receive chemical disinfection throughout the 12-week study, and the PCHS-treated train, where chlorine-based disinfection was replaced by PCHS sanitation. Daily and weekly sanitation protocols are represented by different colors: light blue for daily ethanol/chlorine disinfection, dark blue for ethanol/chlorine disinfection performed four times per day, yellow for weekly ammonium-based disinfection, and green for the introduction of PCHS (ethanol/PCHS daily and PCHS weekly). Sampling campaigns at time points T0–T5 are indicated by red arrows.

Train	*Time periods*				
	T0-T1	**T1-T2**	**T2-T3**	**T3-T4**	**T4-T5**
CTR	77,631	72,799	80,070	120,743	117,277
PCHS	78,074 (+ 0.57%)	84,644 (+ 16.27%)	83,132 (+ 3.82%)	126,333 (+ 4.63%)	126,284 (+ 7.68%)

Table 4. Passengers flow data for control and PCHS trains. Data are reported as total number of passengers in the indicated time-period. The percentage difference in PCHS-train vs. control train are also reported in parentheses.

4.1.2. Characterization of basal contamination

Based on the contamination levels in collected samples, microbiome characterization of surfaces and air was carried out using floor and air filter samples. Taxonomic and community composition analysis at T0, determined by 16S rRNA NGS performed as described in Methods, revealed that the *Proteobacteria* and *Actinobacteria* phyla were dominant in both trains, with relative abundances of 42% and 25% on surfaces, and 80% and 11% in the air, respectively. The same five most abundant phyla were found in both surface and air samples, with similar order of abundances: *Firmicutes*, *Bacteroidota*, and *Cyanobacteria* representing respectively 10%, 7%, and 5% in surface samples, and 3%, 3%, and 1% in air samples.

However, surface samples exhibited greater diversity and higher representation of less abundant phyla compared to air samples. Additional phyla detected in surface samples included *Deinococcota*, *Acidobacteria*, *Fusobacteria, Chloroflexi, Myxococcota,* and *Patescibacteria*, while *Proteobacteria* and *Actinobacteria* together represented 91% of the total microbiome in air filters. At the genus level, *Burkholderia–Caballeronia–Parabulkolderia* spp. were the most prevalent on surfaces (11%), followed by *Massilia* (4.6%), *Deinococcus* (4.4%), *Chloroplast* (2.3%), *Sphingomonas* (2.3%), *Staphylococcus* (2.2%), *Friedmanniella* (2.1%), and *Paracoccus* (2%), collectively accounting for 31% of the total taxa identified. Additionally, human-associated genera such as *Cutibacterium* (1.7%), *Corynebacterium* (1.1%), and *Streptococcus* (0.8%) were present. Potential human pathogens were also detected in low abundance, including *Escherichia– Shigella* (up to 1.5%), *Acinetobacter* (1.1%), *Pseudomonas* (0.9%), and *Enterococcus* (0.7%). In air filters, similarly to the surface microbiome, *Burkholderia–Caballeronia–Parabulkolderia* spp. were the most abundant (47%), followed by *Methylobacterium* (12.1%), *Novosphingobium* (4.4%), *Massilia* (3.2%), *Rhodanobacter* (2.8%), and *Nesterenkonia* (2.4%), collectively making up 72% of the identified taxa. Other notable genera included *Paracoccus* (1.5%), *Spirosoma* (1.5%), *Cutibacterium* (1.4%), and *Sphingomonas* (1.4%), while *Micrococcus* (0.7%) and *Staphylococcus* (0.6%) were less abundant compared to surfaces.

The results from surface and air samples collected at T1–T5, following PCHS implementation showed considerable variability in the microbial community composition for both the control and PCHS trains. Despite this variability, the dominance of the *Proteobacteria* and *Actinobacteria* phyla remained consistent throughout the study. However, the differences observed between different sampling times within the same train, as well as between the control and PCHS trains at specific timepoints, were not statistically significant. To assess similarities between the collected samples, NGS data were analyzed using the PERMANOVA assay, which considers both microbial composition and relative abundance. The results (**Figure 22**) revealed distinct clustering of surface

(floor) and air samples, with no significant differences observed between the control and PCHS trains at T0. At timepoints T1–T5, air filter samples continued to cluster closely in both the control and PCHS trains, with no significant differences detected. For surface (floor) samples, no significant variations in microbial composition were found between the trains at T1, T2, and T3.

However, at T4 and T5, while control train samples continued to cluster with earlier timepoints, samples from the PCHS train formed a separate group, though the differences in diversity were not statistically significant (D'Accolti et al., 2023).

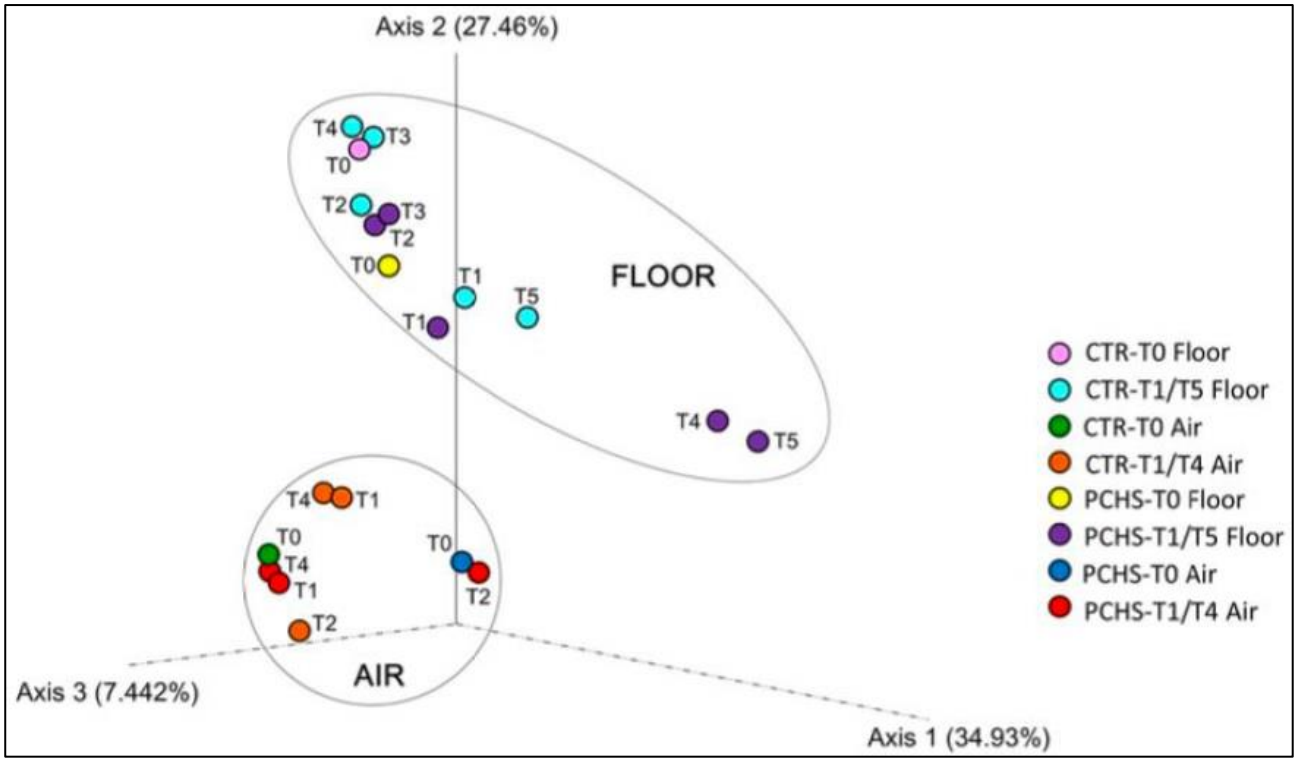


Figure 22. PERMANOVA analysis of collected samples showing differences in clustering of surface (floor) and air samples. CTR, control train; PCHS, PCHS-treated train (D'Accolti et al., 2023).

Overall, the NGS results did not reveal significant changes in the microbiome composition of the PCHS train compared to the control, indicating that PCHS implementation did not cause major shifts in the overall microbiome. Instead, it primarily affected potential human pathogens, which were relatively less abundant in the train's microbiome compared to the most prevalent environmental bacteria. Therefore, these pathogens were more effectively detected through direct CFU counts rather than NGS analysis. In the case of the *Bacillus* group, NGS showed a low abundance of *Bacillus* species, with relative abundance values below 1%. However, after PCHS implementation, a notable increase in *Bacillus* abundance was observed in the PCHS train compared to the control one ($p < 0.05$) supporting the results obtained from direct CFU counts and culture-based detection methods. For those reasons, conventional microbial monitoring of train surfaces and air filters aimed to the

detection of six groups of potential human pathogens, including bacterial (*Staphylococcus* spp., *Enterobacteriaceae* spp., *Pseudomonas* spp., and *Clostridium difficile*) and fungal species (*Candida* and *Aspergillus* spp.). Samples were collected using RODAC plates, followed by CFU counting after appropriate incubation, as described in Materials and methods. A total of 1,632 samples were collected and analyzed.

The results obtained at T0 in both trains showed a very similar level of pathogenic contamination, expressed as the sum of the assayed pathogens in all tested surfaces, corresponding to 10,737 CFU/m² (median value, range 421–178,105 CFU/m²) in the control train and 11,368 CFU/m² (median value, range 421–69,895 CFU/m²) in the train assigned for PCHS treatment. However, the various sampled surfaces showed very different levels of pathogenic contamination, with floors being significantly more contaminated than seats, handrails, and doors ($p < 0.001$) (**Figure 23a**). In detail, pathogens amounted to 96,631 CFU/m² (median value; range 18,105–189,057 CFU/m²) on floors, 19,368 CFU/m² (median value; range 4,211–43,368 CFU/m²) on seats, 5,894 CFU/m² (median value; range 2,947– 10,105 CFU/ m²) on handrails, and 1,739 CFU/m² (median value; range 0–5,474 CFU/m²) on doors. Air filters also showed high level of contamination, evidencing a total pathogenic load corresponding to 86,316 CFU/m² (median value, range 52,632–124,211 CFU/m²) and 97,474 CFU/m² (median value, range 70,737–101,053 CFU/m²) in the control and PCHS train, respectively. At T0, contamination was predominantly due to coagulase-negative *Staphylococcus* spp., which accounted for up to 56% of all detected pathogens (median value of 4,526 CFU/m², range 0–156,211 CFU/m²) (**Figure 23b**). Gram-negative *Enterobacteriaceae* family was less abundant (median value 0 CFU/m², range 0–1263 CFU/ m²), and other bacterial genera, including *Pseudomonas* spp., *Clostridium difficile*, and *Klebsiella* spp., were not detected (median value 0 CFU/ m², range 0–0 CFU/m²). Mycetes were however present, although at a relatively low level, with *Aspergillus* spp. representing the most prevalent (median value 0 CFU/m², range 0–1263 CFU/ m²) followed by *Candida albicans* (median value 0 CFU/ m², range 0–421 CFU/ m²). No significant differences were observed for any of the assayed pathogens at T0 in the two enrolled trains ($p = 0.13$).

4.1.3. Impact of PCHS on microbial contamination

Following the implementation of PCHS sanitation in place of chlorine disinfection (**Figure 23c–d**), a significant reduction in pathogens was observed in both surface and air samples collected from the PCHS-treated train compared to the control train. Such a decrease was already evident at T1, two weeks after the introduction of PCHS, and remained consistent, with further reductions at later times, leading to the virtual disappearance of the assayed surface pathogens at T5 (12 weeks after the introduction of PCHS). Specifically (**Figure 23c**), at T1, the total surface pathogens corresponded

21,053 CFU/m² (median value, range 0–139,789 CFU/m²) in the control train, while in the PCHS-treated train the pathogens amounted to 8,842 CFU/m² (median value, range 0–26,105 CFU/m²), representing a 58% reduction ($p < 0.01$). Notably, during the first two weeks of the study, the control train received an erroneously increase in chlorine disinfection to four times daily due to emergency guidelines. Despite this, the once-daily PCHS application was still significantly more effective at reducing pathogens than the intensified chlorine regimen. At later times, chlorine disinfection was reverted to its original schedule of once per day, making the difference between the control and PCHS-treated trains even more pronounced. At T2 (after 4 weeks of PCHS use), surface pathogen levels were 9,263 CFU/m² (median value, range 0–58,869 CFU/m²) in the control train, compared to 3,579 CFU/m² (median value, range 0–25,689 CFU/m²) in the PCHS train, reflecting a 61% reduction ($p < 0.05$). At T3 (6 weeks of PCHS), the surface pathogen load amounted to 11,158 CFU/m² (median value, range 421–48,000 CFU/m²) in the control train, while the PCHS train showed 2,526 CFU/m² (median value, range 0–9,684 CFU/m²), a 77% decrease ($p < 0.001$). At T4 (9 weeks), surface pathogens were 10,526 CFU/m² (median value, range 0–112,842 CFU/m²) in the control train, compared to 1,895 CFU/m² (median value, range 0–17,263 CFU/m²) in the PCHS train, (- 82%; $p < 0.05$). Finally, at T5 (12 weeks), surface pathogens in the control train reached 2,526 CFU/m² (median value, range 0–84,832 CFU/m²), while the PCHS train recorded 0 CFU/m² (median value, range 0–11,368 CFU/m²), showing a complete 100% pathogens reduction ($p < 0.01$). The analysis of air filters produced similar results (**Figure 23d**). Filters were available for the analysis at times T0, T1, T2, and T4. At T0, both trains exhibited comparable levels of pathogenic contamination. However, at later timepoints, significant differences emerged between the two trains. Specifically, in the control train, the number of pathogens trapped in air filters reached 150,737 CFU/m² at T1 (range 90,526–190,316 CFU/m²), 146,105 CFU/m² at T2 (range 79,158–297,684 CFU/m²), and 292,632 CFU/m² at T4 (range 170,526–444,632 CFU/m²). In contrast, the PCHS-treated train showed lower pathogen levels: 91,789 CFU/m² at T1 (range 18,526–147,789 CFU/m²), 84,842 CFU/m² at T2 (range 66,947–121,263 CFU/m²), and 74,526 CFU/m² at T4 (range 43,789–146,105 CFU/m²). These results evidenced a stabilization of the total amount of potential pathogens in the air and a trend toward decrease, with statistically significant reductions at all timepoints: 39% at T1, 42% at T2, and 75% at T4 ($p < 0.001$). Panels **e–f** of **Figure 23** shows the results with control values normalized to 100%, to better evidence the percentage decrease in the PCHS train compared to the control one.

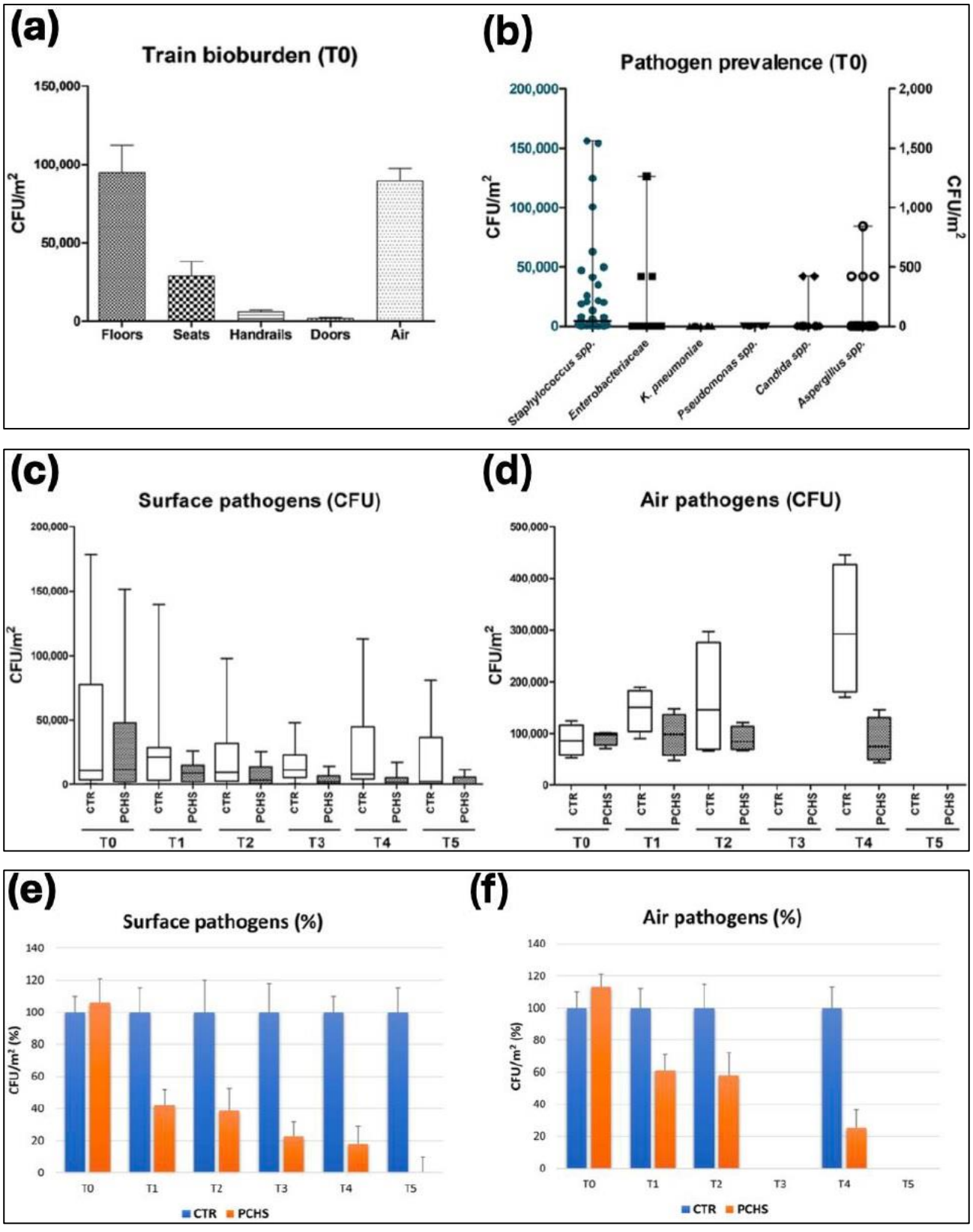


Figure 23. Pathogen contamination in enrolled trains. Surfaces (floor, door, handrails, and seats) and air filters were sampled using RODAC plates. Assayed pathogens included *Staphylococcus* spp., *Enterobacteriaceae* spp., *Pseudomonas* spp., *Clostridium* spp., *Candida* spp., and *Aspergillus* spp. **(a)** Contamination levels in tested surfaces (floors, seats, handrails, and doors) and air filter samples. Results are expressed as median values of CFU/m^2 ± S.D. **(b)** Prevalence of assayed pathogens in total surface and air samples. Results are expressed as median values of CFU/m^2 ±

S.D. Values on the left Y-axis refer to *Staphylococcus* spp., whereas values on the right Y-axis refer to the other microbes. **(c, d)** Pathogenic contamination levels before (T0) and after (T1–T5) the introduction of PCHS sanitation on surfaces **(c)** and air filters **(d)**. The results are expressed as CFU/m^2: median values (lower part of the box) and Q3 values (upper part of the box, representing the 75% percentile values) are shown, together with min and max values. **(e, f)** Comparison between the median levels of contamination detected in the control train (CTR), normalized to 100%, and those detected in the PCH Strain, expressed as percentage values of the PCHS train versus the number of CFU/m^2 detected in the control train, taken as 100%.

4.1.4. Colonization by PCHS-Bacillus in enrolled trains

As expected, the count of PCHS-*Bacillus* significantly increased in the PCHS-treated train following the introduction of PCHS sanitation, while it remained consistently low in the control train (**Figure 24a–b**). In the treated train surfaces, *Bacillus* levels increased from 0 CFU/m² (median value, range 0–16,842 CFU/m²) at T0 to 4,421 CFU/m² (median value, range 0–21,895 CFU/m²) at T1, 7,789 CFU/m² (median value, range 0–29,053 CFU/m²) at T2, 7,158 CFU/m² (median value, range 842–229,895 CFU/m²) at T3, 16,421 CFU/m² (median value, range 421–242,947 CFU/m²) at T4 ($p < 0.05$), and 25,263 CFU/m² (median value, range 0–274,526 CFU/m²) at T5 ($p < 0.001$). Similar trends were observed in air filters, where *Bacillus* counts increased from 2,526 CFU/m² at T0 (median value, range 1,263–5,895 CFU/m²) to 8,421 CFU/m² (median value, range 8,421–23,158 CFU/m²) at T1, 24,421 CFU/m² (median value, range 9,263–24,842 CFU/m²) at T2, and 10,737 CFU/m² (median value, range 4,632–17,263 CFU/m²) at T4 ($p < 0.001$ at all timepoints). In contrast, no significant increase in *Bacillus* counts was observed in the control train at any sampling time. Namely, surface *Bacillus* median values in the control train were 632 CFU/m² at T0, 213 CFU/m² at T1, 0 CFU/m² at T2, 210 CFU/m² at T3, 421 CFU/m² at T4, and 211 CFU/m² at T5. On the air filters of the control train, *Bacillus* counts were 2,737 CFU/m² at T0, 2,526 CFU/m² at T1, 2,947 CFU/m² at T2, and 1,684 CFU/m² at T4.

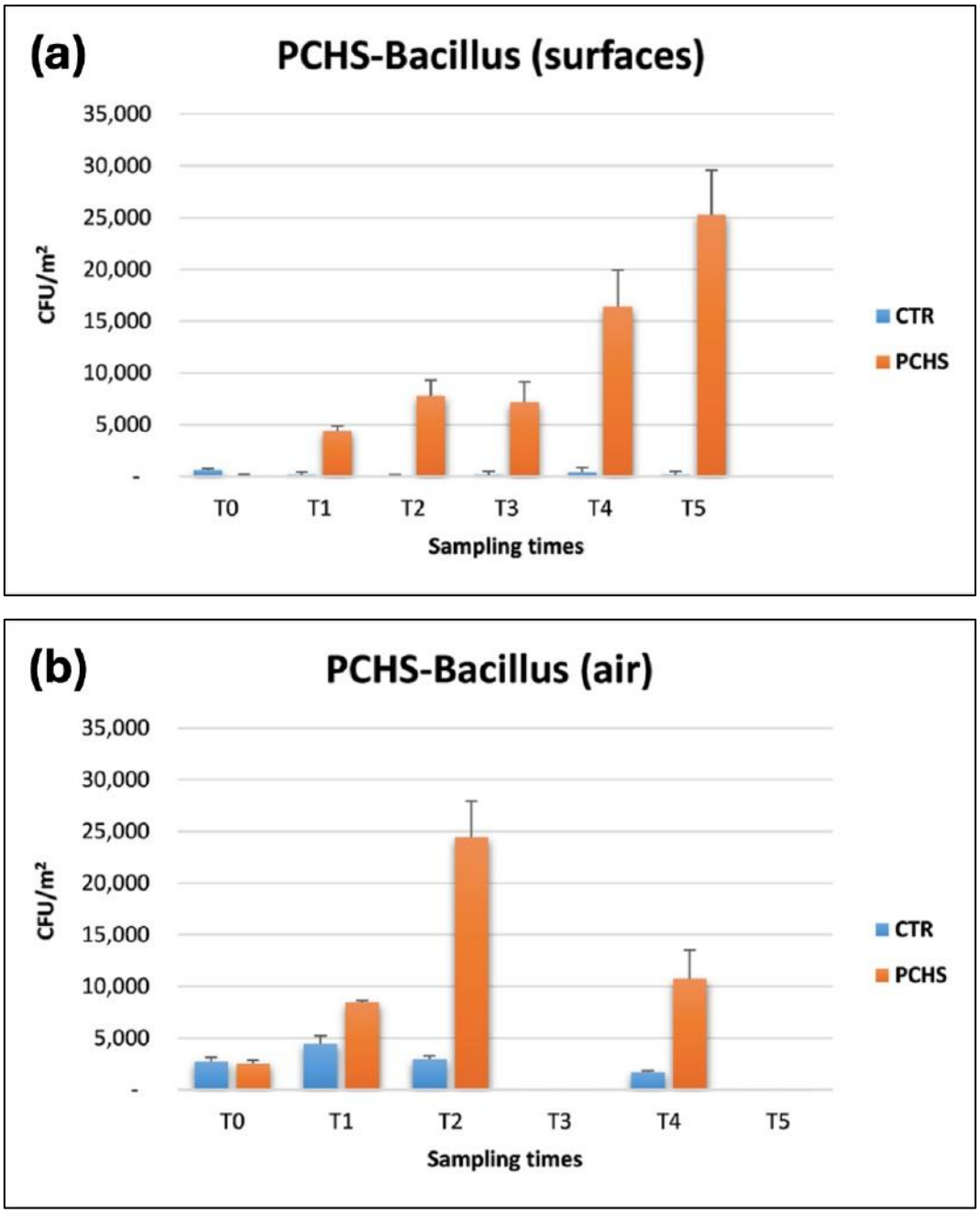
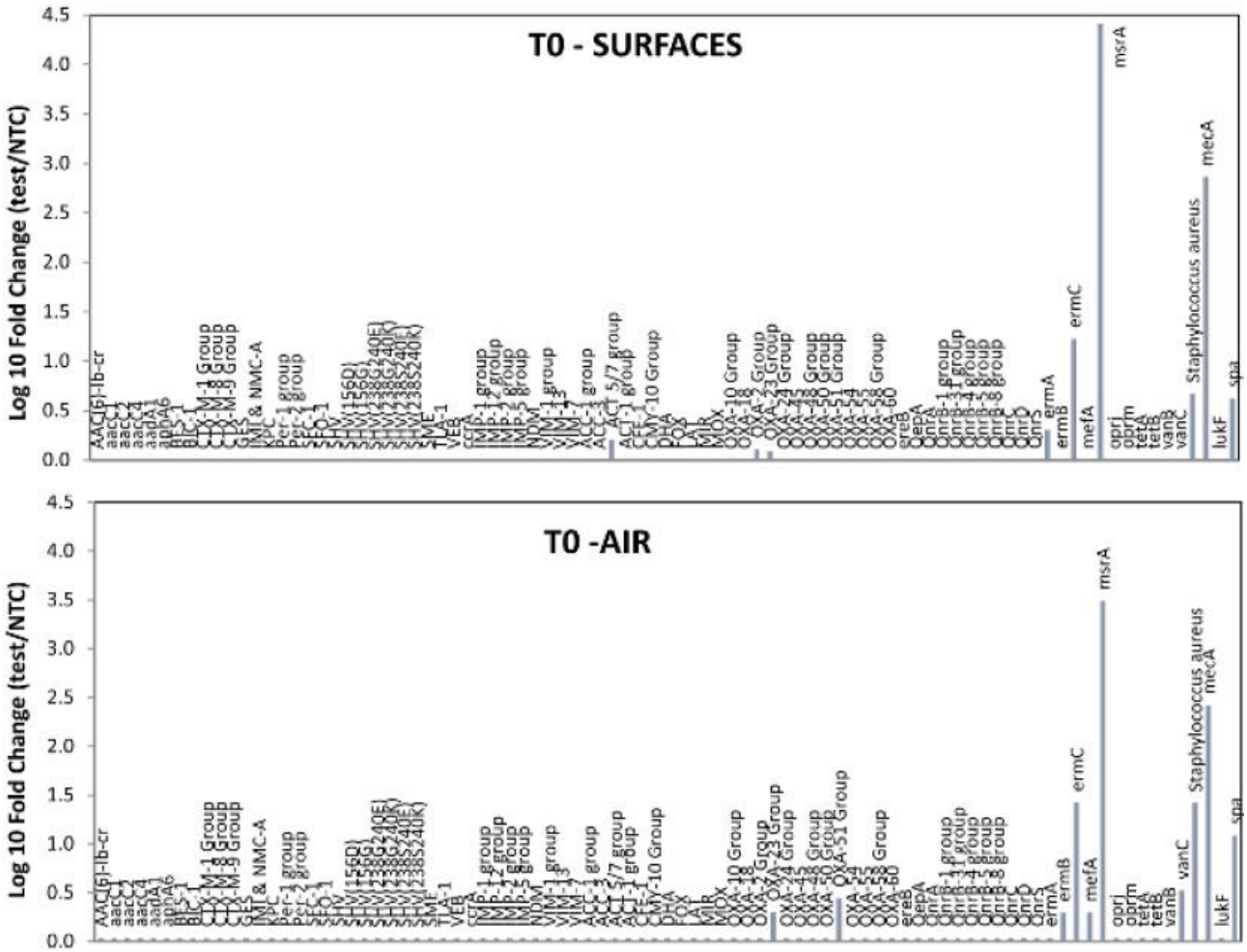


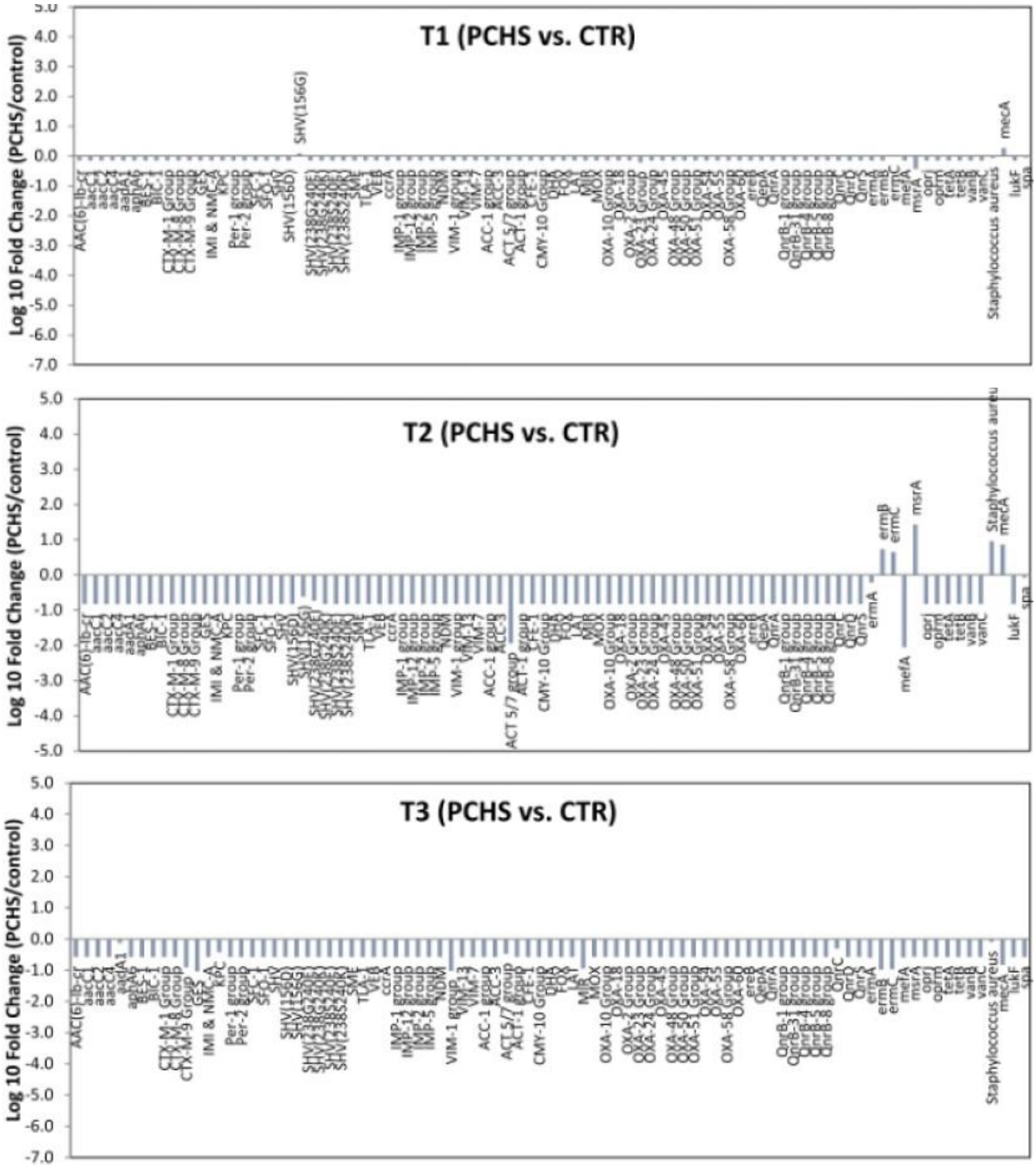
Figure 24. PCHS-*Bacillus* amount on surfaces **(a)** and air **(b)** of enrolled trains. *Bacillus* spp. CFUs were counted on Baird Parker RODAC agar plates. Results are expressed as median CFU value per m² ± SD.

4.1.6. Characterization of the microbial resistome

The surface and air microbiomes of the trains were analyzed also for AMR gene content using a qPCR microarray, which simultaneously identified and quantified 84 AMR genes, as described in Methods. At T0, both train microbiomes contained several resistance genes conferring resistance to various antibiotic classes, including macrolides, methicillin, and class-C/class-D β-lactamases (**Figure 25**). The most abundant AMR genes were *ermC*, *msrA*, and *mecA*, followed by detectable but lower levels

of *OXA-2*, *OXA-23*, *OXA-51* groups, *ACT 5/7* group, *ermA/ermB*, *mefA*, and *vanC* genes. The bacterial species *S. aureus* and its virulence gene *spa* (included in the resistoma microarray) were also detected at moderate levels. Although both trains had comparable levels of AMR genes at T0, subsequent timepoints revealed substantial differences. In the control train, AMR gene levels remained largely unchanged from T0, while in the PCHS train, most AMR genes showed a significant decrease. In detail, at T1 nearly all AMR genes detected at T0 had dropped to undetectable levels in the PCHS train compared to the control one ($pc < 0.01$). At later timepoints, the PCHS train maintained these reduced AMR levels or saw further decreases, while the control train, which underwent chemical disinfection maintained higher levels of AMR genes (**Figure 25**).




T1 (PCHS vs. CTR)
Log 10 Fold Change (PCHS/control)
T2 (PCHS vs. CTR)
Log 10 Fold Change (PCHS/control)
T3 (PCHS vs. CTR)
Log 10 Fold Change (PCHS/control)

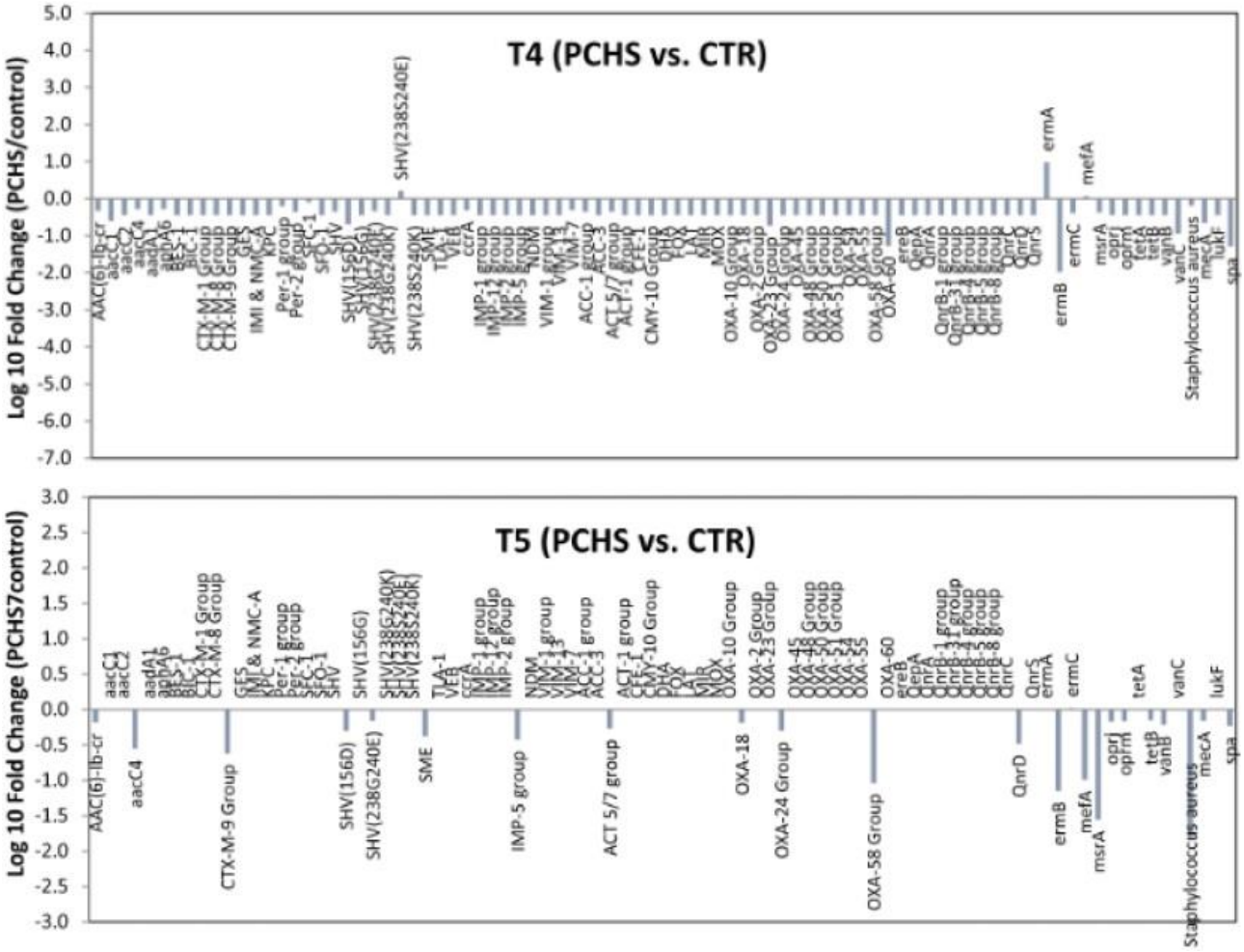


Figure 25. Characterization of the resistome of the train microbiome. Results obtained by qPCR microarray analysis performed in samples collected at the indicated times (T0-T5). Original T0 resistome in surface samples and air filters are shown together with the cumulative results refer to surface and air at T1–T5 timepoints. Results are expressed as mean values ± S.D. of Log_{10} fold changes for each R gene when compared with controls.

4.2. Shaping the indoor microbiome of the high-traffic subway BE: a pre-post study during the non-pandemic period

4.2.1. Study design

Since during the pandemic period the application of PCHS had to be mandatorily combined with chemical disinfectants, we performed a subsequent study aimed to assess the PCHS effectiveness when used in total replacement of disinfection. Similarly to the previous study performed in the subway BE, two trains were enrolled (n° 4404 and n° 4435) of the M4 subway line at ATM in Milan, comparing it with the results obtained with conventional chemical disinfection. The study was

subdivided in two phases: pre-PCHS and PCHS, during which microbiological monitoring was conducted at various time points. Specifically, 4 sampling campaigns were carried out:

- T0 – pre-PCHS phase, in which conventional sanitation was used;
- T1 - 2 weeks after PCHS introduction
- T2 - 4 weeks after PCHS introduction
- T3 - 4 months after the introduction of PCHS

Microbial contamination was assessed on both surfaces and in the air, including the *Bacillus* presence, the residual potentially pathogenic microbial community, and the presence of AMR genes in the microbiome.

4.2.2. Microbiological analyses

Based on previous findings, the microbiological analysis of train surfaces and air filters aimed to assess the presence of four microbial groups, identified as the major human pathogens: *Staphylococcus* spp., *Enterobacteriaceae*, *Pseudomonas* spp., and fungi (with specific identification of *Aspergillus* spp.). The analysis was performed by sampling with RODAC plates and subsequent CFU counting, after appropriate incubation. A total of 440 samples were collected and analyzed.

At T0, which included sampling during chemical disinfection, the results obtained showed high microbial contamination, consistent with the findings of the previous study. At time T0, most of the pathogenic microorganisms were detected on air filters rather than on floor and seat surfaces. In fact, the median counts for the sum of detected pathogens in the air filters were 482,315.79 CFU/m³ in train 4404 and 482,947.37 CFU/m³ in train 4435. A high concentration of *Staphylococcus* spp. was found in the air filters, with a median of 8,000 CFU/m³ for train 4404 and 6,526.315 CFU/m³ for train 4435. The presence of *Bacillus* spp. was found to be low, as expected, recording a median value of 2,947.37 CFU/m³ in train 4404 and 2,736.845 CFU/m³ in train 4435. Microorganisms belonging to the *Enterobacteriaceae* and *Pseudomonas* spp. families, on the other hand, reported very low or zero median values. Conversely, on surfaces (floors and seats), the median values relating to the sum of pathogens were found to be significantly lower than those detected in air filters, despite highlighting a similar trend in terms of distribution of microorganisms. In fact, 14,105.26 CFU/m² were detected in train 4404 and 11,368.42 CFU/m² in train 4435. Also, in this case, contamination was mainly attributable to *Staphylococcus* spp., with median values equal to 7,157.895 CFU/m² in train 4404 and 9,052.63 CFU/m² in train 4435. The presence of *Bacillus* spp. was also insignificant on surfaces, with a median equal to 421.055 CFU/m² in train 4404 and 210.525 CFU/m² in train 4435. Like the results obtained for the filters, *Enterobacteriaceae* and *Pseudomonas* spp. showed negligible median values in both trains. The data obtained are represented in **Figure 26**.

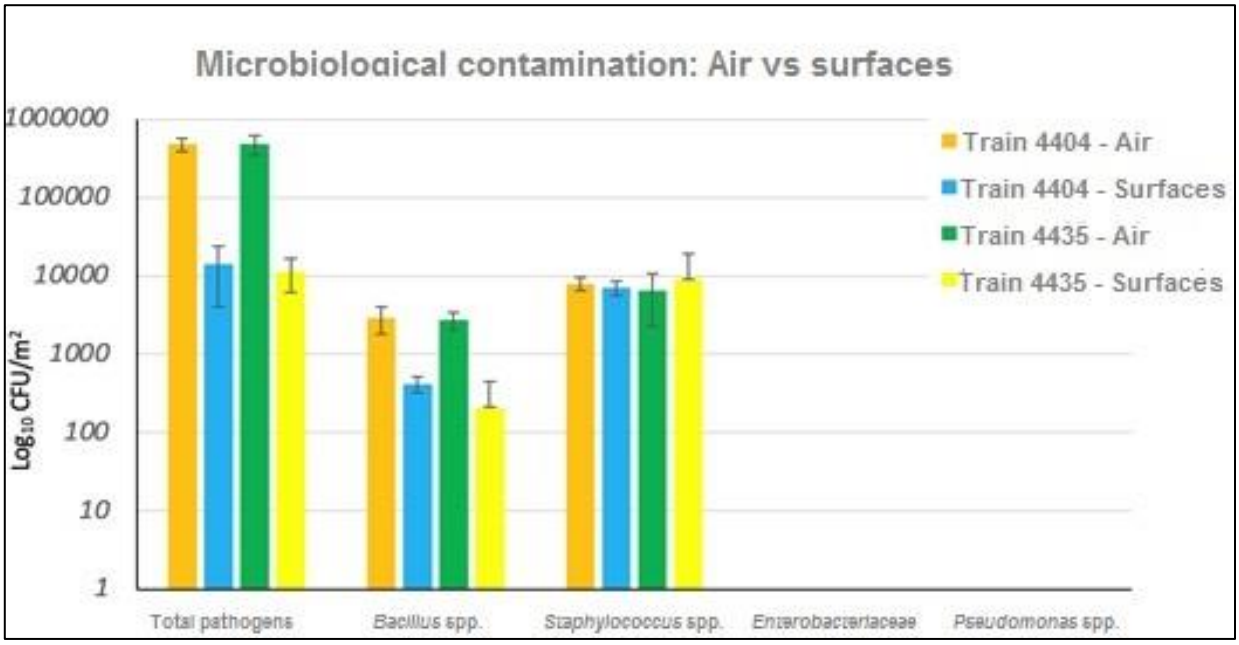


Figure 26. Pathogen contamination at T0. Data are expressed as the logarithmic value of the median CFU/m² ± standard deviation (SD), recorded on surfaces and in the air of trains before the adoption of the PCHS.

At time T1, after 2 weeks of PCHS use, a significant reduction in the pathogenic microbial load was observed on the surfaces of both trains analyzed. Specifically, train 4404 recorded a decrease in the median from 14,105.26 CFU/m² (T0) to 3,789.48 CFU/m² (T1), corresponding to a reduction of approximately 73%. Similarly, in train 4435, a decrease in the median from 11,368.42 CFU/m² (T0) to 3,157.90 CFU/m² (T1) was observed, with a reduction of approximately 72%. In parallel, an increase in the presence of *Bacillus* spp. was observed, with a recorded median of 5,052.64 CFU/m² in train 4404 and 6,736.84 CFU/m² in train 4435. This increase is indicative of the surface colonization process by probiotic microorganisms introduced through the PCHS protocol. Analyzing the pathogen data, in train 4404 the median for *Staphylococcus* spp. was 4,000 CFU/m², while in train 4435 it was 2,105.26 CFU/m². Regarding *Enterobacteriaceae* spp. and *Pseudomonas* spp., no significant presence was detected in either train, with median values equal to zero (**Figure 27**). During phase T1, it was not possible to perform sampling of the air filters on both trains.

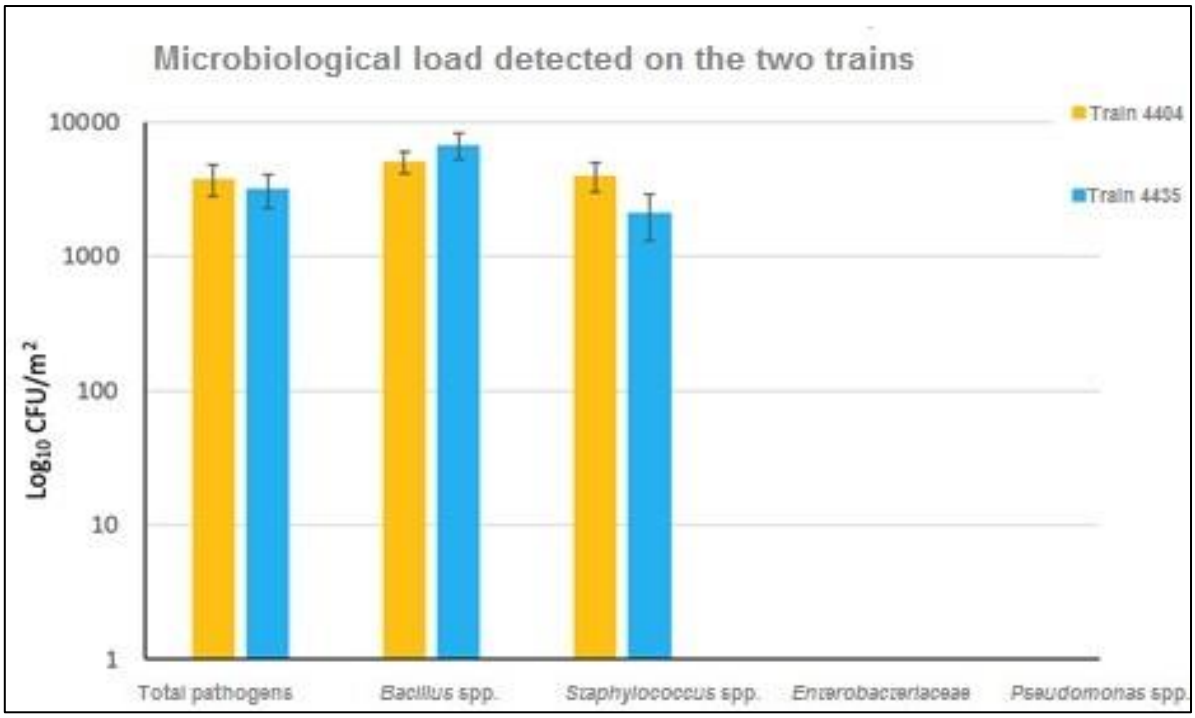


Figure 27. Pathogen contamination at T1. Data are expressed as the logarithmic value of the median CFU/m² ± SD, recorded in the trains 2 weeks after adopting the PCHS protocol.

It should be emphasized that the occupant flow was comparable across all periods, and therefore the decrease in contamination cannot be attributed to a reduction in occupant numbers. During the first month of the study, at time T0 (December 2024), 90,230 passengers/day were recorded, and in the following month (T1), 95,453 passengers/day were recorded. At time T2, corresponding to 7 weeks after the introduction of PCHS, a further reduction in the pathogenic microbial load was observed in both surface and air samples. Specifically, in train 4404, the median of total pathogens detected on surfaces decreased to 1,052.63 CFU/m² compared to 3,789.48 CFU/m² recorded at T1 and 14,105.26 CFU/m² at T0. Similarly, in train 4435, the values decreased to 842.11 CFU/m² at T2, compared to 3,157.90 CFU/m² at T1 and 11,368.42 CFU/m² at T0. Overall, the reduction reached approximately 92.5% (**Figure 28**).

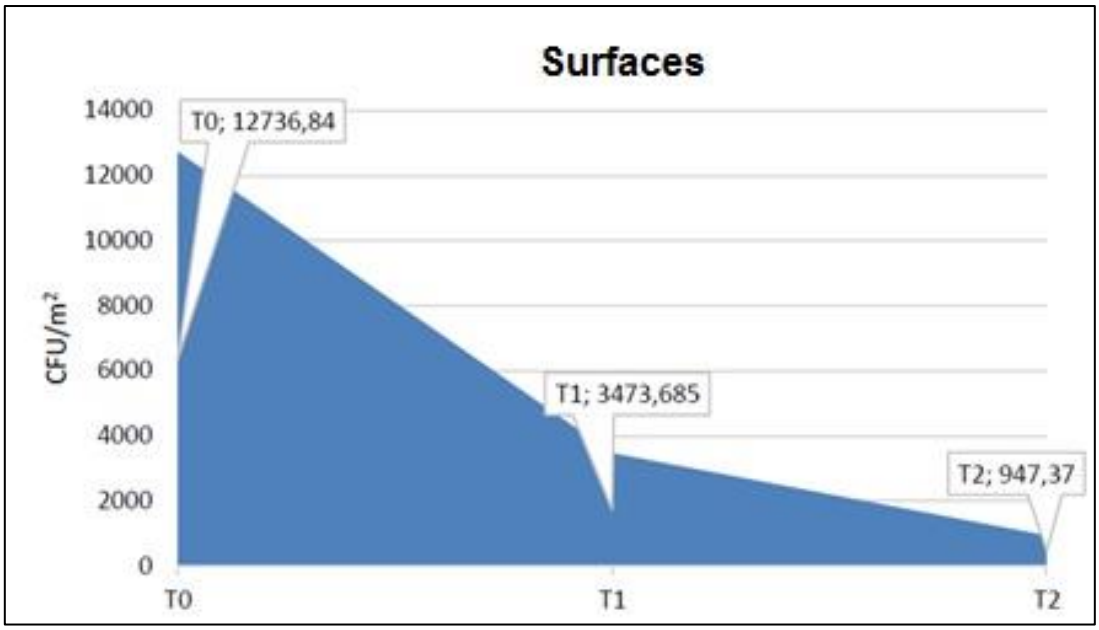


Figure 28. Pathogen contamination on surfaces, at times T0, T1, and T2. Data are expressed as the median value of CFU/m² measured on both trains enrolled in the study.

For air samples, a similar decrease was found: in train 4404 the median total bacterial load decreased significantly, going from 482,315.79 CFU/m³ at T0 to 1,684.21 CFU/m³ at T2; in train 4435 the values decreased from 482,947.37 CFU/m³ to 1,684.21 CFU/m³ equal to a decrease in the total pathogenic load of 99.1% (**Figure 29**).

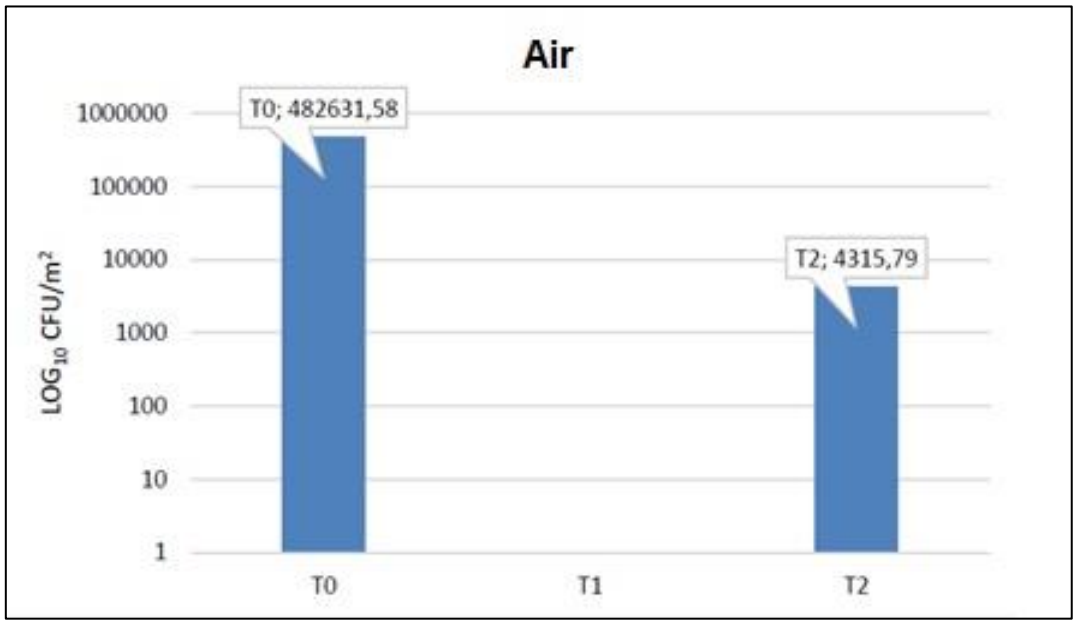


Figure 29. Pathogen contamination in air filters, at times T0 and T2. Data are expressed as the median value of CFU/m³ measured on both trains enrolled in the study.

Analyzing individual bacterial species, *Staphylococcus* spp. showed a progressive reduction over time. In train 4404, the median decreased from 7,157.90 CFU/m³ at T0 to 4,000 CFU/m³ at T1 and finally to 2,736.85 CFU/m³ at T2. In train 4435, the values followed a similar trend, going from 9,052.63 CFU/m³ at T0 to 2,105.26 CFU/m³ at T1 and then to 1,052.63 CFU/m³ at T2. Regarding *Enterobacteriaceae* and *Pseudomonas* spp., no significant presence was detected in surfaces or air samples at any of the sampling phases, with medians consistently equal to zero. In parallel with the reduction in pathogen load, an increase in colonization by probiotic microorganisms was observed, in line with the objectives of the PCHS protocol. The genus *Bacillus* spp., in fact, showed a clear increase in both surfaces and air samples. In train 4404, the surfaces showed median values of *Bacillus* spp. increasing from 421.05 CFU/m² at T0 to 5,052.63 CFU/m² at T1 and finally to 69,052.63 CFU/m² at T2; for the air samples, the increase was even more significant, going from 2,947.37 CFU/m³ at T0 to 118,736.84 CFU/m³ at T2. Similar dynamics were observed in train 4435: on the surfaces the values increased from 210.53 CFU/m² at T0 to 6,736.84 CFU/m² at T1 and then to 48,631.58 CFU/m² at T2, while in the air samples they went from 2,736.85 CFU/m³ at T0 to 118,947.37 CFU/m³ at T2. The results showed that after just two weeks, PCHS resulted in a significant decrease in pathogenic microorganisms (T0 vs. T1, $p<0.01$). After seven weeks of PCHS, pathogenic contamination further decreased both on surfaces and in the air, with significant differences between both T0 and T1 ($p<0.001$). A final sampling (T3) was conducted four months later to assess the stabilization of the results obtained at the first two time points. Data collected at T3 highlighted a further significant reduction in pathogen contamination on both trains analyzed. On surfaces, the median pathogen count was 0 CFU/m² in both trains. Among the microorganisms detected, *Staphylococcus* spp. were the predominant ones, with medians of 1263.16 CFU/m² for train 4404 and 1052.63 CFU/m² for train 4435. *Enterobacteriaceae* and *Pseudomonas* spp. were not detected. Conversely, levels of *Bacillus* spp., considered an indicator of probiotic colonization, remained high on both trains. The median values recorded were 186,947.37 CFU/m² in train 4404 and 173,263.1 CFU/m² in train 4435. The results also showed a significant decrease in the presence of pathogens in the air. In train 4404, the median was 6,526.31 CFU/m³, and in train 4435, it was 1,263.16 CFU/m³. The presence of *Bacillus* spp. was also high in the air, with values of 190,105.26 CFU/m³ in train 4404 and 160,842.10 CFU/m³ in train 4435. To facilitate a more immediate understanding of the system's effectiveness, the results detected at time T3 were compared with those recorded at time T0. As can be seen from the graphs below, the quantity of pathogenic microorganisms drastically decreased, both in surface and air samples, reaching values close to zero (**Figure 30** and **Figure 31**).

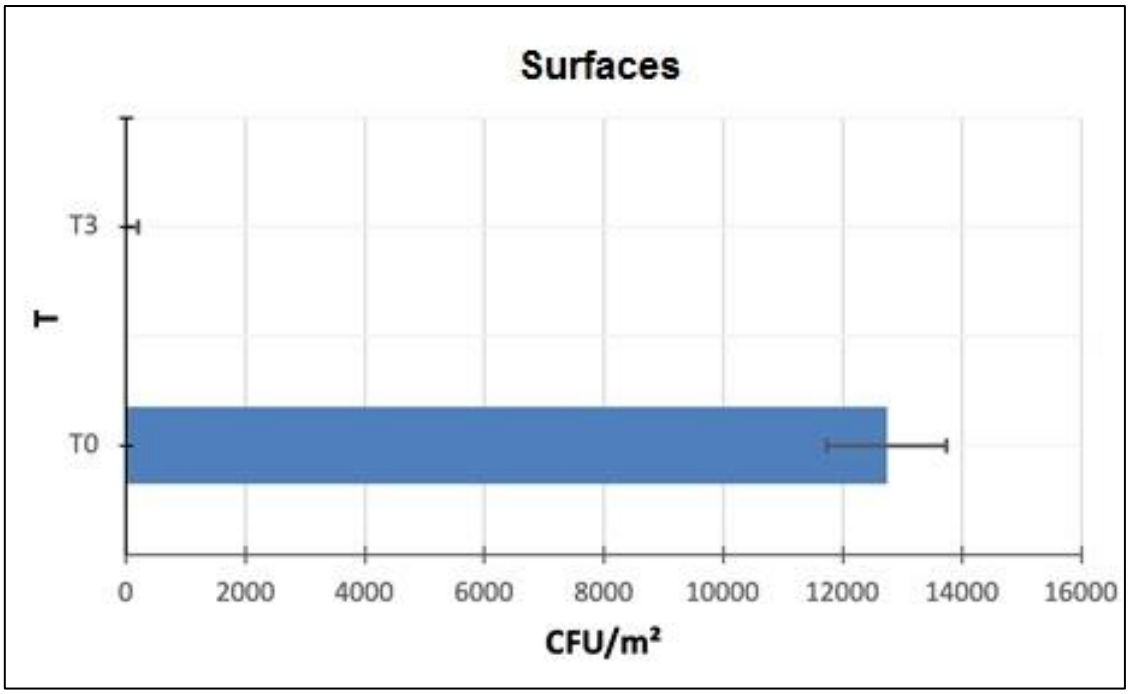


Figure 30. Comparison of pathogen contamination on surfaces, at beginning (T0) and end (T3) of the study. Data are expressed as median value of CFU/m² ± SD, measured on both trains enrolled in the study.

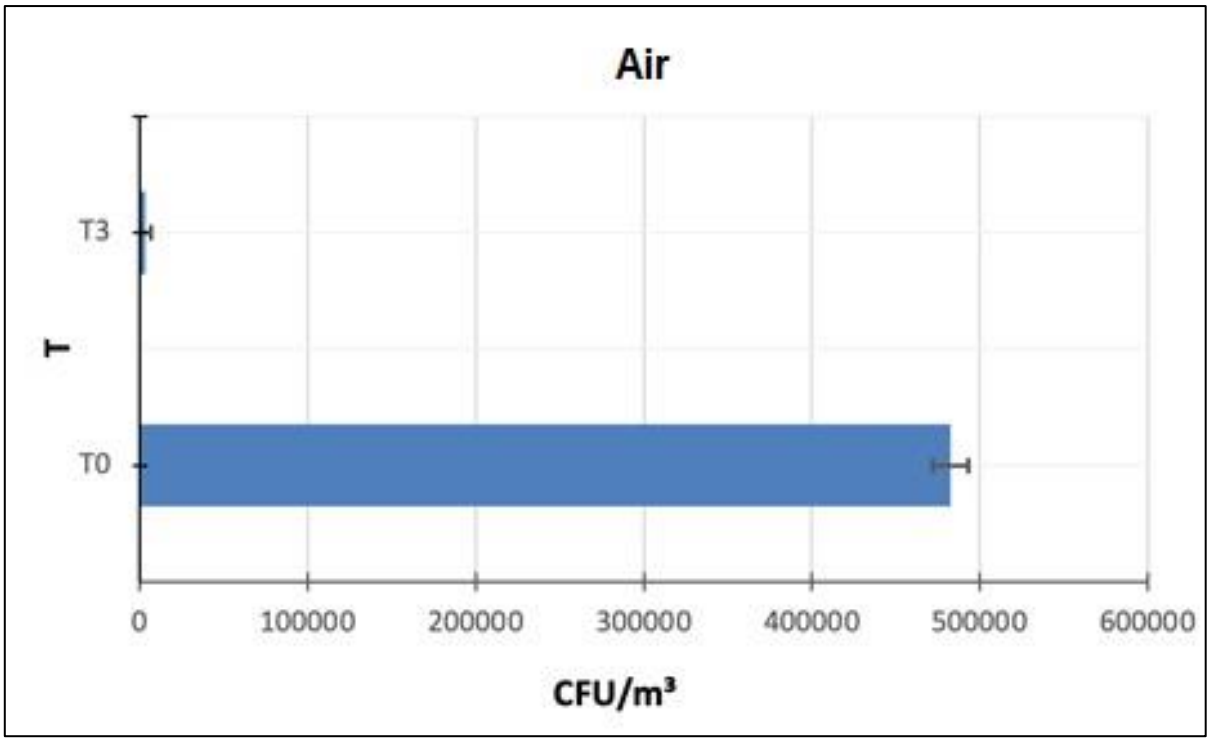


Figure 31. Comparison of pathogen contamination in air filters, at beginning (T0) and end (T3) of the study. Data are expressed as median value of CFU/m³ ± SD, measured on both trains enrolled in the study.

In parallel, the analysis of the levels of *Bacillus* spp. at different time intervals (T0-T3) showed a progressive increase in the probiotic species present in the PCHS, as expected (**Figure 32, 33, 34 and**

35), confirming the ability of these probiotics to colonize the treated environment, displacing and replacing the pathogenic component through competitive exclusion. In train 4404 (Figure **31**), a progressive increase in CFU/m² of *Bacillus* spp. was observed on the monitored surfaces over the different monitoring times, accompanied by a marked decrease in the total pathogen count (excluding Bacillus spp.). At time T0, the presence of *Bacillus* spp. was 421.05 CFU/m², while with the introduction of PCHS, *Bacillus* spp. increased to 5052.63 CFU/m² at T1, 69052.63 CFU/m² at T2, and 186947.37 CFU/m² at T3. In parallel, the total microbial count minus Bacillus decreased from 32842.10 CFU/m² initially (T0) to 421.05 CFU/m² at the end of the observation period (T3).

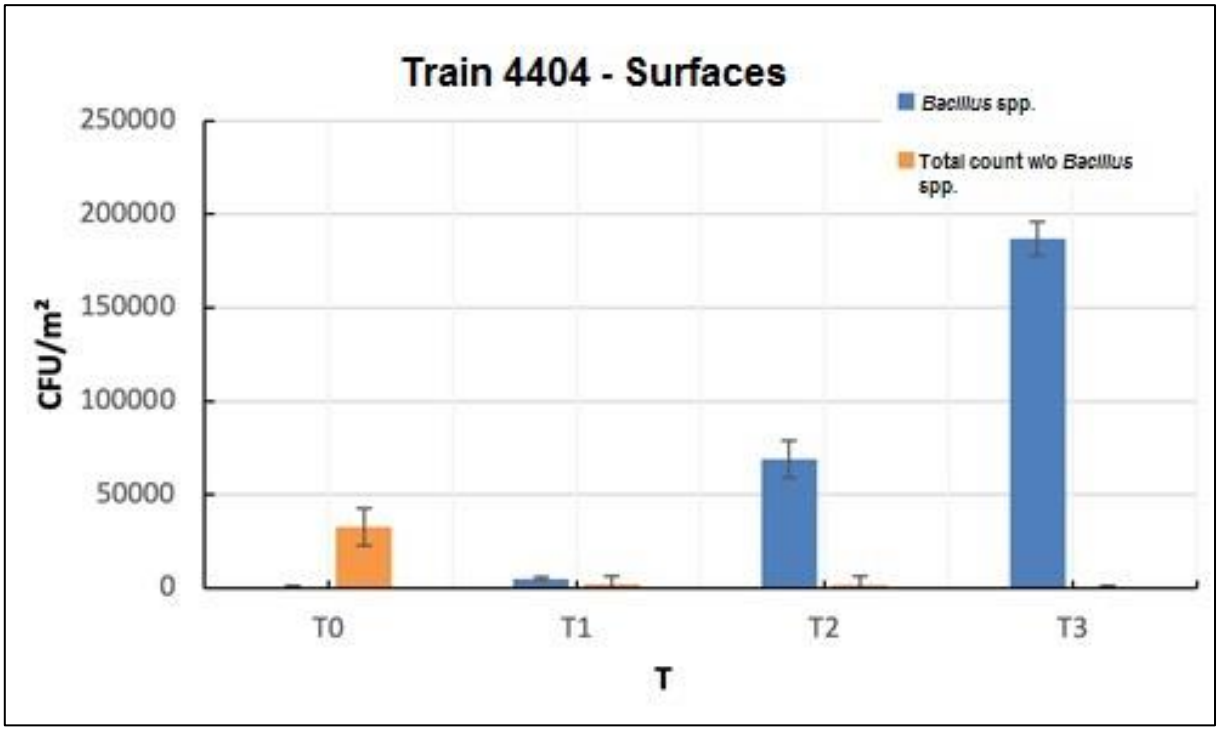


Figure 32. Train 4404, surface samples. Presence of *Bacillus* and total microbial contamination (excluding *Bacillus*). Data are expressed as median CFU/m² ± SD.

Similarly, in the air samples taken from train 4404, at T0 the presence of *Bacillus* spp. was 2947.37 CFU/m³, while the remaining microbial flora was equal to 88,842.10 CFU/m³. No data relating to phase T1 are available in this study. At time T2, the Bacillus reached 118,736.84 CFU/m³, while the remaining microbial flora was practically zero (0 CFU/m³). Finally, at T3, *Bacillus* spp. further increased, reaching 190105.26 CFU/m³, while the total count minus *Bacillus* spp. remained almost zero, with a recorded value of only 210.52 CFU/m³ (**Figure 33**).

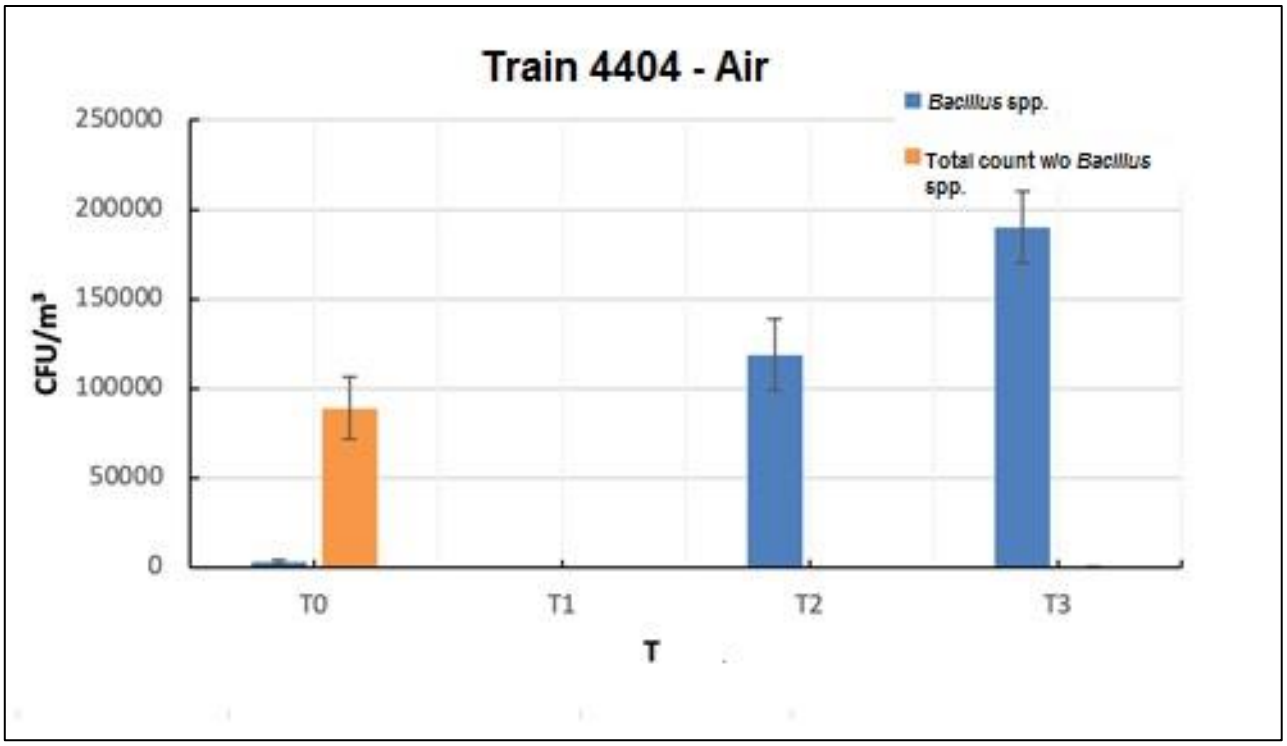


Figure 33. Train 4404, air samples. Presence of *Bacillus* spp. and total microbial contamination (excluding *Bacillus*). Data are expressed as median values of CFU/m^3 ± SD.

In train 4435, the results obtained had a similar trend to those observed in train 4404. Initially, at T0, a quantity of *Bacillus* spp. equal to 210.52 CFU/m^2 was recorded, while at T1 the quantity of *Bacillus* spp. increased to 6736.84 CFU/m^2, at T2 it reached 48631.58 CFU/m^2, and finally at T3 it reached 173263.16 CFU/m^2, exceeding the data recorded at time T2 by more than 3.5 times. This exponential growth of *Bacillus* spp. was accompanied by a drastic decrease in the total count without *Bacillus* spp. which went from 47578.95 CFU/m² at time T0 to 0 CFU/m² at time T3 (**Figure 34**).

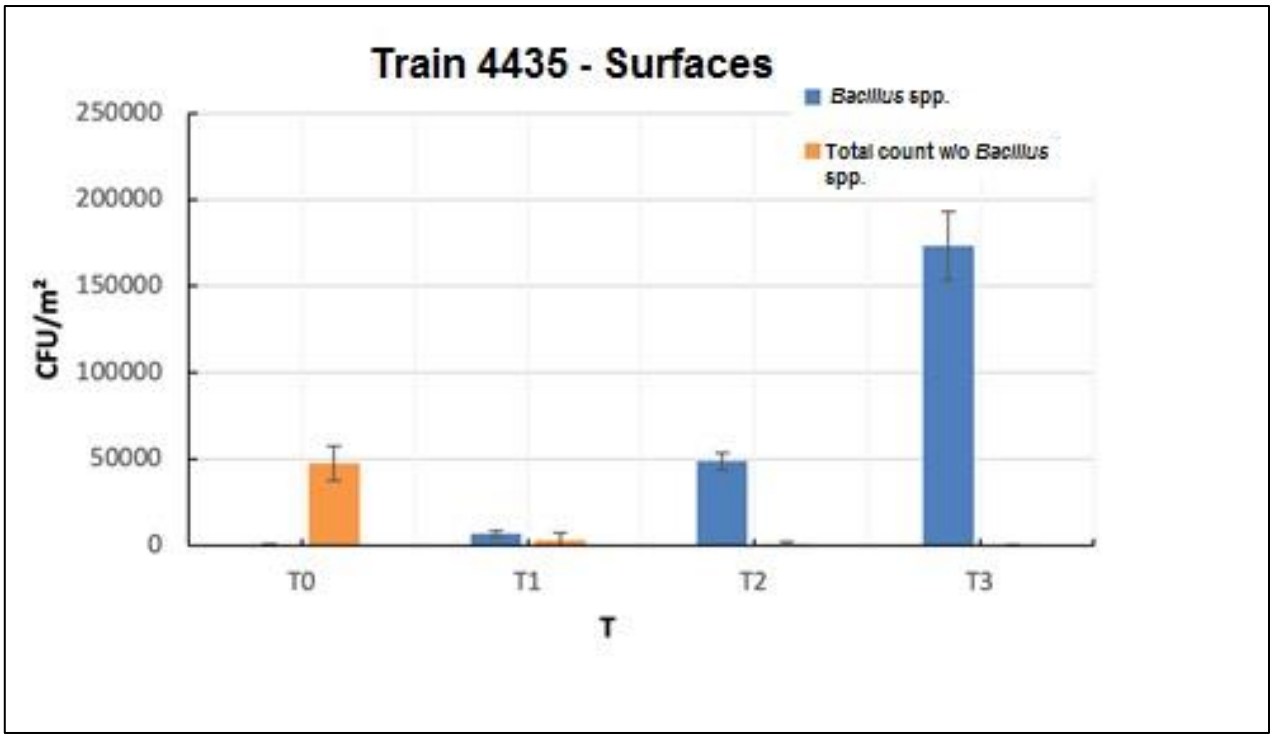


Figure 34. Train 4435, surface samples. Presence of *Bacillus* spp. and total microbial contamination (excluding *Bacillus*). Data are expressed as median CFU/m2 ± SD.

Also, in the air samples, the values of *Bacillus* spp. increased from T0 to T3 (**Figure 35**). At the beginning (T0) the *Bacillus* spp. were equal to 2736.84 CFU/m³, but at T2 and T3 they increased to 118947.37 and 160842.10 CFU/m³, respectively. As for Train 4404 for this train it was not possible to analyze the air samples at time T1. Similarly, a complete elimination of the total microbial count excluding *Bacillus* spp. was observed, going from 85684.20 CFU/m³ to 0 CFU/m³.

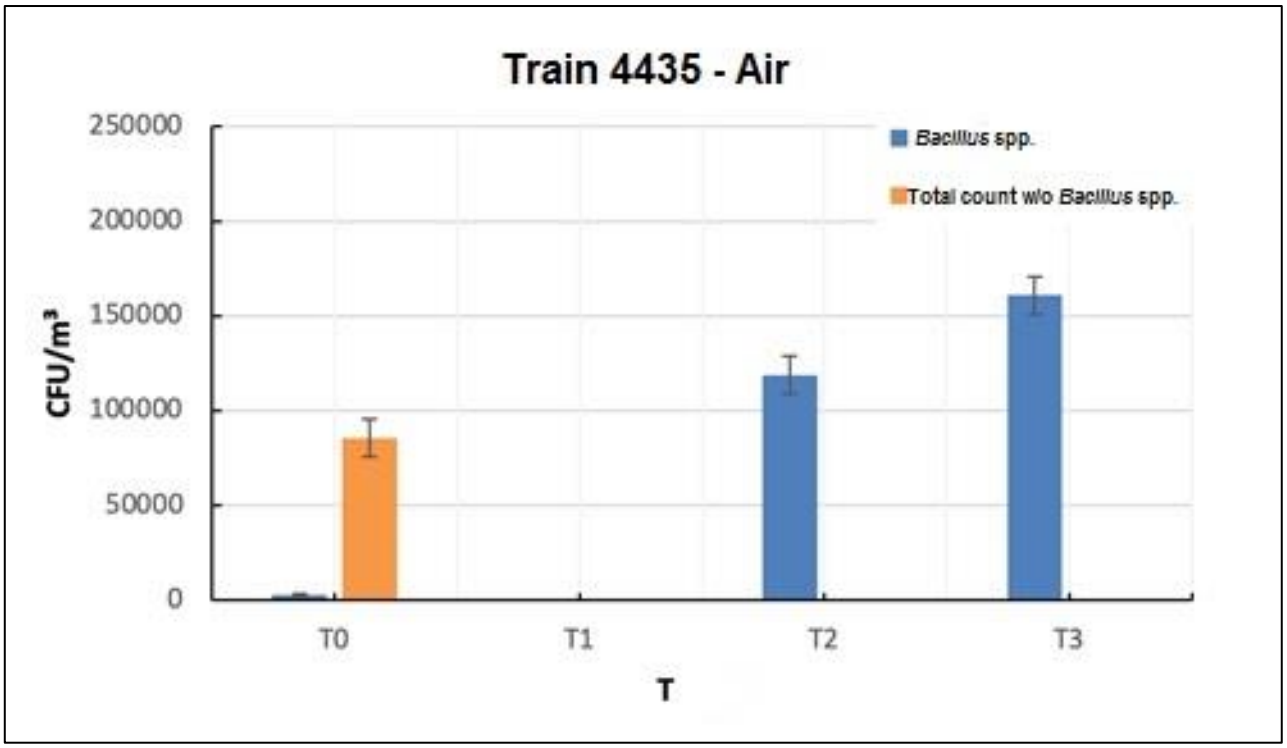


Figure 35. Train 4435, air samples. Presence of *Bacillus* spp. and total microbial contamination (excluding *Bacillus*). Data are expressed as median values of CFU/m^3 ± SD.

In addition to the trend of potentially pathogenic microbial species, in this case-study the presence of fungi associated with indoor fungal infections was also monitored. At T0, results showed high median values of fungal contamination, especially in the air. On train 4404, the air contained 482,315.79 CFU/m^3 of total fungi, of which 0 CFU/m^3 were *Aspergillus* spp. Similarly, on train 4435, 482,947.37 CFU/m^3 of total fungi were found, of which 0 CFU/m^3 were *Aspergillus* spp. Surfaces were found to be less contaminated, with median values of 14,105.26 CFU/m^2 of total fungi, of which 842.11 CFU/m^2 of *Aspergillus* spp., in train 4404, and values equal to 11,368.42 CFU/m^2 of total fungi, of which 421.05 CFU/m^2 of *Aspergillus* spp. in train 4435. At time T1, a significant decrease in fungal loads on surfaces was observed (air filters could not be examined at this time). On train 4404, 3789.47 CFU/m^2 of total fungi and 0 CFU/m^2 of *Aspergillus* spp. were found; on train 4435, 3157.89 CFU/m^2 of total fungi and 0 CFU/m^2 of *Aspergillus* spp. were found. At time T2, median values were further decreased on surfaces. On train 4404, total fungi were 1052.63 CFU/m^2 and *Aspergillus* spp. were absent, while on train 4435, 842.10 CFU/m^2 were recorded for total fungi and 0 CFU/m^2 for *Aspergillus* spp.. Inside train 4404, a concentration of 1,684.21 CFU/m³ of total fungi was recorded in the air, while *Aspergillus* spp. was completely absent (0 CFU/m³). Conversely, in train 4435, the survey revealed a higher quantity of total fungi, equal to 6,947.37 CFU/m³, again with the absence of *Aspergillus* spp. Finally, at time T3, fungal contamination values were found to be consistently low. Stable values of 0 CFU/m² were recorded on surfaces for both parameters, on both trains. In the air,

median values of 6526.31 CFU/m^3 of total fungi and 0 CFU/m^3 of *Aspergillus* spp. were recorded on train 4404; on train 4435, 1263.16 CFU/m^3 of total fungi and 0 CFU/m*3* of *Aspergillus* spp. were observed. In summary, the results showed a significant decrease in fungal contamination from T0 to T3. The reduction was almost total on surfaces starting from T1, while the air maintained greater variability and a residual presence of fungi, although decreasing significantly compared to the initial levels detected at T0 (**Figure 36**).

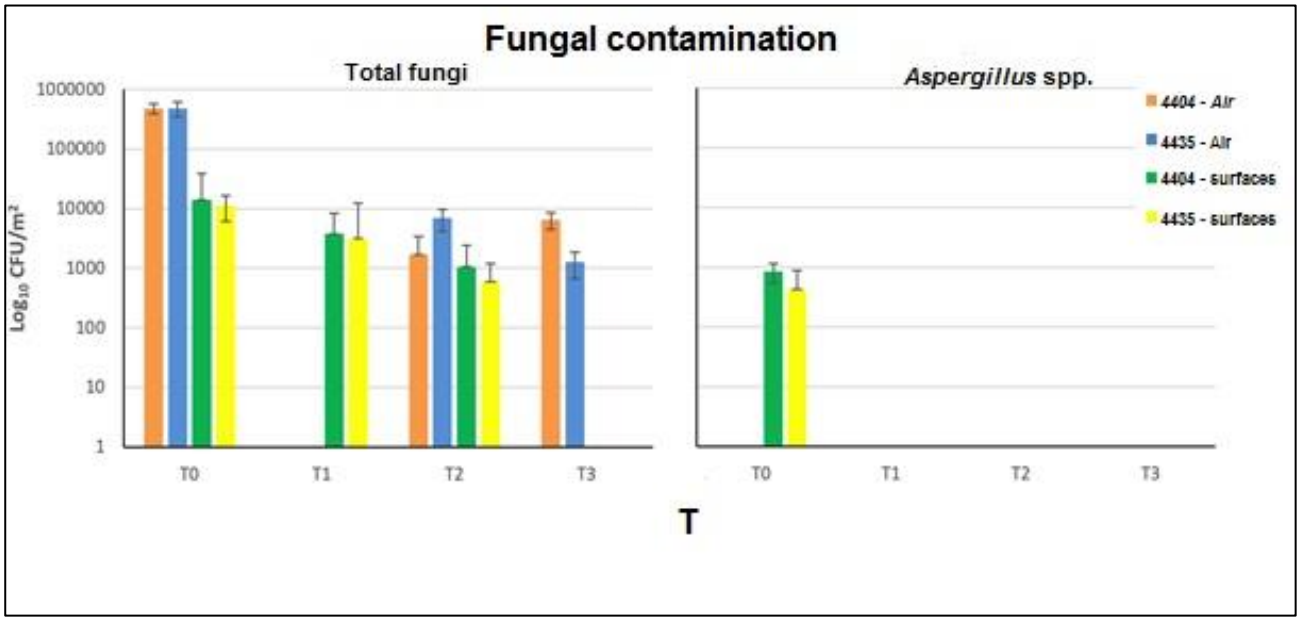


Figure 36. Fungal contamination in the enrolled trains. Data are expressed as Log_{10} of the median values ± SD for total fungi (left) and *Aspergillus* spp. (right), in the air and surface samples collected at times T0–T3.

4.2.3. Molecular analyses

The microbial population contaminating surfaces and air was characterized for the presence of AMR genes. For this purpose, a molecular investigation method based on quantitative real-time PCR microarray (qPCR microarray) was used. The analysis was performed on DNA extracted from the total microbial population present in the samples. The qPCR microarray used identified and quantified the presence of ARGs to aminoglycosides, tetracyclines, beta-lactams, and macrolides in the microbial population of the trains. This demonstrates how antibiotic-resistant microorganisms can also exist outside of hospital settings and how AMR can spread into the community, becoming a public health concern. As schematically shown in **Figure 37**, at the initial time point T0, both trains showed the presence of several resistance genes directed against different classes of antibiotics, including macrolides, methicillin, and β-lactams. The most frequently detected ARGs were *ermC* (2.5 Log_{10} Fold Change, FC), *msrA* (>3 Log10 FC), and *mecA* (>1.5 Log_{10} FC). In particular, the identification of the *mecA* gene (encoding for methicillin resistance) is likely attributable to the

presence of MRSA (Methicillin-Resistant *Staphylococcus* aureus) strains, highlighting a possible health risk linked to MRSA infections even outside the hospital environment. In smaller but detectable quantities, the following ARGs were found: *OXA-2*, *OXA-23*, *OXA-51*, *ACT 5/7*, *ermA/ermB*, *mefA*, and *vanC*. The presence of the *vanC* gene, associated with resistance to vancomycin, a last-line antibiotic used to treat serious infections caused by Gram-positive bacteria, is a concern. Its early detection, both in the air and on surfaces, highlighted the spread and persistence of resistant microorganisms within the metropolitan environment. The simultaneous identification of the *vanC* and *mecA* genes gave these findings significant significance, indicating the possible presence of multiply resistant microorganisms. This condition could pose a serious risk to both the ecosystem and human well-being.

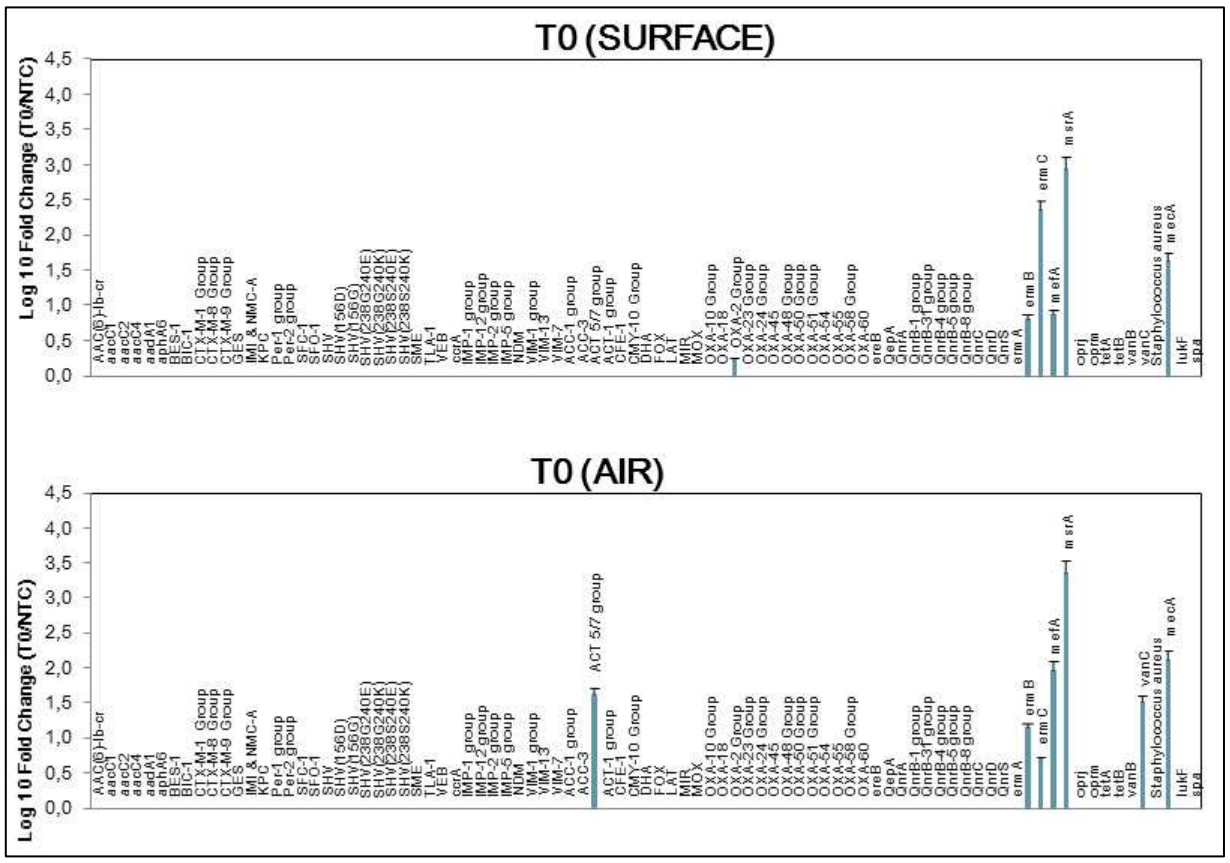


Figure 37. Presence of ARGs at T0 in surface and air samples from both enrolled trains. Data are expressed as Log_{10} of the mean Fold Change (FC) values, evaluated with respect to controls, ± SD.

Following the introduction of PCHS, a decrease in ARM genes was observed in both trains. In fact, at T1, a reduction in all R genes that had been identified in the microbial population at T0 was observed in surface samples. In particular, a decrease in *ermC* and *msrA* was observed, with values <-1.5 Log_{10} FC and <-1 Log_{10} FC. The only gene not decreased was *ermB*, maintaining values above 1 Log_{10} FC. This could be associated with the fact that the *ermB* gene is naturally present in the

genome of the *Bacillus* spp. strains included in the PCHS probiotic system formulation. This does not constitute a direct clinical risk, as it is inherent in the physiology of the probiotic and presents a low probability of transfer to other bacteria. In both trains, it was not possible to perform the study on air samples at time T1 (**Figure 38**).

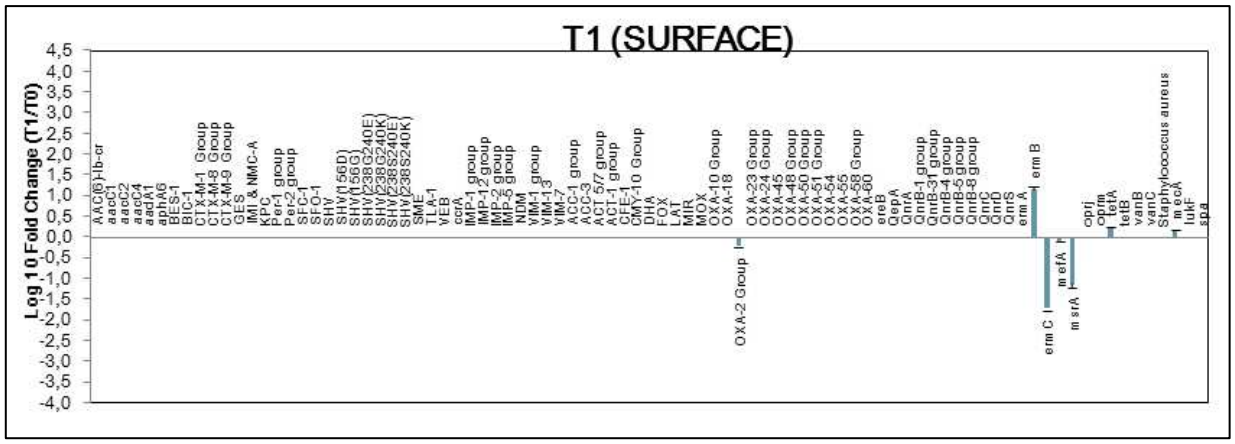


Figure 38. Presence of ARGs at T1 in surface samples from both enrolled trains. Data are expressed as Log_{10} of the mean Fold Change (FC) values, evaluated with respect to those obtained at T0, ± SD.

At time T2, the results confirmed the decrease in AMR genes that had been identified at T0, both on the surface and in the air. In particular (**Figure 39**), a significant decrease in the *mecA* (<-1 Log_{10} FC), *msrA* (<-1 Log_{10} FC), *ermC* (<-1.5 Log_{10} FC), and *OXA-2* group (<0 Log_{10} FC) genes was observed on the surfaces. The *OXA-50* group gene was not decreased, remaining at levels similar to those measured at T0 (+ 0.2 Log_{10} FC). The presence of *ermB* also remained stable, with a slight increase compared to T0 (+ 0.2 Log_{10} FC). In air samples, a decrease in the genes *mecA* (<-2 Log_{10} FC), *vanC* (<-1.5 Log_{10} FC), *mefA* (-2 Log10 FC), *ermC* (<-0.5 Log_{10} FC), *ermB* (<-1 Log_{10} FC), and *ACT 5/7* group was observed. In these samples, the most significant decrease after PCHS was found in the *msrA* gene (<-3.5 Log_{10} FC, equal to 99.9%).

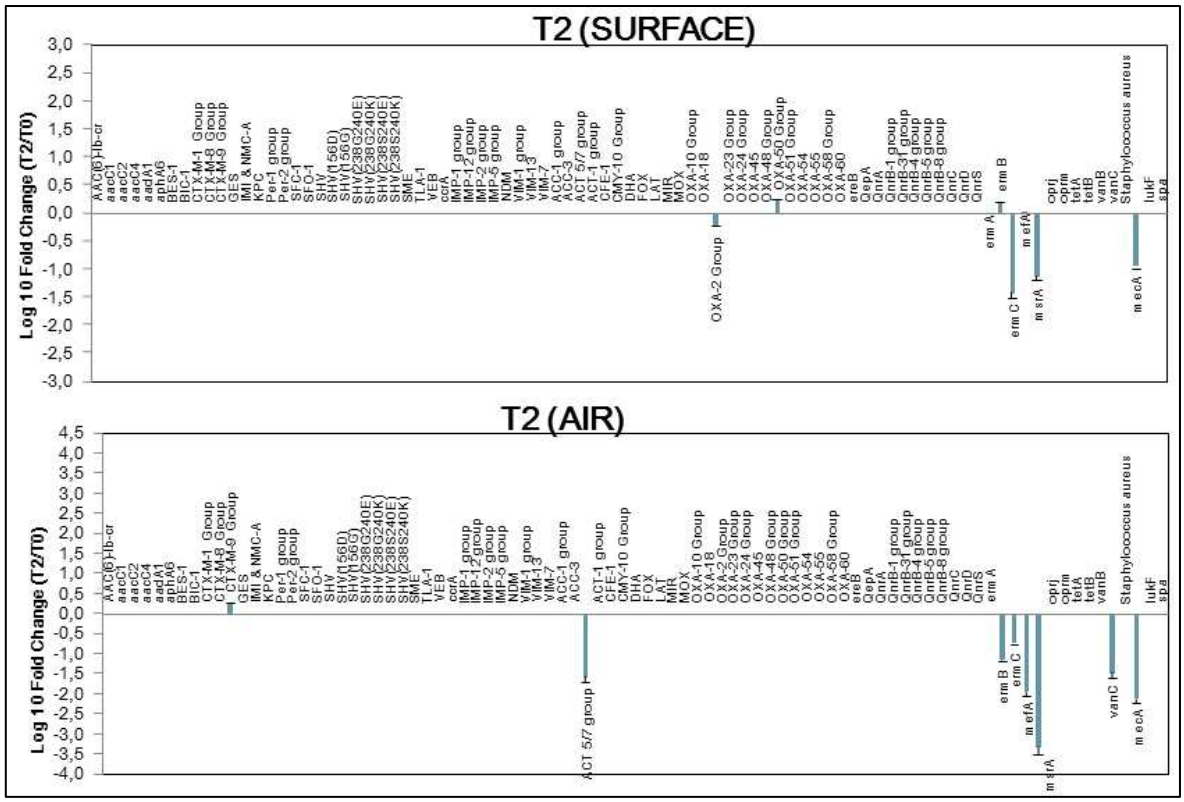


Figure 39. Presence of ARGs at T2, in surface and air samples from both enrolled trains. Data are expressed as Log_{10} of the mean Fold Change (FC) values, evaluated with respect to those obtained at T0, ± SD.

Finally, at time T3 (**Figure 40**), a further decrease was observed in the ARM genes that had already shown a decrease at time T2 (*OXA-2* group, *ermB*, *ermC*, *mefA*, *merA*, *mecA*), while the presence of the *OXA-50* gene slightly increased (+0.6 Log_{10} FC) and AMR genes were not present at previous times it were found to be present: *oprJ* and *oprM*, probably following the appearance of carrier bacteria. Among all the decreased ARM genes, the *ermC* gene is the one with the greatest decrease (-2.5 Log_{10} FC). As regards the *mefA* gene, the values are approximately -1 Log_{10} FC, while those of *merA* are <-1.5 Log_{10} FC. The *mecA* gene also shows a value of <-1.5 Log_{10} FC. In air samples, a marked decrease was observed in the genes ACT 5/7 group (<-1.5 Log_{10} FC), *ermB*, *ermC*, *mefA* (all with values equal to -2 Log_{10} FC), *msrA* (-2.5 Log_{10} FC), *vanC* (-1.5 Log_{10} FC), and *mecA* (-2.0 Log_{10} FC). Conversely, at T3 the appearance of some AMR genes not initially present and not present in surface samples was detected, such as *INP-5* group (+1.1 Log_{10} FC), *FOX* (+0.9 Log10 FC), and *NIH* (+1 Log_{10} FC).

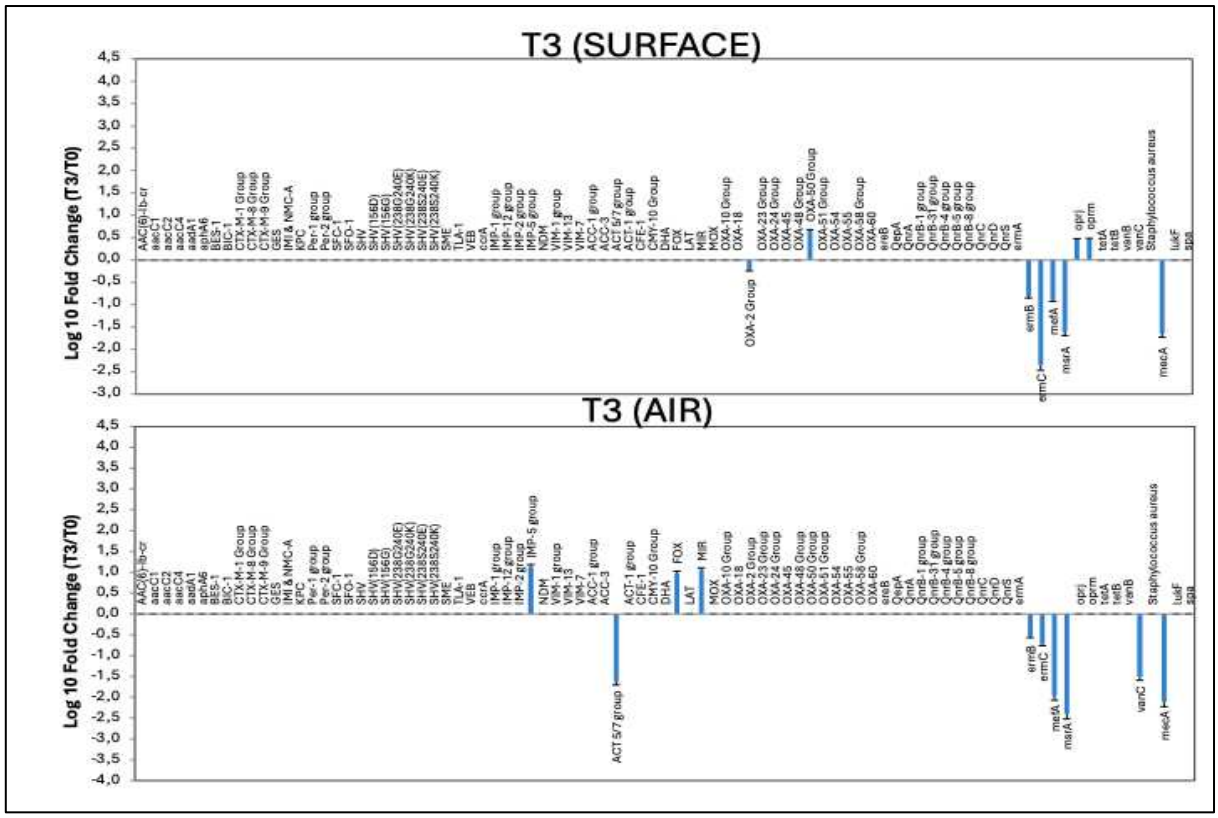


Figure 40. Presence of ARGs at T3, in surface and air samples from both enrolled trains. Data are expressed as Log_{10} of the mean Fold Change (FC) values, evaluated with respect to those obtained at T0, ± SD.

4.3. Shaping the microbiome of the school BE: a pre-post and case-control study assessing the effects of plants, mechanical ventilation, and PCHS introduction

4.3.1. Study design

To characterize the microbiological quality of the indoor school environment and evaluate the impact of green interventions aimed to improve it, a pre–post case–control study was performed in an Italian high school from March 2023 to May 2024, excluding the summer holiday period, for a 10-month total survey. Two classrooms with superimposable features were enrolled in the study, one receiving three experimental interventions (test room, TR) and the other not receiving any interventions and serving as a control (control room, CR). The study included four consecutive phases during which the interventions were implemented in the TR:

- T0, providing basal values detected with usual conventional sanitation and ventilation via window opening.
- T1, during which greenhouse plants were added to the TR.
- T2, during which mechanical air ventilation was installed in the TR.

- T3, during which the TR received PCHS, probiotic-based sanitation, in substitution for the conventional chemical-based one.

4.3.2. Features of the enrolled school BE

The classroom analyzed is part of the "Liceo Ludovico Ariosto" school complex (located in Ferrara, a municipality in Northern Italy). During lessons, the classroom is occupied by 17 students and one teacher**. Figures 19 a, b** and **Figure 41 a, b** show the classroom layout and sensor and MVHR position, in terms of a sketch and photos, while **Figure 41** presents a photograph taken inside the room in the absence of people and before the installation of the controlled mechanical ventilation system that will be analyzed. **Figure 19b** shows that only one wall, shown on the left- hand side, separates the classroom from the unheated environment. The key characteristics of the analyzed environment are provided **in Table 5**. Specifically, **Figure 42a** shows that the classroom features large windows facing outward, as well as a ceiling shield characterized by additional windows, reaching a maximum height of 5.15 m at this point. Moreover, additional internal windows are facing the main corridor (shown on layout sketch in **Figure 19**).

(a) (b)

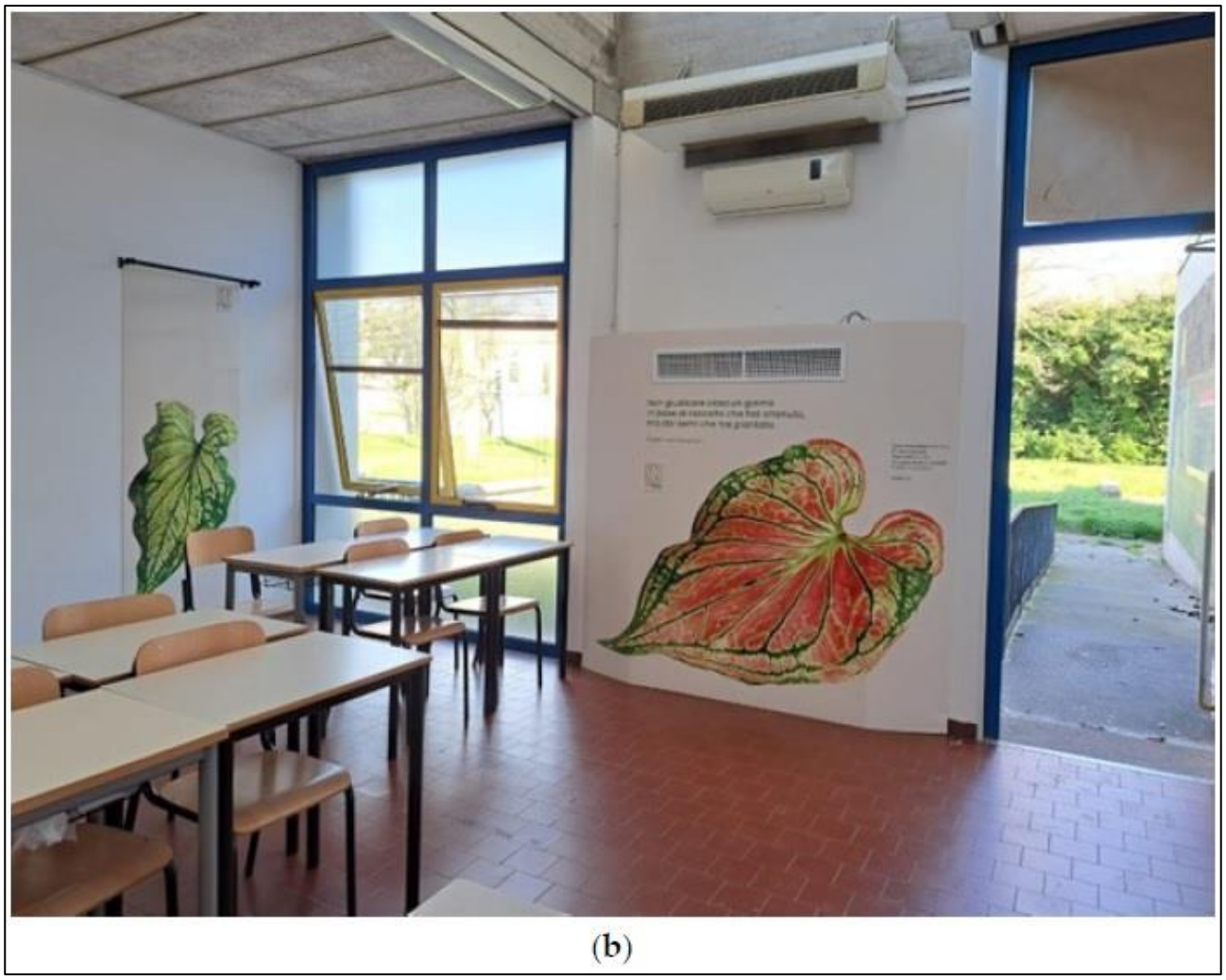
Figure 41. A photo of the school (**a**) and classroom (**b**). In the classroom, the presence of the shield on the ceiling and the position of the existing heating and cooling system can be observed.

Length	8.47 m
Width	5.75 m
Ceiling height	3.23 m
Maximum height	5.15 m
Floor area	48.7 m^2
Volume	170 m^3

Table 5. Main characteristics of the classroom analyzed.

Winter heating is currently provided by a wall-mounted fan coil with a thermostat, while summer cooling is achieved through an air split system with a condenser unit installed on the building's roof. Additionally, like all other classrooms in the school complex, the classroom under examination was not equipped with any air exchange control system, meaning ventilation was ensured manually by opening doors and windows.

(a)



(b)

Figure 42. (**a**) A view of the machine installed inside the classroom, positioned low between the two glass openings. Above, you can also see the fan coil unit, which is part of the school's installed systems and is used for heating. (**b**) A view of the classroom, with the removable masking of the Aldes unit for aesthetic purposes.

4.3.3. Features of the introduced mechanical ventilation

The controlled mechanical ventilation machine used in the study is a commercial unit produced by Aldes (model VEX 380, Aldes Italia, Modena, Italy), and the unit installed inside the classroom is visible in **Figure 42b**. The machine is designed vertically and features a maximum airflow of 1000 m^3/h. It also includes a 2 kW post-heating coil. This post-heating unit is necessary, as otherwise, the air introduced into the environment could have a temperature lower than 20 ◦C, resulting in thermal discomfort for the occupants. The fans have variable speeds, and the heat exchanger operates on the counterflow principle. The main technical specifications, as well as the performance efficiency curves of the exchanger, are presented in **Table 6** and **Figure 43** based on volumetric flow rate. The post-heating coil ensures that the air supplied is at a fixed temperature of 20.8 ◦C at nominal flow, assuming an external air temperature of 15 ◦C.

Heat recovery efficiency [1]	80%
Nominal power (excluding electrical post-heating)	377 W
Nominal external pressure	50 Pa
Fan efficiency	49.3%
Specific power (SFP_{int})	1189 W/(m^3/s)
Filters	F7/ePM1 70% for extraction and fresh air
Sound power level [1]	56 dB(A)

[1] According to EU n1253/2014.

Table 6. Main prameters of the mechanical air ventilation system.

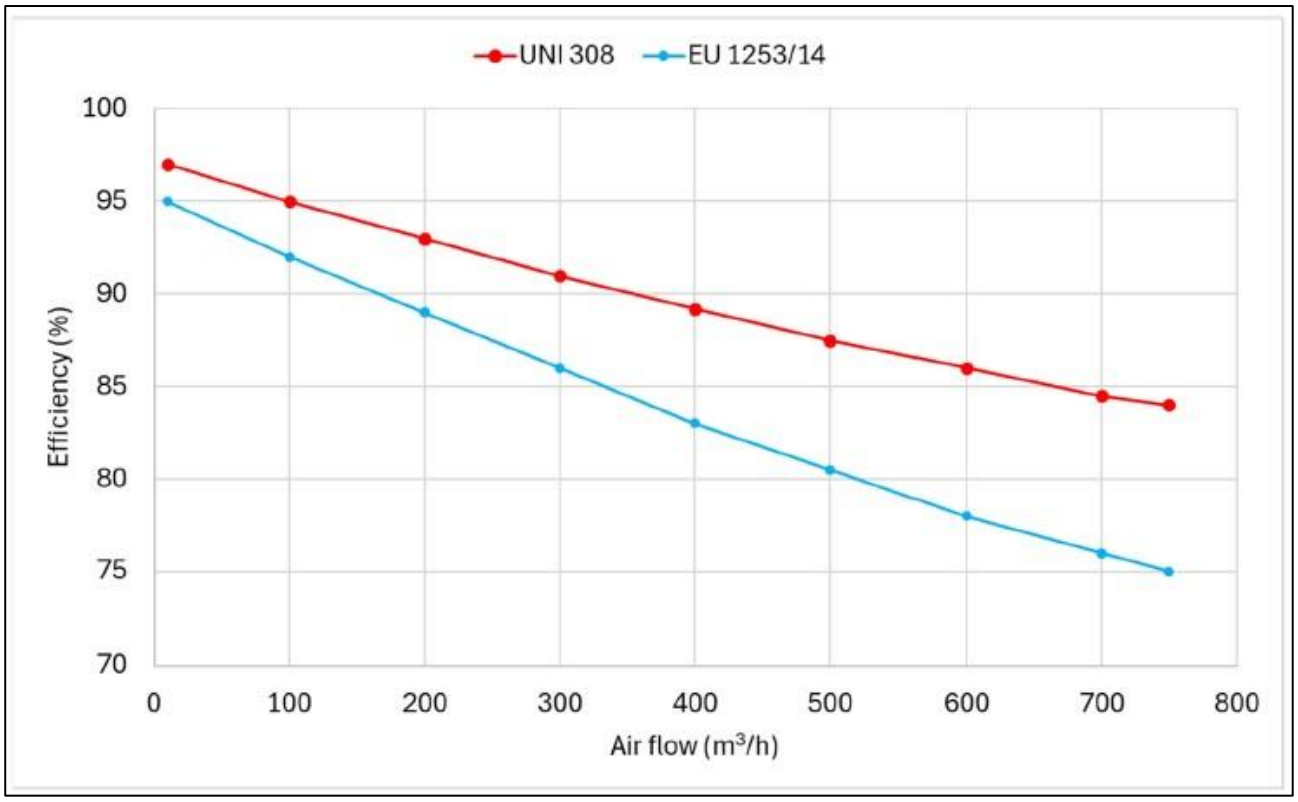


Figure 43. Heat recovery efficiency according to UNI 308 (external temperature/ relative humidity −5°C/80%; internal conditions: 20 °C/50%) and according to EU 1253/14 (external temperature 7°C, internal temperature 20 °C.

4.3.4. Analysis set-up and sensors

The analyses included measurement of CO_2 concentration during:

- The first phase (from mid-September 2023 to December 31st, 2023), with only natural ventilation in the classroom (Sensor 1 active).
- The second phase (from January 6th, 2024 and onwards), with mechanical ventilation implemented (Sensor 1, Sensor 2 and MVHR actives)

For the first phase, the openings of the windows and the main door facing the corridor were recorded as well. It is also noted that, for the scenario involving controlled mechanical ventilation, the airflow rate of the unit was limited to a constant value of 400 m^3/h, estimating a necessary renewal rate of 22

m^3/h per person (17 Students and 1 Professor—6.1 L/s per person). Subsequently, from April 8th 2024, the airflow was increased by 10% to 440 m^3/h (24.4 m^3/h per person, i.e., 6.7 l/s per person) to analyze its effects on daily CO_2 concentration; the airflow was not increased further to keep noise levels within acceptable limits in the classroom. The daily operating schedule of the unit was set to 6 h, from 7:00 a.m. to 1:00 p.m., so it would only be used during periods of actual occupancy and for 6 days a week (excluding Sundays, as there are no classes on Sundays). Additionally, during analyses with controlled mechanical ventilation, the fresh air from the recuperator entering the classroom was heated by the post-heating coil to achieve an outlet temperature of 20 ◦C.

4.3.5. Microbiological analyses

The classroom microbiome was monitored throughout the whole study via bimonthly sampling campaigns uniformly distributed during the study period. At each sampling campaign, five environmental samples were collected in duplicate from the air and surfaces (floor, student tables, and teacher's desk), before and after the students' classes. Surface points were simultaneously sampled by RODAC contact plates and swabs for microbiological culture-based analyses and PCR-based molecular analyses, respectively. The results of the microbial monitoring were similar in the multiple sampling campaigns carried out within each period. Moreover, the environmental samples collected from the different surfaces (floor, student tables, desks) were considered together, since they expressed the overall contamination of the classroom environment. Based on this, the bioburden results were calculated and expressed as median values of those detected in the different sampling campaigns included in each period, showing the surface and air results separately. At the basal level (T0), the results collectively evidenced the presence of potential human pathogens in the classroom environment, as judged by RODAC CFU counts (**Figure 44**). The microbial bioburden, expressed as the sum of searched pathogens, included fungi (including *Aspergillus* spp.) and bacteria (mostly *Staphylococcus* spp., including *S. aureus*), and different levels of microbial contamination were detected on surfaces and in the air, and at pre- and post-lesson timepoints. No significant differences were observed between the TR and CR in any sample at T0 (p = n.s.); thus, a median value was collectively calculated for both enrolled classrooms.

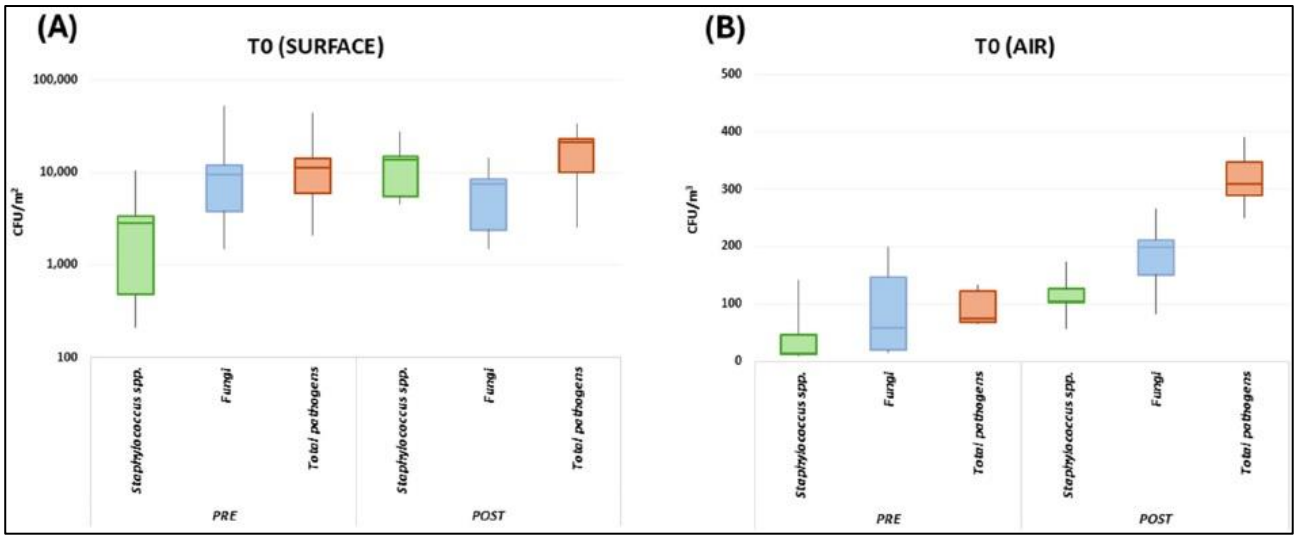


Figure 44. Basal level of pathogen bioburden (T0). Prevalent pathogens and the sum of all searched pathogens are shown. Contamination levels are illustrated as box plots with median, Q1, Q3, minimum, and maximum values of CFU/m2 or CFU/m3 detected in surface (**A**) and air (**B**) samples, before (pre) and after (post) lessons. Being superimposable, CR and TR values were considered together. *Staphylococcus* spp. are represented in green, fungi in blue and the total pathogens in red.

In detail, before lessons, the T0 surface bioburden corresponded to 12,421 CFU/m^2 (median value, range 3098–45,236 CFU/m^2). After the lesson period (6 h), the surface contamination increased as expected, likely due to the presence of students, reaching 21,412 CFU/m^2 (median value, range 2540–33,947 CFU/m^2). Besides the expected quantitative differences attributable to the presence of human occupants, qualitative differences were also observed in the bioburden composition before and after lessons. In detail, before lessons, the surface bioburden essentially consisted of fungi (76.7% of the total surface population, corresponding to a median value of 9526 CFU/m^2), followed by *Staphylococcus* spp. (23.2% of the total surface bioburden, corresponding to a median value of 2881.7 CFU/m^2). After lessons, fungi were not any more prevalent on surfaces, representing 35.6% of the total pathogens (7622.7 CFU/m^2, median value). Instead, *Staphylococci* increased, accounting for 64.4% of the total bioburden (corresponding to a median value of 13,789.3 CFU/m^2). The contamination of the air was significantly lower since the total airborne bioburden corresponded to 75 CFU/m^3 (median value, range 65–134 CFU/m3) before lessons and 310 CFU/m^3 (median value, range 250–391 CFU/m3) after lessons. The airborne population was mainly attributable to fungi before and after lessons. Of note, *Aspergillus* spp. were detectable in air samples, though at a low level (0.01% of the total mycetes), highlighting the presence of this important human pathogen in this community environment. Overall, mycetes represented 76.5% of the total microbial population before lessons (corresponding to a median value of 57,4 CFU/m^3) and represented 64% of the total airborne microbes after lessons (corresponding to a median value of 198.4 CFU/m^3). *Staphylococcus* spp. were

also well represented in the airborne microbial population, accounting for 19.8% of the total airborne pathogens before lessons (14.9 CFU/m^3, median value) and 33.7% after lessons (104.5 CFU/m^3, median value). Neither bacteria of the *Enterobacteriaceae* family nor mycetes of the *Candida* genus were detected at T0 in surface or air samples of the TR or CR (median value 0 CFU/m^2, range 0–0 CFU/m^2). At T1 (**Figure 45**), no significant variations were observed in the control CR compared to the basal values detected at T0 (CRT1 vs. CRT0, p = n.s.). The total surface bioburden corresponded in fact to 12,689 CFU/m^2 before lessons and 17,898 CFU/m^2 after lessons. As observed at T0, before lessons, the surface microbial community was mainly represented by fungi (71.5%, 9072.6 CFU/m^2), followed by Staphylococci (28.5%, 3616.4 CFU/m^2), whereas after lessons, Staphylococci became prevalent (64.9%, 11,615.8 CFU/m^2) and fungi decreased in relative abundance (35%, 6264.3 CFU/m3). Also, the total airborne bioburden levels were very similar to those observed at T0, corresponding to 74 CFU/m^3 before lessons and 290 CFU/m^3 after lessons. Fungi were prevalent in the air both before (75.1%, 55.6 CFU/m^3) and after lessons (63.6%, 184.4 CFU/m^3), as also detected at T0.

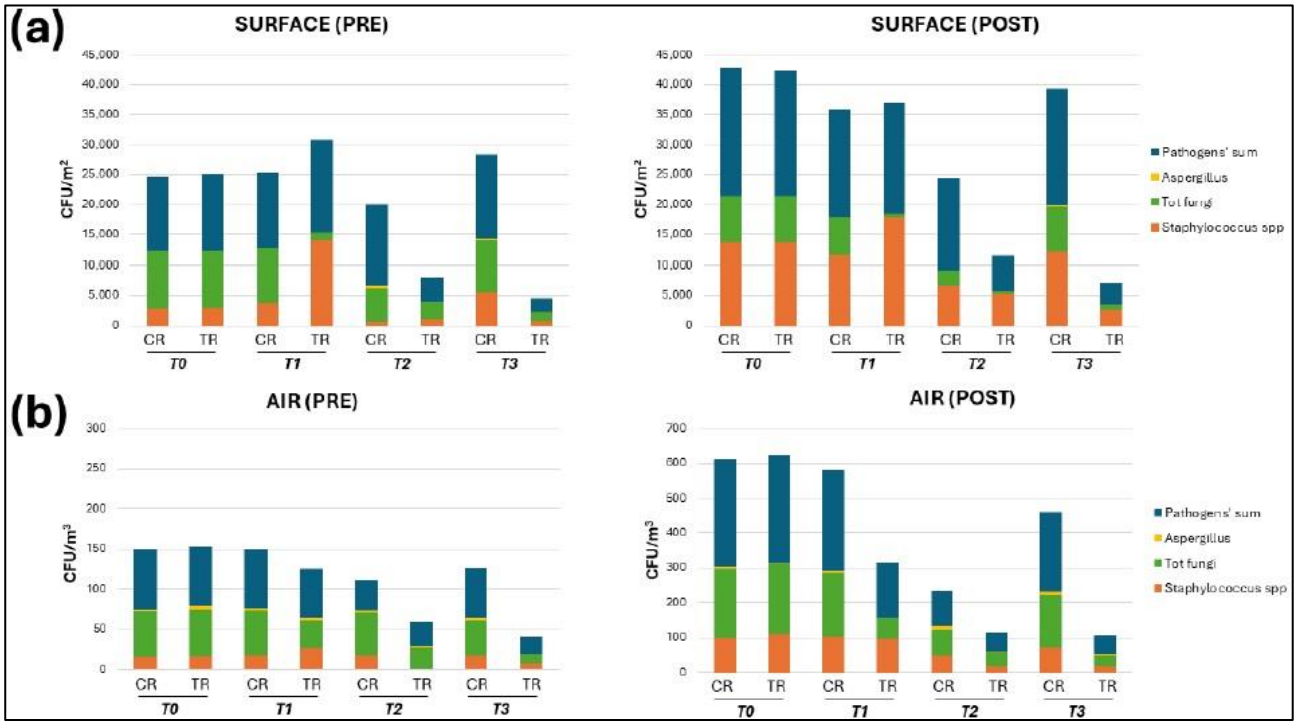


Figure 45. Pathogen bioburden in the CR and TR rooms. The presence and proportion of total and individual pathogens are shown during all the study periods (T0, T1, T2, and T3). The results are expressed as the sum of the median values obtained by RODAC sampling and CFU counts on surfaces (**a**) and in the air (**b**) before (pre) and after (post) lessons.

By contrast, some microbial alterations were observed in the TR, following the placement of green plants (T1). First, an overall increase in bioburden on surfaces was observed compared to T0

(15,473.7 vs. 12,421 CFU/m^2), which was mainly attributable to a higher presence of Staphylococci, whose amount corresponded to a median value of 14,130.2 CFU/m_2 (compared to 2881 CFU/m^2; p <0.001), thus representing the vast majority of surface bioburden even before lessons (91.3%). After lessons, the total surface bioburden further increased, with 18,549 CFU/m^2 (median value), and *Staphylococcus* spp. represented 96.6% of the total microbial population (17,918.3 CFU/m^2, median value). Fungi thus represented a very small fraction of the surface bioburden, both before (7.8%; 1206.9 CFU/m^2) and after lessons (2.5%; 463.7 CFU/m^2). In contrast, the total airborne contamination appeared reduced in the TR at T1 compared to the control values both before lessons (61 CFU/m^3 vs. 74 CFU/m^3; p = n.s.) and after lessons (157.5 CFU/m^3 vs 290 CFU/m^3; p <0.05). Of note, the fungal component was particularly affected, representing only 56% of the total bioburden before lessons, corresponding to 34 CFU/m^3 (instead of 71.5% and 55.6 CFU/m^3 for the control. After lessons, the relative abundance of fungi was further decreased, as it represented 36.9% of the total bioburden, corresponding to 58.1 CFU/m^3, compared to 184.4 CFU/m^3 for the control.

The introduction of green plants in the TR was thus associated with a whole reduction in fungal contamination corresponding to 38.8% ($p < 0.05$) before lessons and 68.4% ($p < 0.01$) after lessons, in comparison with the control CR values.

At T2, a mechanical ventilation system was installed in the TR, allowing a constant flow rate of 440m^3/h of fresh air (the same flow rate for the expelled air) during lesson time. The presence of mechanical ventilation kept temperature and humidity levels constant, at 21–23 ◦C and 40–45%, respectively, whereas in the CR, the temperature and humidity fluctuated continuously, increasing up to 25 ◦C and 65%, respectively. The microbial monitoring evidenced a clear reduction in pathogens associated with this intervention. Specifically, before lessons, the TR surface bioburden corresponded to 3998.9 CFU/m^2 (median value; range 1052.63–4578.94 CFU/m^2), compared with 13,368.4 CFU/m^2 detected in the CR (median value; range 2549–25,621.5 CFU/m^2), with a significant 70% reduction ($p < 0.01$). After lessons, the median value of surface contamination in the TR corresponded to 5789.5 CFU/m^2 (median value, range 3578.9–6889.5 CFU/m^2), compared with the 15,263.2 CFU/m^2 measured in the CR (median value; range 6521.7–23,121 CFU/m^2), confirming that the introduction of mechanical ventilation significantly reduced the surface contamination even in the presence of students (−62.1%; $p < 0.05$). The relative abundance of the main microbial components was similar to that observed at T0, despite the continuous presence of plants. Namely, in the TR, fungi were prevalent before lessons, representing 73% of total surface pathogens (2919.2 CFU/m^2), whereas Staphylococci became prevalent after lessons (89%) (5152.7 CFU/m^2). A clear decrease was also observed in the airborne bioburden in the TR compared to the control. In detail, the TR air bioburden corresponded to 28.5 CFU/m^3 before lessons (median value, range 21–32 CFU/m^2) and 58

CFU/m^3 after lessons (median value, range 45–65 CFU/m^3), whereas it corresponded to 38 CFU/m^3 before lessons (range 29–61 CFU/m^3) and 101 CFU/m^3 after lessons (range 75–221 CFU/m^3) in the CR. By comparing the TR and CR values, the airborne bioburden decreased by 26% at pre-lesson and by 42.63% at post-lesson timepoints ($p < 0.05$). In the air of the TR, fungi remained prevalent both before (93%) and after lessons (66.6%), as also observed in the CR environment, though the CFU number was diminished (26.5 vs. 52.2 CFU/m^3 and 38.6 vs. 70.7 CFU/m^3 in TR vs. CR values before and after lessons, respectively).

At T3, PCHS was implemented in the TR in substitution for the conventional chemical one. As expected, based on previous results, this intervention was associated with significant changes in the TR environmental bioburden compared to the control (**Figure 46**). In the CR, surface contamination was in line with what was observed at the previous timepoints, corresponding to 14,105 CFU/m^2 before lessons (median value; range 11,256–28.456.3 CFU/m^2) and 19,631.8 CFU/m2 after lessons (median value; range 12,589.6–31,102 CFU/m^2). Fungi were prevalent before the arrival of students (60% of total surface bioburden, corresponding to 8463 CFU/m^2), whereas Staphylococci became prevalent afterward (62%; 12,173.6 CFU/m^2). In contrast, the levels measured in the TR showed a significant decrease in all surface pathogens, which corresponded to 2315.8 CFU/m^2 before lessons (median value, range 1473.7–4263.2 CFU/m^2) and 3578.9 CFU/m^2 (median value, range 1136.8–5105.3 CFU/m^2) after lessons. The TR values were thus diminished compared to the CR ones, with 83.5% before lessons and 81.8% after lessons ($p < 0.0001$). On the other hand, the proportions of different microbial components were maintained, with fungi representing 68% of the total surface bioburden before lessons (1574 CFU/m^2, median value) and Staphylococci becoming prevalent after lessons (75%; 2684 CFU/m^2, median value).

The decrease in pathogenic bioburden was paralleled by a gradual increase in PCHS-derived *Bacillus* spp., which reached 7578.9 CFU/m^2 at the end of the T3 period, thus representing 76.6% and 67% of the total microorganisms detected in the classroom before and after lessons, respectively.

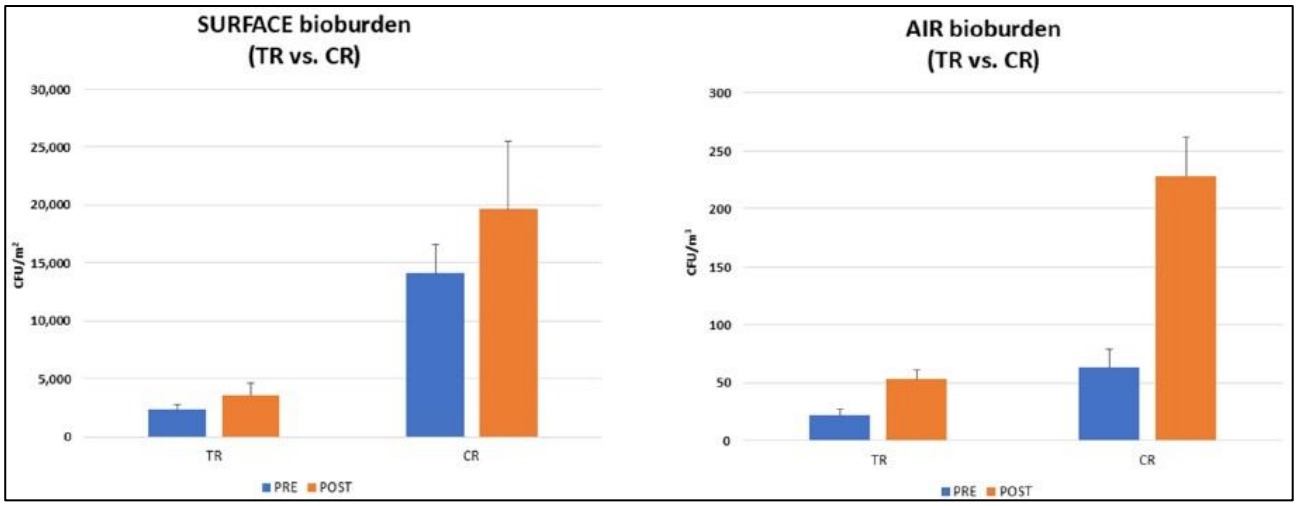


Figure 46. T3 surface and air bioburden in the TR and CR rooms. Results are expressed as median values of CFU/m2 ± SD (surface) and CFU/m3 ± SD (air) detected before (pre) and after (post) lessons.

Also, air contamination levels remarkably decreased in comparison with the CR, since the TR air pathogens corresponded to 22 CFU/m^3 before lessons (median value, range 9–69 CFU/m^3) and 53 CFU/m^3 after lessons (median value, range 15–81 CFU/m^3). In contrast, in line with previous periods, the CR exhibited 63 CFU/m^3 of airborne pathogens before lessons (median value; range 18–101 CFU/m^3) and 228 CFU/m^3 after lessons (median value; range 141.2–352 CFU/m^3). Fungi were prevalent both before and after lessons in the CR (68% and 66.8%, respectively) and in the TR, where they represented 51% and 64% of total airborne pathogens before and after lessons, respectively. Of note, while *Aspergillus* was detected in the CR, with a median value of 3 CFU/m^3 (range 0–5 CFU/m^3), no *Aspergillus* was found in the TR (median value 0 CFU/m^3, range 0–0 CFU/m^3). Based on the measured CFU numbers, the decrease in air contamination was 65.1% before lessons and 76.8% after lessons ($p < 0.001$). **Figure 46** specifically evidences the differences between TR and CR bioburden levels at the final T3 study period.

4.3.6. Molecular analyses

Floor samples, being the most abundant in terms of microbial load, were also analyzed by qPCR microarray to profile the antibiotic resistance genes (ARGs) harbored by the microbial population persistently colonizing the classroom environment. The results evidenced a low but detectable presence of different ARGs in the classroom microbes, conferring resistance against antibiotics belonging to different classes, including aminoglycosides, tetracycline, beta-lactams (including methicillin), and macrolides. Most detected ARGs and their functions are summarized in **Table 7**.

ARGs	Antibiotic/Gene Function	Reference
aadA1	Aminoglycoside	Hollingshead et al., 1985 [44]
aphA6	Aminoglycoside	Aris et al., 2019 [45]
CTX-M-9 Group	Class A beta-lactamase	Sun et al., 2010 [46]
GES	Class A beta-lactamase	Lee et al., 2005 [47]
IMI & NMC-A	Class A beta-lactamase	Walther-Rasmussen et al., 2007 [48]
SFO-1	Class A beta-lactamase	Matsumoto et al., 1999 [49]
SHV (238G240K)	Class A beta-lactamase	Caselli et al., 2016 [39]
Per-1 group	Class A beta-lactamase	Aly et al., 2016 [50]
VIM-7	Class B beta-lactamase	Toleman et al., 2004 [51]
IMP-5 group	Class B beta-lactamase	Brízio et al., 2006 [52]
ACT 5/7 group	Class C beta-lactamase	Guan et al., 2024 [53]
FOX	Class C beta-lactamase	Gonzalez Leiza et al., 1994 [54]
LAT	Class C beta-lactamase	Tzouvelekis et al., 1994 [55]
MIR	Class C beta-lactamase	Papanicolaou et al., 1990 [56]
MOX	Class C beta-lactamase	Oguri et al., 2014 [57]
OXA-2 Group	Class D beta-lactamase	Bhattacharjee et al., 2015 [58]
OXA-23 Group	Class D beta-lactamase	Smith et al., 2013 [59]
OXA-55	Class D beta-lactamase	Héritier et al., 2004 [60]
QnrB-8 group	Fluoroquinolone	Rezazadeh et al., 2016 [61]
ermA	Macrolide lincosamide streptogramin_b	Malhotra-Kumar et al., 2009 [62]
ermB	Macrolide lincosamide streptogramin_b	Min et al., 2008 [63]
ermC	Macrolide lincosamide streptogramin_b	Shivakumar et al., 1981 [64]
mefA	Macrolide lincosamide streptogramin_b	Daly et al., 2004 [65]
msrA	Macrolide lincosamide streptogramin_b	Poole et al., 2005 [66]
tetB	Tetracycline efflux pump	Warburton et al., 2013 [67]
mecA	Methicillin	Utsui et al., 1985 [68]

Table 7. Most detected ARGs with encoded functions.

In the CR, the abundance of ARGs detected at T0 remained nearly unchanged throughout the whole study, whereas some noteworthy variations were observed in the TR following the introduction of the planned interventions (**Figure 47**). At T0, both the TR and CR showed the same ARGs, without any relevant differences. The prevalent ARGs before lessons included, in order of abundance (expressed as Log_{10} fold change, FC, with respect to the negative control, NTC), *msrA* (4.2 Log_{10} FC), *ermB* (1.9 log_{10} FC), *aphA6* (1.7 Log_{10} FC), and *mecA* (1.16 Log_{10} FC), followed by aadA1 (0.9 Log_{10} FC), *Per-1* group (0.5 Log_{10} FC), and *OXA-2* group (0.45 Log_{10} FC). *S. aureus* and its virulence genes *spa* and *luk* (included in the microarray) were also detected at high frequency (4.47 Log_{10} FC). Notably, *mecA* was found in the classroom environment, evidencing the likely presence of MRSA (methicillin-resistant *S. aureus*) in the school environment. After the attendance of students, further ARGs appeared, likely as a consequence of the spread of students' bacteria. The most prevalent ARGs after lessons included *mefA* (5 Log_{10} FC), *msrA* (3.1 Log_{10} FC), *ACT-5/7* group (3.03 Log_{10} FC), *ermB* (1.9 Log_{10} FC), *FOX* (1.7 Log_{10} FC), *ermC* (1.7 Log_{10} FC), and *tetB* (1.3 Log_{10} FC).

At T1, following plant introduction, a decrease in some ARGs was observed in the TR, whereas *ermB* (4.5 Log_{10} FC) and *mecA* (2.9 Log_{10} FC) appeared slightly increased, probably as a consequence of the increased number of Staphylococci observed in the TR during the T1 phase of the study. No statistical significance was detected in any of the observed differences. After classes, compared to

what was observed at T0, some ARGs were decreased up to 2 Logs (*ACT-5/7* group, *FOX*, *ermB*, *mefA*), but new ARGs appeared, including *aphA6* (2.08 Log_{10} FC), *CTX-M-9* group (4.9 Log_{10} FC), and *VIM-7* (4.7 Log_{10} FC).

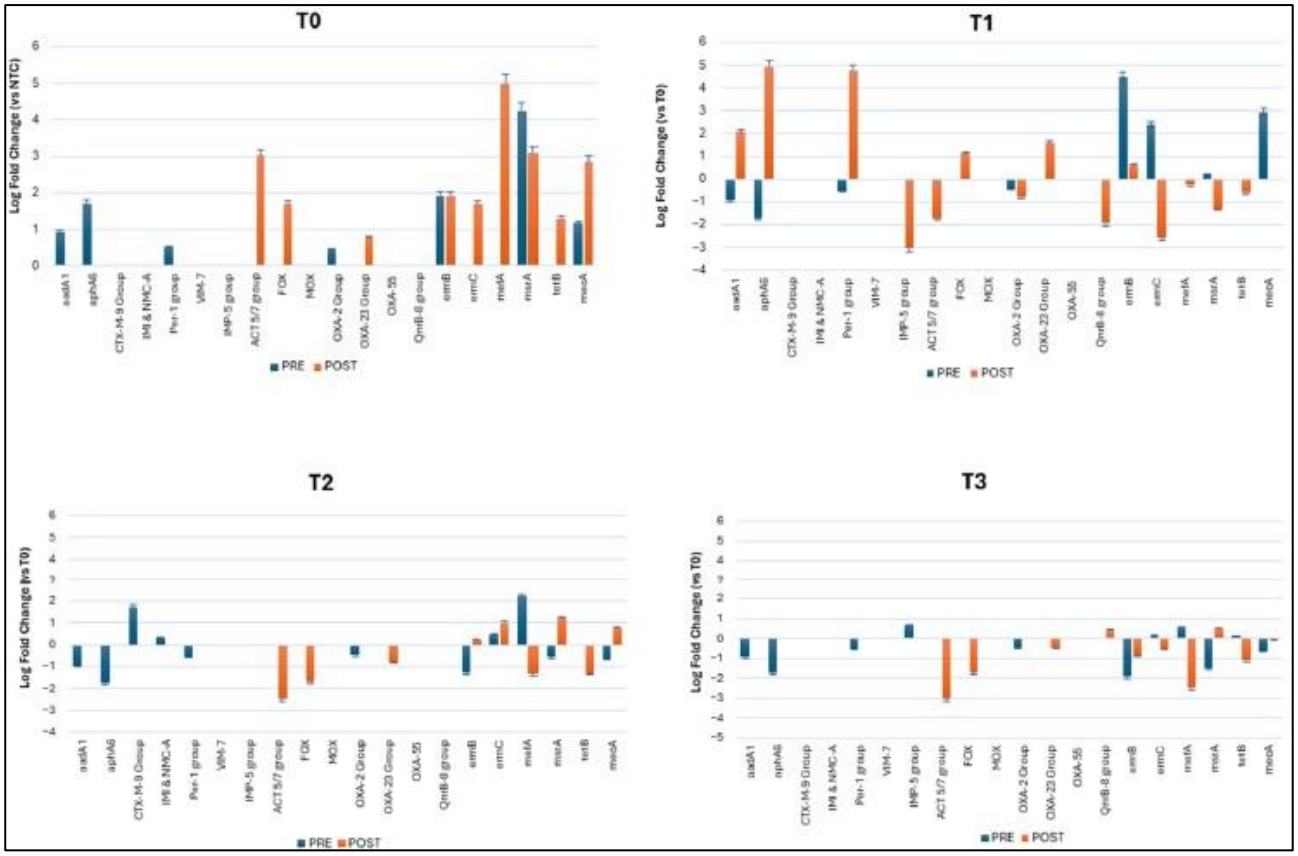
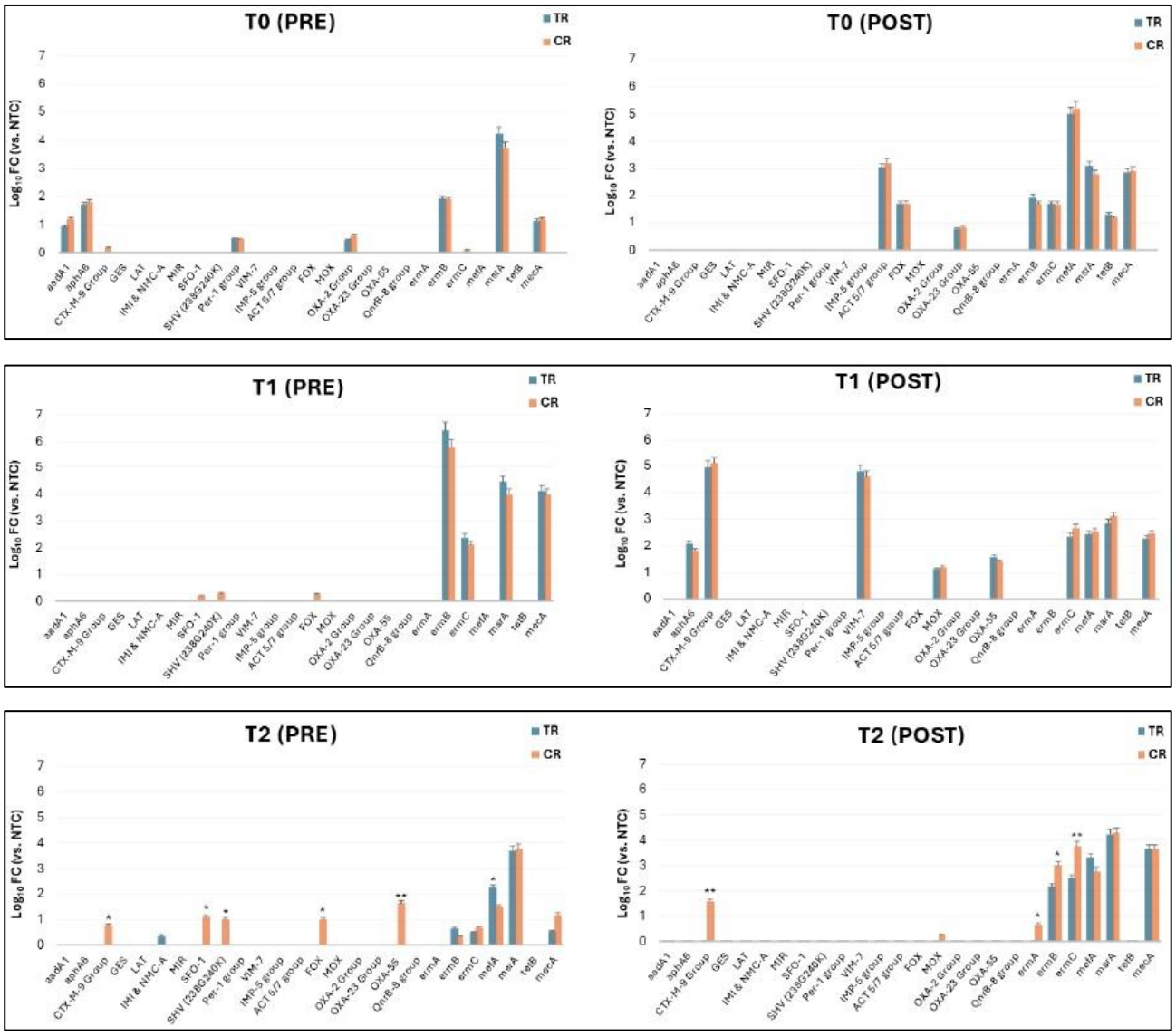


Figure 47. Resistome characterization in the TR classroom. ARGs were evidenced by qPCR microarray, performed on floor samples at the indicated times (T0, T1, T2, and T3) before (pre) and after (post) lessons. Results are expressed as mean values ± SD of the Log_{10} fold change (FC) for every ARG. T0 values were obtained for comparison with the negative control (NTC) values, whereas T1, T2, and T3 values were obtained for comparison with the T0 values, as indicated on the y-axis.

At T2, upon the installation of a mechanical ventilation system, the number of ARGs appeared to decrease compared to T0, both before and after lessons, likely in association with the decreases observed in the whole bioburden by CFU counts. However, while *aadA1*, *aphA6*, and *ermB* were decreased by up to −1.5 Log_{10} FC before lessons, *CTX-M-9* group and *mefA* were increased by around 2 Log FC compared to T0. After lessons, a remarkable drop in almost all ARGs detected at T0 was recorded, except for *ermC*, *msrA*, and *mecA*, which were increased by about 1 Log_{10} each. In contrast, at T3, the introduction of PCHS sanitation was associated with an overall decrease in all the ARGs detected at T0, T1, and T2 before lessons (up to −99.9%, $p_c < 0.001$). The decrease was also evident after lessons, when all the ARGs identified in previous periods appeared to be significantly

diminished (up to −3 Log_{10}), thus confirming the significant impact of probiotic-based sanitation in preventing the diffusion of drug-resistant microbes in both sanitary and non-sanitary environments. Of note, while the differences observed between the TR and CR were not significant at T0 and T1, they became statistically significant at both T2 and T3 ($0.05 < p_c < 0.001$) (**Figure 48**).

At T3, the differences were significant for all the ARGs detected at both pre- and post-lesson timepoints. Similarly, the presence of virulent *S. aureus* (associated with the virulence genes *spa* and *luk F*) appeared to be significantly diminished only at T3, in the TR vs. the CR.



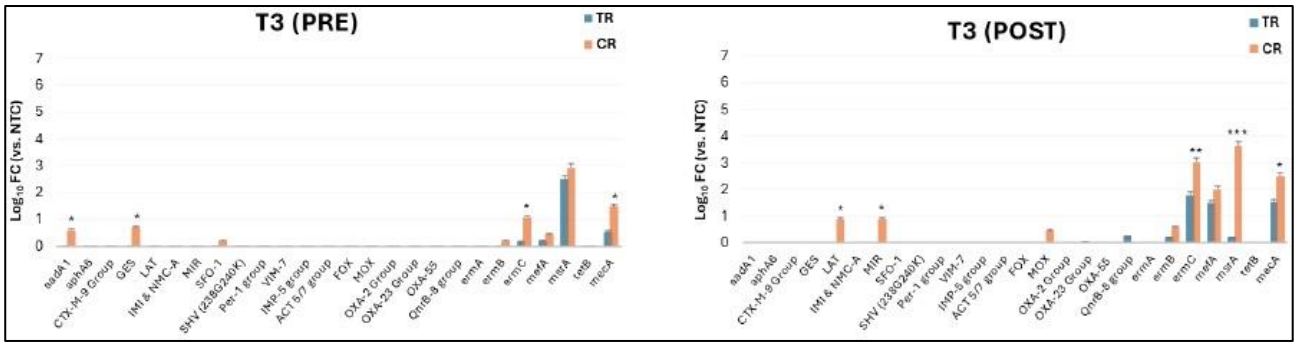


Figure 48. Resistome characterization in TR and CR classrooms. ARGs were quantified by qPCR microarray performed on floor samples at the indicated times (T0, T1, T2, and T3) before (pre) and after (post) lessons. Results are expressed as mean values ± SD of the Log_{10} fold change (FC) vs. the NTC for every indicated ARG. Asterisks indicate statistically significant differences between TR and CR values (*, $p_c < 0.05$; **, $p_c < 0.01$; ***, $p_c < 0.001$).

4.3.7. Thermohygrometric and CO_2 concentration analysis

In **Figure 49**, carbon dioxide concentration, temperature, and relative humidity values, recorded for two Sundays (October 29th, 2023, and December 17th, 2023), are presented. Focusing on CO_2 (shown with measurement uncertainty according to the technical datasheet), we observe that, on both days, within the selected monitoring period from 7:00 a.m. to midnight, the indoor CO_2 concentration in the classroom exhibits a decreasing trend, dropping from about 650 ppm(v) to approximately 550 ppm(v). In contrast, outdoor CO_2 levels are lower than indoors and show more fluctuations. This trend (CO_2_INT) is typical in spaces where, due to external infiltration and no internal generation (as the room is unoccupied), CO_2 concentration tends to decrease. Additionally, we note that the indoor concentration does not reach the outdoor level; this equilibration is expected to occur in the early hours of Monday morning (not reported here for brevity). Regarding relative humidity, we observe stability within the classroom on both days: around 70% on the Sunday in November and between 55% and 60% on the Sunday in December. Indoor temperatures also remained stable over the two days, but were lower in December (16–17 ◦C) compared to October's 19–20 ◦C. Outdoor temperatures, however, were notably different, with December's minimum around 7 ◦C compared to October's minimum of 14 ◦C. The temperature trend in December highlights how the heating system is correctly turned off when the school is unoccupied.

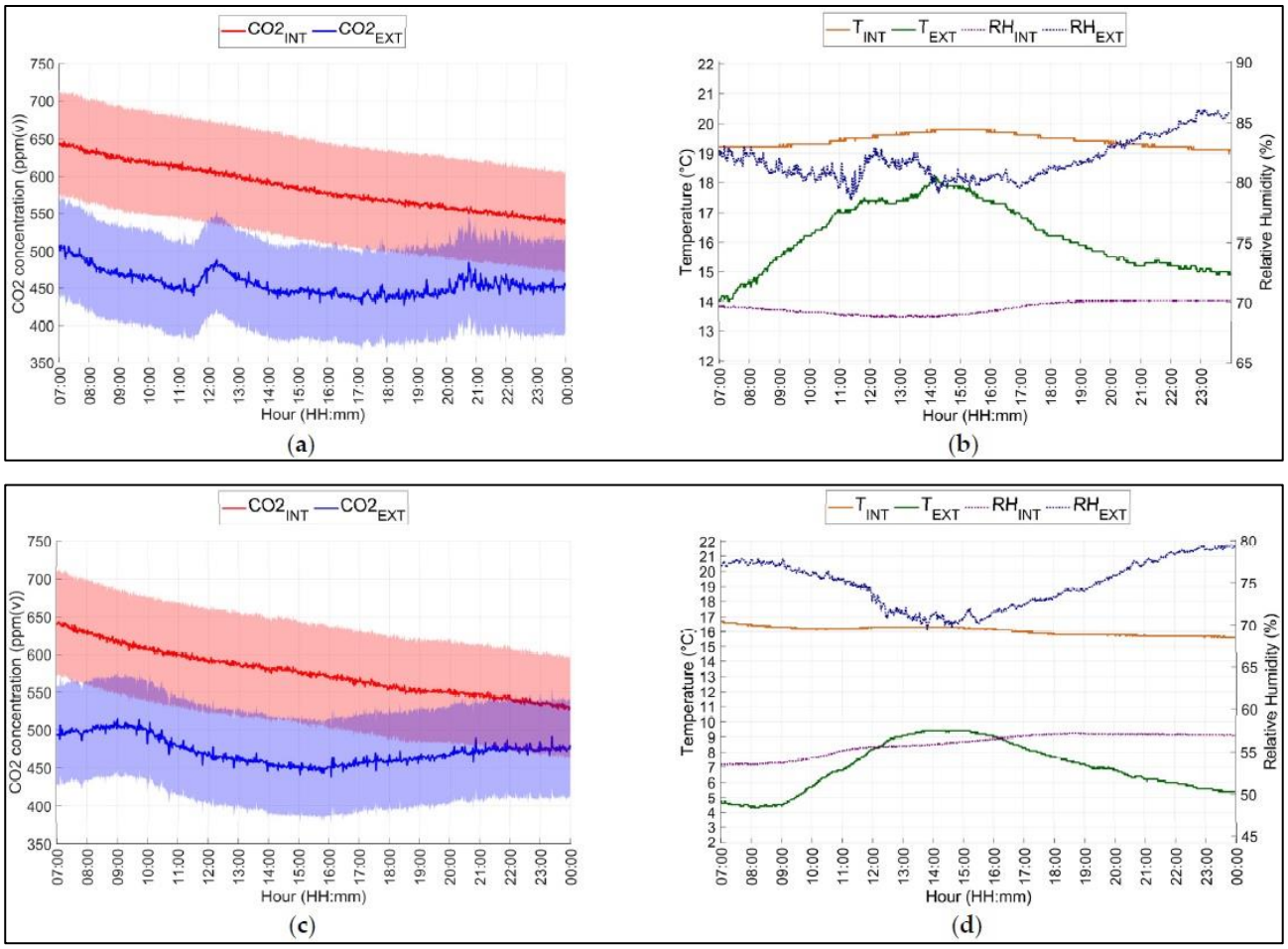


Figure 49. The CO_2 concentrations in the classroom (CO_2_INT) and outdoors (CO_2_EXT), internal and external temperatures (T_INT and T_EXT), and internal and external relative humidity (RH_INT and RH_EXT) for two different days (Sundays, without students): October 29th 2023 (**a**,**b**) and December 17th 2023 (**c, d**)

In **Figure 50**, the trends of the CO_2 concentrations recorded over the two analyzed days in October and December are shown, in the absence of internal CO_2 production due to the lack of anthropogenic activities. It is observed that at 7:00 a.m. on both days, the concentrations are very close: 642 ppm(v) on the October day and 641 ppm(v) on the December day. Additionally, the two curves are perfectly superimposable, with deviations within the accuracy of the internal sensor.

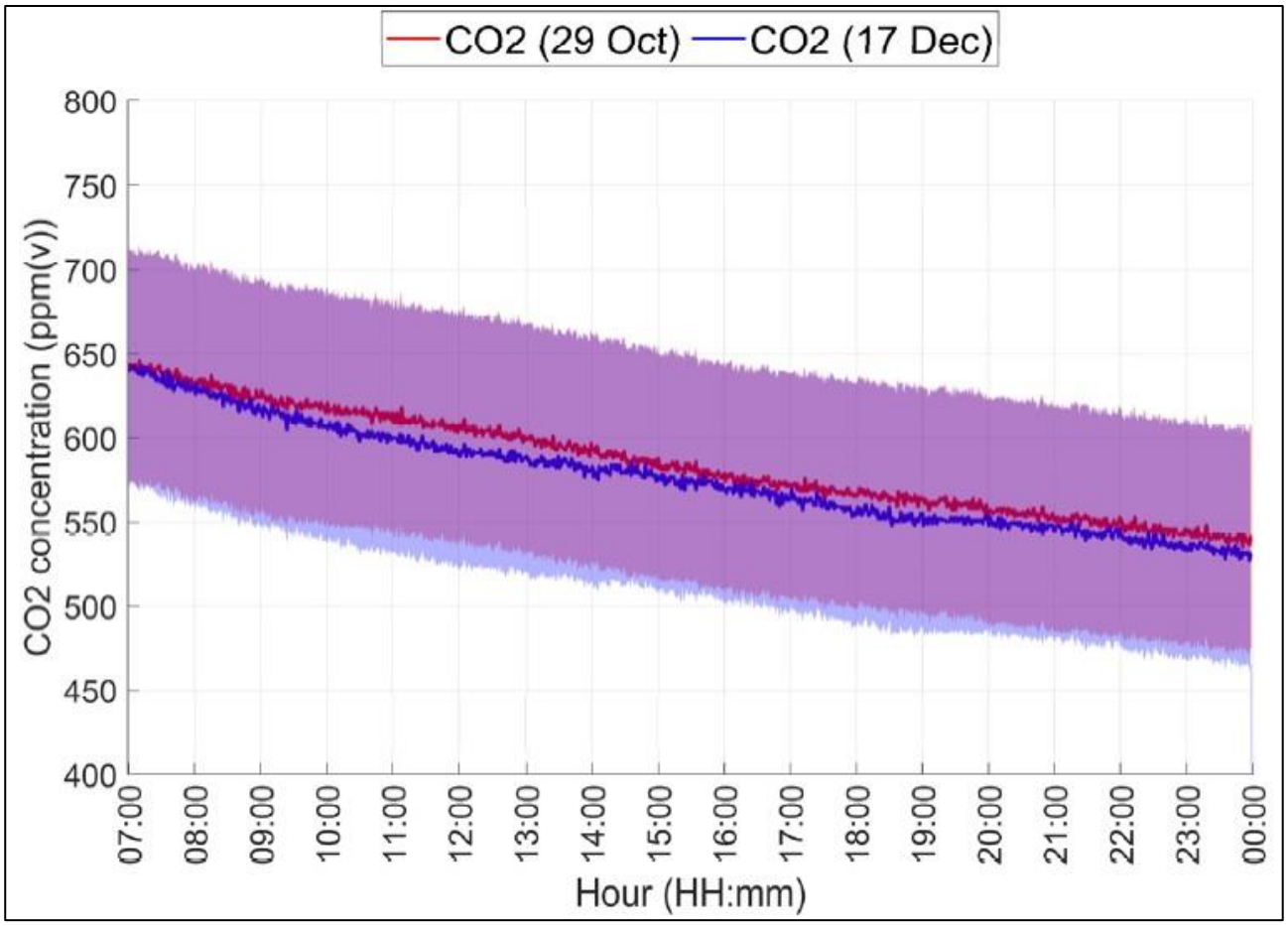


Figure 50. Trends of CO_2 concentrations for two Sundays: October 29th, 2023 and December 17th, 2023. In the figure the shaded areas represent the uncertainties related to the measurements of the two sensors.

Let us now consider the measurements taken without controlled mechanical ventilation, i.e., when ventilation and air exchange are manually managed by opening and closing windows during class hours. **Figure 51a,b**, refer to Monday October 23rd, a day when the heating system was not yet active, as the external temperature combined with internal loads was sufficient to maintain a comfortable climate inside the classroom (the average indoor temperature during student occupancy from 8:00 a.m. to 1:00 p.m. was 20.5 ◦C, with a minimum of 19.3 ◦C and a maximum of 21.7 ◦C). From the CO_2 graph in **Figure 51a**, significant variations in CO_2 levels in the environment were observed (the average value during the usual period of occupancy is 1334 ppm(v), with extremes of 618 and 2205 ppm(v). Rapid changes occur in correspondence with the opening of windows and/or doors to carry out natural ventilation. Moreover, comparing the measured CO_2 values with temperature and indoor humidity, it is observed that an increase in CO_2 corresponds to an increase in relative humidity in the classroom, due to the presence of people; however, relative humidity quickly decreases following the opening of doors and windows. This effect is also visible in the indoor temperature, although to a lesser extent, due to the position of the installed sensor within the classroom, which is installed 1.70

m above the floor. These data show that natural ventilation was carried out, but the exchanges were still insufficient to ensure adequate CO_2 levels for the presence of people in the environment. Now, in the case of **Figure 51c, d**, referring to November 23rd, 2023, the heating system was on, and during the time interval of classroom occupation by students, an average temperature of 21.5 ◦C was recorded, with extremes of 18–23.5 ◦C. Even in this case, from the CO_2 graph, it was observed that natural ventilation was carried out, and the corresponding trends related to humidity and indoor temperature discussed previously are observed. Also, in this case, natural ventilation was entirely insufficient to ensure adequate air quality in the environment during the hours of occupation (with an average concentration of 1504 ppm(v), with a maximum of 2485 ppm(v) and a minimum of 442 ppm(v), the latter corresponding to the opening of windows). Considering the carbon dioxide concentration shown in **Figure 51a, c** in the absence of mechanical ventilation, a characteristic "sawtooth" pattern can be observed. This pattern is typical of the Lotka–Volterra model (Volterra, 1926), commonly used in ecology to estimate the dynamics of two interacting populations, prey and predator, over time. This time-dependent model describes population trends through two differential equations, where key factors include the prey's growth rate (in this case, the increase in CO_2 when windows are kept closed), the prey's mortality (CO_2 decreasing rate due to infiltration), and the predator's growth rate (represented by the opening of windows). Finally, analyzing **Figure 51e, f**, referring to Wednesday December 13th, which was characterized by a more severe external climate (with an average of 11.2 ◦C and minimum of 8.4 ◦C), there were very limited air exchanges with the outside and other environments: this situation led to poor air quality inside the classroom, reaching a peak CO_2 level at 12:15 of 4063 ppm(v), corresponding to a peak in relative humidity of 62%. Furthermore, an abnormal trend is observed when attention is focused on indoor temperature. Even in this case, the heating system was operational. Still, unlike the previously analyzed day, when the system was turned off at the end of classroom use, in this case, the system remained on until 6:00 p.m., leading to excessively high internal air temperatures of over 27 ◦C. This abnormal behavior of the system was also detected on other days of the heating season.

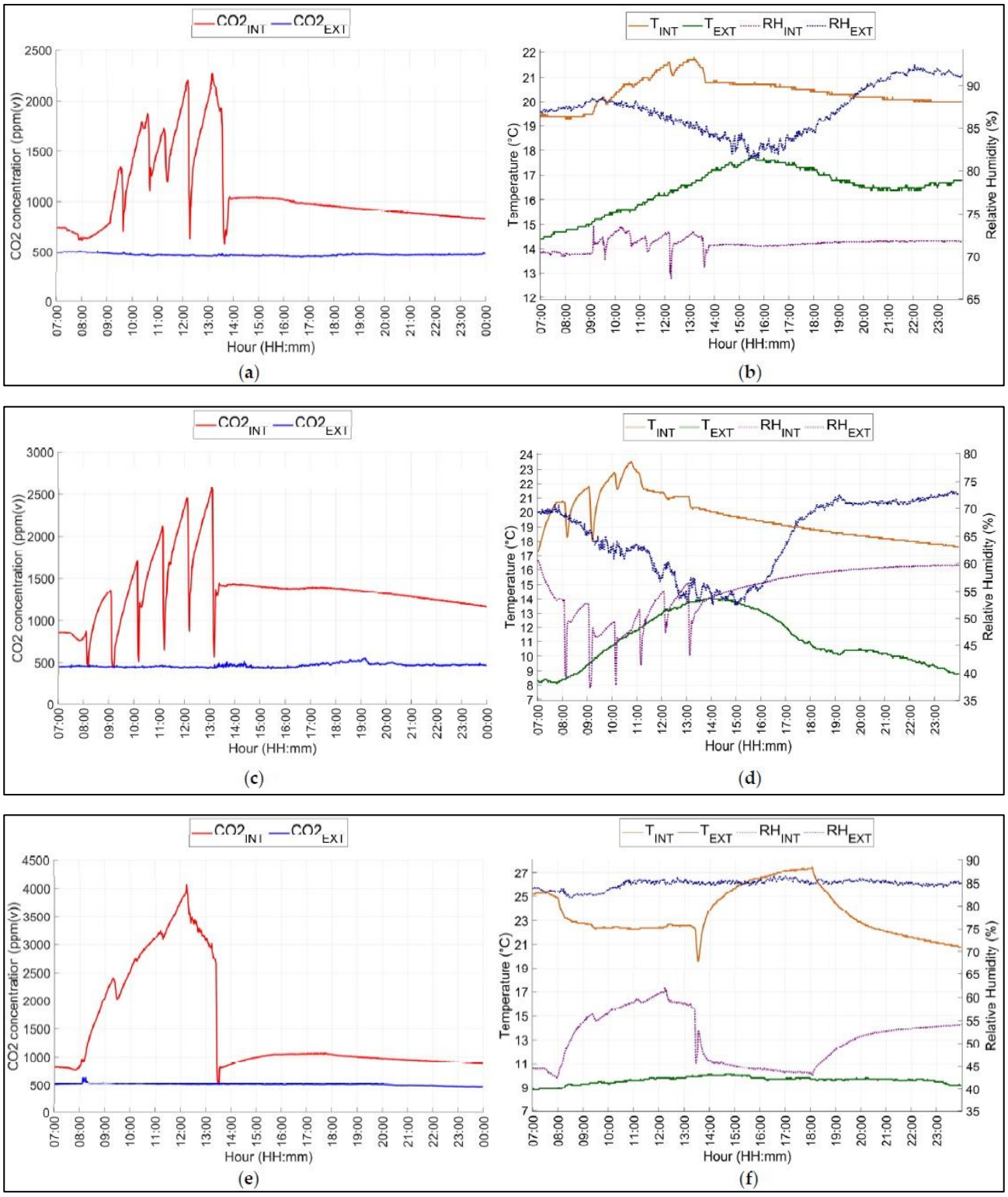


Figure 51. CO_2 concentration, temperature, and humidity for three different days in which natural ventilation is considered: Monday October 23rd 2023 (**a**,**b**); Thursday November 23rd 2023, (**c**,**d**); and Wednesday December 13th 2023 (**e**,**f**).

In **Figure 52**, the graphs relating to the CO_2 concentration, temperature, and relative humidity for two days when the controlled mechanical ventilation system was operational are shown. From **Figure 52**

a,b, referring to January 19th 2024, a day when both the heating system was operational and controlled mechanical ventilation was set at a fixed flow rate of 400 m^3/h from 7:00 a.m. to 1:00 p.m., a rising CO_2 trend is observed, with a peak at 11:30 a.m. of 1552 ppm(v) and an average during the occupancy period of 1204 ppm(v).

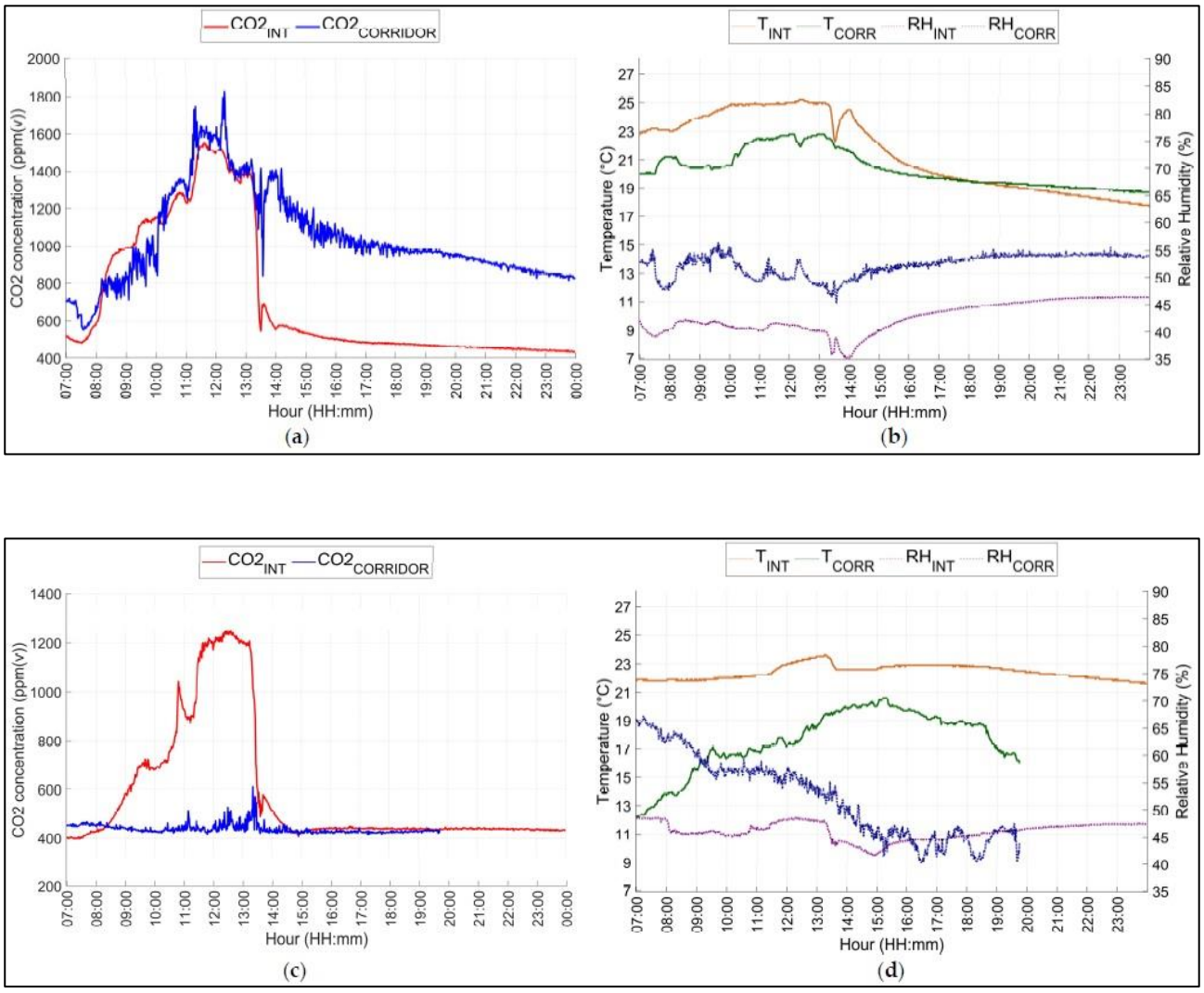


Figure 52.CO_2 concentration, temperature, and humidity for two different days in which mechanical ventilation is considered: January 19th 2024 (**a**,**b**) and April 4th 2024 (**c**,**d**). The data refer to conditions in the classroom (subscript "INT") and in the corridor (subscripts "CORR" and "CORRIDOR").

In fact, if carbon dioxide is seen as gas tracker, **Figure52** highlights that an efficient air renew occurs. These values are lower than those obtained with natural ventilation; moreover, during the occupancy period, they are comparable to the values detected in the adjacent corridor (the Testo sensor previously positioned outside was moved to the adjacent corridor). It is also observed that after the students leave the school, CO_2 shows a decreasing trend both in the corridor and the classroom; however, in the classroom, the CO_2 levels are close to those normally found outside. During the

occupancy period, the temperatures were maintained at around 23 ◦C in the classroom while ranging between 20 and 23 ◦C in the corridor; the humidity also remained stable at values between 50 and 55% in the classroom. **Figure 52**c, d refers to April 4th, 2024, when the heating was off, but the controlled mechanical ventilation was operating at a fixed rate of 440 m^3/h. Also, in this case, the peak of CO_2 increased during the occupancy period, with a peak of 1250 ppm(v) and an average during the occupancy period of 844 ppm(v). It is also noted that during the period from 11:30 a.m. to 1:00 p.m. a plateau was reached; the values obtained are still lower compared to January 19th, 2024, due to the 10% increase in the air renewal rate. The sensor placed in the corridor, however, measured a lower concentration, close to what should be present outside. It is likely that some corridor windows were open, while the classroom door to the corridor was closed. Inside the classroom, temperatures and humidity were maintained constantly at ranges of 21–23 ◦C and 45–50%, respectively. It is clearly visible that the concentration of CO_2 decreased following the implementation of controlled mechanical ventilation, as well as the stabilization of the relative humidity level. Moreover, **Figure 53** shows the trend of thermohygrometric parameters when using only scheduled natural ventilation, achieved by opening the windows for 5–10 min at each hour change. The aim was to experiment with a simple procedure carried out by the students to reduce CO_2 concentration.

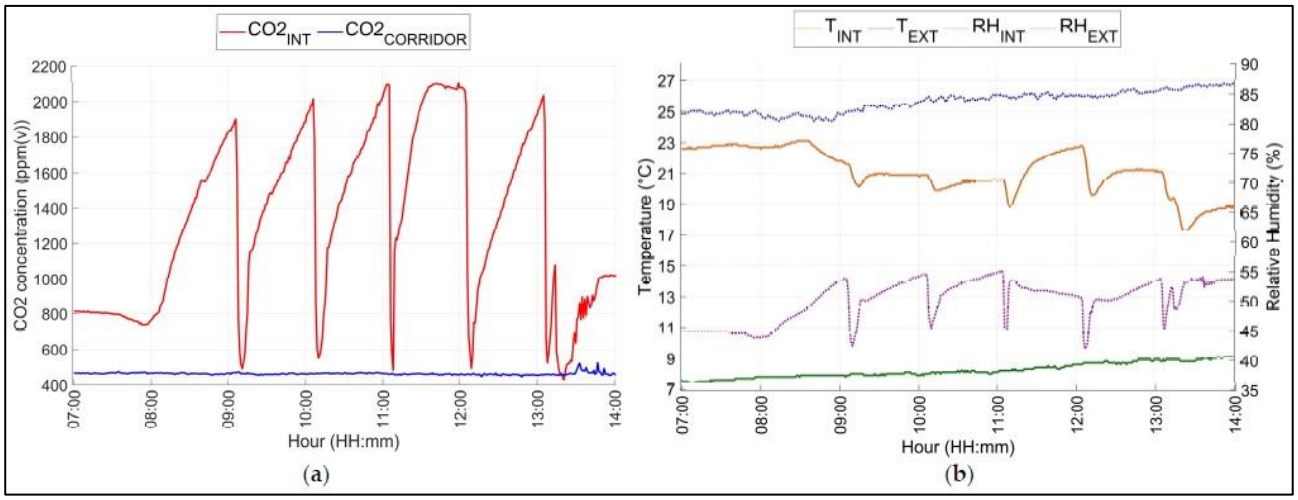


Figure 53. CO_2 concentration (**a**), temperature, and humidity (**b**) for one day in which natural ventilation is considered, on December 1st, 2023. The typical trend of the Lotka–Volterra model is visible (Khovalyg et al., 2020).

This behavior highlights that the vast majority of schools in Italy, as well as in many other European countries lack classrooms equipped with mechanical ventilation systems. Therefore, it is essential to identify indirect criteria for controlling IAQ and CO_2 concentration. A distinct sawtooth pattern can be observed. The presented results demonstrate that, although such an intervention is theoretically feasible and can lead to improvements in both average and peak CO_2 concentrations, temperature

control becomes more challenging. This results in complaints from students and teachers about temperature fluctuations. In this case, the ambient temperature ranged between 18 and 23 ◦C, compared to much smaller fluctuations (21–23 ◦C) in the case of **Figure 52d** (mechanical ventilation). Additionally, considering **Figure 52c, d**, when mechanical ventilation is employed, not only is a stabilization of CO_2 concentration observed during the occupancy period, remaining below 1330 ppm(v), but also a stabilization of the indoor temperature between 8:00 a.m. and 1:00 p.m., ranging from 22.0 to 23.3 ◦C. The same behavior was not observed in the case of natural ventilation (**Figure 53a, b**). Although periodic air changes kept the CO_2 concentration below 2200 ppm(v), greater temperature variability was recorded in the classroom, ranging from 23.3 to 18.9 ◦C during window openings. This resulted in an important increase in thermal discomfort associated with windows opening for ventilation. In **Figure 54a, b**, the daily values of average and maximum CO_2 inside the classroom for when students are present in the classroom of are reported, respectively. Focusing on **Figure 54a**, it was observed that the average maximum values occurred in presence of natural ventilation, with values reaching up to 2500 ppm(v). In the case of controlled mechanical ventilation, however, the average values are much lower and rarely exceed 1200 ppm(v). Furthermore, the effect of increasing the renewal rate of the machine in the final part of the measurement campaign is observed: in the last part, the average CO_2 values resulted in an average concentration almost always below 1000 ppm(v). Similar considerations can be made for the maximum daily concentration during the occupancy period reported in **Figure54b**: it is particularly of note that the peaks of maximum concentration in the presence of natural ventilation reach up to 4500 ppm(v), while in the case of controlled mechanical ventilation the maximum peaks rarely exceed 1500 ppm(v). Considering Equation 1:

$$\Delta CO_2(\%) = 100\frac{C_{NV} - C_{MV}}{C_{NV} - 500}$$

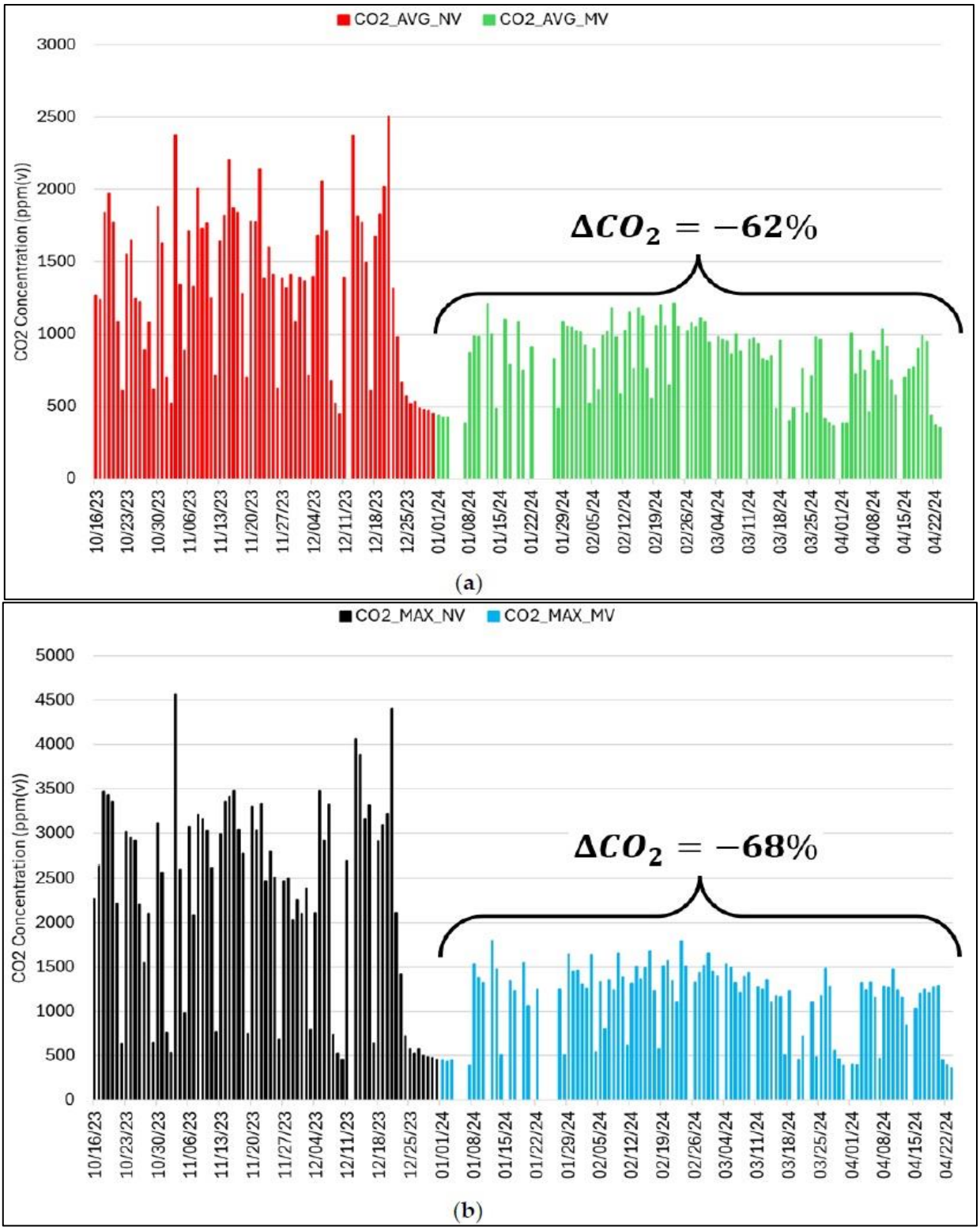


Figure 54. The trends of daily average CO_2 during the occupancy period (**a**) and of the maximum peaks (**b**) in the case of natural ventilation (NV) and controlled mechanical ventilation (MV). In Figure (**a**), the mean percentage reduction in carbon dioxide considering MV with respect to NV is reported as well.

In expressing the percentage reduction in CO_2 concentration following the adoption of mechanical ventilation, CMV represents the average concentration recorded during occupancy periods under mechanical ventilation, while CNV refers to the average concentration recorded during occupancy

periods under natural ventilation. Analyzing the data reported in **Figure 54a**, excluding non-occupancy periods such as Easter and Christmas holidays and Sundays, a significant reduction in CO_2 concentration is observed. Specifically, the daily average reduction is −62% for mean daily values. More precisely, under natural ventilation, the daily average CO_2 concentration reaches 1576 ppm, while it reaches 900 ppm under mechanical ventilation. Furthermore, considering the maximum concentrations obtained during the same observation period, a CNV value of 4573 ppm(v) was recorded on November 3rd, 2023, and a CMV value of 1802 ppm(v) was recorded on 12 January 12th, 2024. This results in a concentration reduction of 68%, as calculated using Equation 1. It is possible to express the CO_2 emission rate inside the classroom, starting from the concentration in ppm relative to volume yCO_2 (ppm(v), as read from Testo sensors) and then expressing it as a weight concentration (xCO_2, ppm by mass, Equation 2). Additionally, the CO_2 concentration in mg/m^3 of air (C_{CO2}) can also be calculated within a given environment:

$$x_{CO2} = y_{CO2}\frac{\mu_{CO2}}{\mu_{air}} \tag{2}$$

$$C_{CO2} = \frac{n_{CO2}}{n_{air}}\frac{\mu_{CO2}}{v_{mol,air}} = f_{CO2}\cdot\frac{\mu_{CO2}}{v_{mol,air}} \tag{3}$$

In these equations, the variables are as follows:

- μ_{CO2} and μ_{air} are the molar masses of CO_2 and air, which are 44 g/mol and 29 g/mol, respectively.
- $v_{mol,air}$ is the molar volume of air, which depends on temperature. At 20 ◦C, it is24.05×10^{-3} m^3/mol.
- f_{CO2} is the molar (or volume) fraction expressed as a percentage, representing the ratio of n_{CO2}/n_{air} divided by 10^6. For example, at a concentration of 1000 ppm(v) in the environment, the volume fraction is 0.1%, as shown in Equation 4:

$$f_{CO2} = \frac{y_{CO2}}{10^6} = \frac{1000}{10^6} = \frac{0.1}{100} = 0.1\% \tag{4}$$

The quantity of CO_2 in mg/m^3 emitted by each person in the room can be estimated using a mass balance (Equation 5) in steady state, which considers the air supply rate into the room through mechanical ventilation (m_{air}), the external concentration (C_{ext}), the internal concentration (C_{int})

measured at the machine's intake, and the internal CO_2 production ($m_{CO2,tot}$) due to people in the room. In this analysis, air change rate since infiltration is neglected:

$$\dot{m}_{air}\, C_{ext} + \dot{m}_{CO2,tot} = \dot{m}_{air}\, C_{int} \quad (5)$$

The infiltration airflow has been considered negligible compared to the airflow of the Aldes machine. This can be inferred from **Figure 51a, c, e**; in fact, the decay of the internal CO_2 curve within the class (with doors closed, no presence, and the machine turned off) is very slow. Assuming an external concentration of C_{ext} = 760 ppm (500 ppm(v)), an internal concentration of C_{int} = 2050 ppm (1350 ppm(v)), and an air renewal flow rate of 480 kg/h, the total internal CO_2 production is calculated to be 622 g CO_2/h, applying Equation 3 to determine the CO_2 concentration per unit of volume of air at 20 ◦C, considering the molar volume of air to be 24.05 *10^{-3} m3/mol. The previous result was obtained by applying Equation 5 to determine $m_{CO2,tot}$. Dividing this value by the number of people in the room (assumed to be 18, 17 students and 1 professor) provides a per-person CO_2 production rate of 34.5 g CO_2/(h · person). This value is in accordance with the scientific literature (Esfehani et al., 2019, Stonner et al.,2018). By introducing a calculated average weight of 54.6 kg per person, the CO_2 emission obtained is 0.63 g CO_2/h per kg of weight. This field data are useful for predicting the airflow required to maintain a predetermined level of carbon dioxide in any environment where people are at rest: of course, higher values of airflow ensure lower CO_2 concentrations, but face other issues, such as noise emissions or simply energy consumption.

4.3.8. IAQ analysis

In this section, the indoor air quality conditions in the environment with and without mechanical ventilation is presented. Consumption values are also presented accordingly. As is well known, electrical energy consumption is strongly related to carbon dioxide emissions (Aly et al., 2016), also regarding a dynamic consumption context (Toleman et al., 2004). **Figure 55** shows the electricity consumption trends for the MVHR system during the measurement period from February 18th to April 18th 2024, alongside the average external temperature recorded in Ferrara by sensors from the Emilia-Romagna Regional Environmental Protection Agency (ARPAE) for a climate station located in the city center (Brizio et al., 2006, Guan et al., 2024). The reported values represent averages from 7:00 a.m. to 1:00 p.m., corresponding to the actual operating hours of the MVHR system. It is noted that, as expected, the ventilation system is turned off on Sundays. While an inverse trend between external temperature and daily energy consumption is observed, the correlation between external temperature

and electricity demand is not particularly strong (R^2 = 0.425). This electricity consumption includes energy used by the system's electric post-heating battery and fans. The low correlation may be attributed to multiple influencing factors, such as the varying amounts of energy supplied by the heating system, exchanges with the surrounding environment, and the potential openings of doors to the outside or adjacent spaces. Additional correlations were also investigated, particularly with the 24 h daily average external temperature and with the internal average temperature during both the 6 h of occupancy and the 24 h daily period. However, the correlation index values were found to be below 0.4.

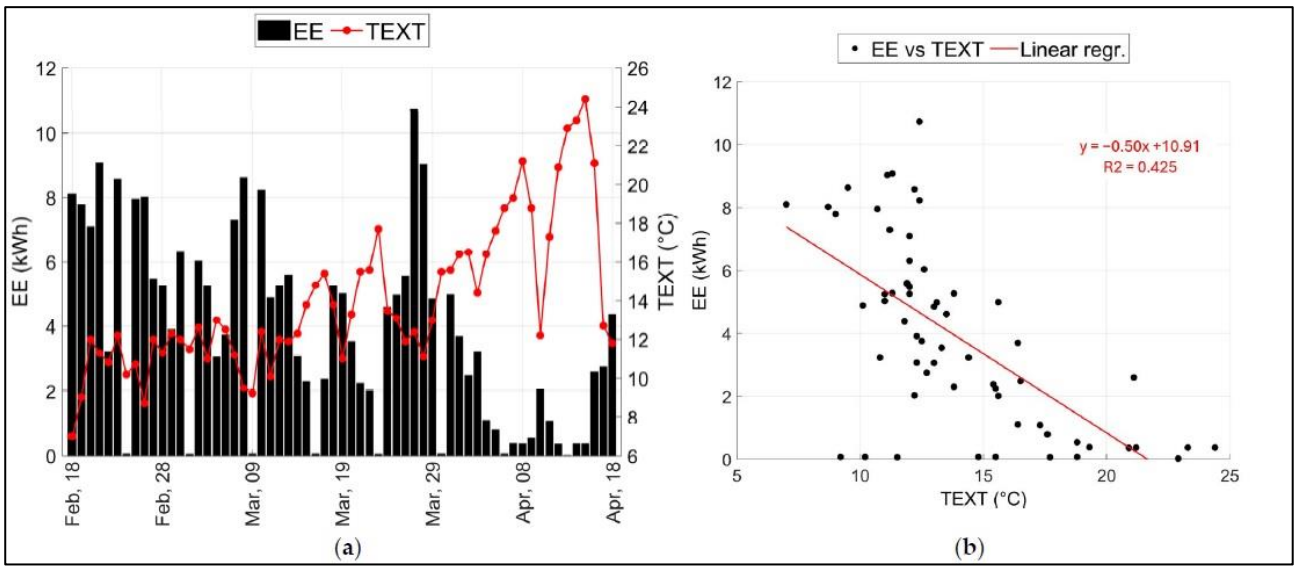
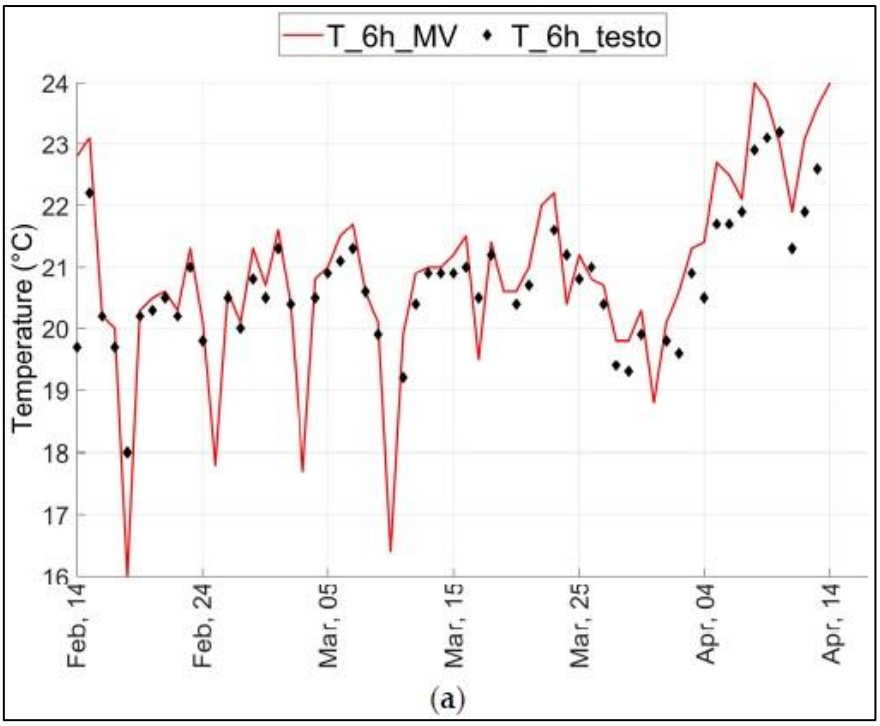
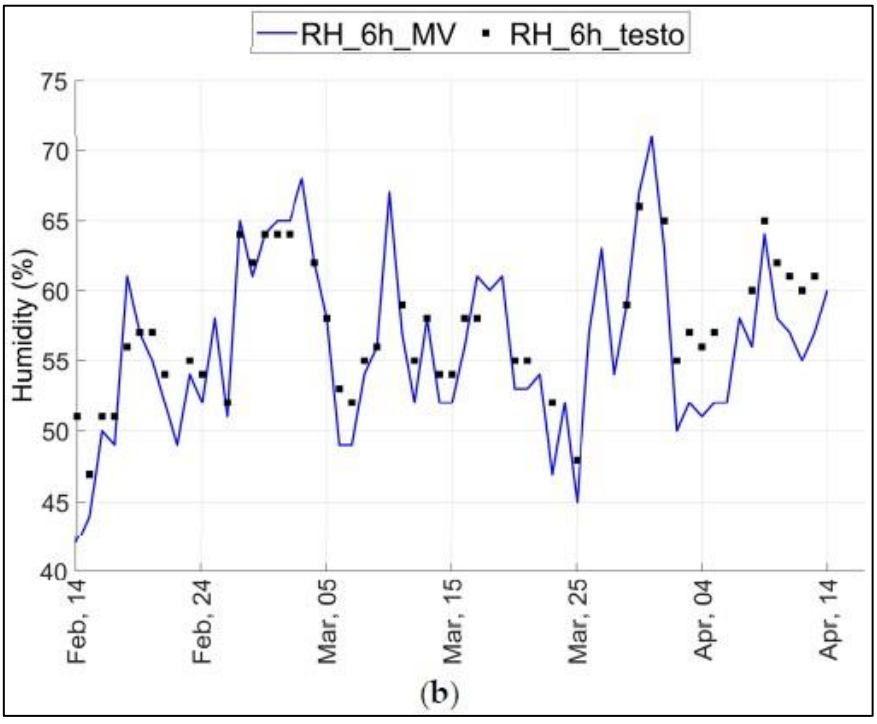


Figure 55. Trend of electricity demand during occupancy period vs. external air temperature (**a**) and correlation (**b**).

In **Figure 56**, the internal trends for temperature, CO_2 concentration, and humidity are shown over a two-month period, comparing measurements from the Testo sensor (positioned 1.70 m above the floor) with those from a sensor located near the return of the controlled mechanical ventilation unit. In **Figure 56a**, it can be observed that the temperature measured near the ventilation unit is close to that recorded by the Testo sensor, with average deviations of 0.8 K over the measurement period (excluding data points where values from either sensor were missing). Minimal discrepancies are also noted for relative humidity, shown in **Figure 56b**. with an average difference of 1% over the period. However, for CO_2 concentration, the average deviation is more significant at 147 ppm (**Figure 56c**), though still within a reasonable range. This discrepancy in CO_2 levels may be attributed to the differing concentrations that can occur at various heights within a room (Tzouvelekis et al., 1994).


T_6h_MV
T_6h_testo
Temperature (°C)
24
23
22
21
20
19
18
17
16
Feb, 14
Feb, 24
Mar, 05
Mar, 15
Mar, 25
Apr, 04
Apr, 14

(a)


RH_6h_MV
RH_6h_testo
Humidity (%)
75
70
65
60
55
50
45
40
Feb, 14
Feb, 24
Mar, 05
Mar, 15
Mar, 25
Apr, 04
Apr, 14

(b)

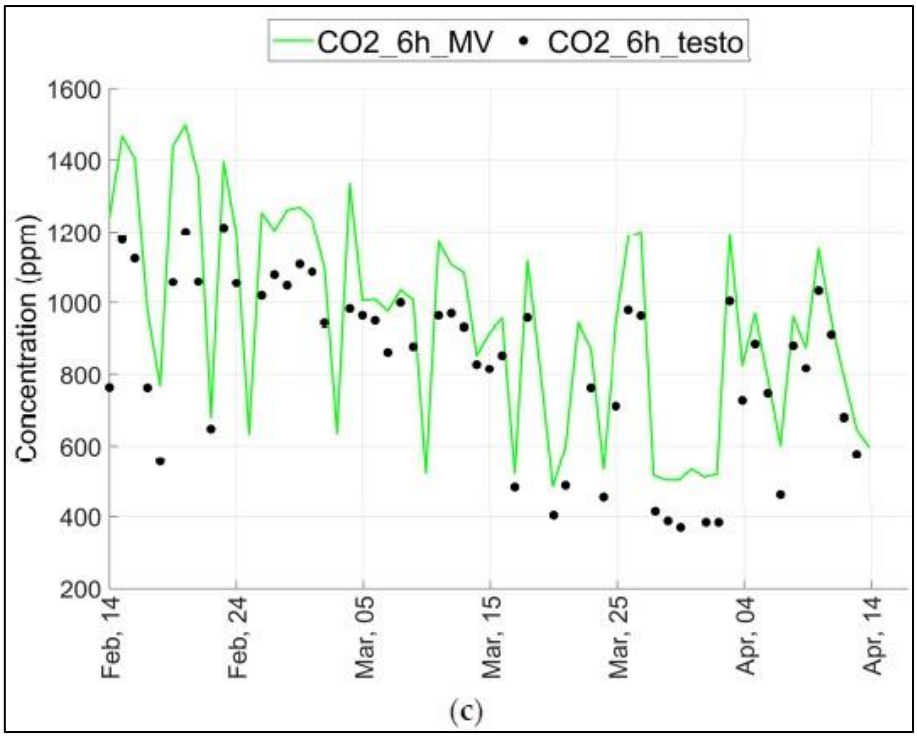


Figure 56. Comparisons of temperature (**a**), relative humidity (**b**), and carbon dioxide concentration (**c**) inside the classroom, measured by the Testo sensor positioned 1.7 m ("testo") above the floor and the AIRTHINGS sensor ("MV") located at the mechanical ventilation outlet.

5. DISCUSSION

5.1. Shaping the microbiome of the subway BE (pandemic period)

In confined BEs, the environment microbiome predominantly stems from human occupants (Li et al., 2021), and can become a reservoir of microbes that can be readily transmitted to individuals, substantially increasing the risk of acquiring infectious diseases (Adams et al., 2015; Mahnert et al., 2019). Hospitals are recognized as paradigmatic examples of this transmission route; in fact, the hospital microbiome serves as a reservoir for potential human pathogens. Indeed, contamination of hospital surfaces by clinically significant pathogens is a critical concern in healthcare settings, as it contributes to the onset of HAIs. In addition, the high selective pressure exerted by the continuous use of disinfectants and antimicrobials in the hospital BE, renders these microbes more and more resistant. Therefore, AMR has spread in the hospitals, becoming a parallel threat for human health, since more and more HAI are difficult to treat with antimicrobials. The frequent surface recontamination process, driven by the presence of colonized or infected patients, hospital visitors, and staff, makes thorough decontamination efforts challenging. Chemical-based detergents and disinfectants are widely used for cleaning hospital surfaces, especially in recent years when they have been widely implemented as a mandatory measure to combat the COVID-19 pandemic, a practice that remains common in both healthcare and non-healthcare settings. However, these chemical methods present several significant limitations. Beyond their substantial environmental impact, they are only effective in short-term pathogen reduction; recontamination often occurs within 30 minutes after application (Vandini, Temmerman, et al., 2014). Furthermore, these chemical methods can drive the selection of resistant microbial strains. Resistance can develop not only against the disinfectants themselves but may also extend to antibiotics, as observed with chlorhexidine, a disinfectant linked to induced resistance against colistin, a critical antibiotic for treating multi-drug-resistant Gram-negative infections (Wand et al., 2017). Recent perspectives have shifted toward viewing environmental health in parallel with human health. Rather than attempting to eliminate all microbes, it may be more effective to replace harmful pathogens with beneficial microorganisms to restore a balanced microbiome and prevent pathogenic colonization (Al-Ghalith & Knights, 2015). In the search for an eco-friendly and effective sanitization strategy that addresses the limitations of conventional chemical disinfection methods, we previously developed a probiotic-based sanitation (PBS), named Probiotic Cleaning Hygiene System (PCHS). This approach employs sustainable detergents containing spores of non-pathogenic, widely distributed probiotic bacteria from the *Bacillus* genus, which have a well-documented history of safe use in human applications. Within hospital settings, this approach achieved sustained pathogen reduction (-80%), a 99.9% decrease in

AMR microbes, and a 52% drop in HAI incidence, without increasing environmental pollution or AMR (Caselli, 2017; Caselli et al., 2019; Caselli et al., 2016; Caselli et al., 2018; D'Accolti et al., 2022; D'Accolti et al., 2021; Soffritti et al., 2022). Besides hospitals and sanitary environments, other indoor community environments can be critical areas for human-to-human, surface-to-human, and human-to-surface microbial exchange. Among these, mass transportation spaces are particularly impacting on human health, as shown during the COVID-19 period, being able to transmit microbes impacting rapidly a high number of people. Urban subways are characterized by high densities and frequent passenger turnover. This prompted government initiatives aimed at reducing passenger density and enhancing disinfection protocols to ensure safer travel during the COVID-19 pandemics, and chemical disinfectants were extensively adopted as a mandatory measure to combat the pandemic (ISS, 2020), raising serious concerns about the risk of future AMR pandemics, as emphasized by the WHO (Getahun et al., 2020). Based on the results obtained in vitro showing the antiviral activity of PCHS against all the enveloped viruses, including SARS-CoV-2 (D'Accolti et al., 2021; Soffritti et al., 2022), its applicability and efficacy was tested in subways compared it to the conventional chemical disinfectants, during the COVID-19 period. A pre–post and case–control study was thus conducted over 12 weeks in two driverless trains (matriculated as train 5508 and 5511) of the M5 subway line of the city of Milan. Due to COVID-19- related restrictions, a full replacement of chemical disinfection with PCHS sanitation was not feasible, so a combined approach was used. This regimen was developed based on preliminary tests assessing the compatibility of PCHS probiotics with chemical disinfectants. Specifically, PCHS was applied 30 minutes after disinfecting with ethanol or ammonium-based products and was used as a substitute for chlorine-based disinfectants. The data collected during the PCHS application revealed significant differences in pathogen levels between the control and PCHS-treated trains. Indeed, probiotic sanitation led to a rapid reduction of pathogens (58% decrease within 2 weeks) and eventually to near-total elimination (100% reduction by week 12). In contrast, the pathogen levels in the control train remained relatively unchanged, despite a four-times-daily application of chlorine disinfectant in the first two weeks, compared to a once-daily application of PCHS in the treated train. Of note, the superior decontamination ability of PCHS, compared to chemical disinfectants, was evident on both tested surfaces and in air, suggesting that its action can provide a more sustained decontamination from both surface and air-dispersed pathogens. Importantly, PCHS use induced a significant reduction in AMR genes, with levels dropping by up to 2-log compared to the control train, aligning with previous observations in PCHS treated sanitary environments (Caselli et al., 2019; Caselli et al., 2018). To better understand the alterations of trains' microbiomes during PCHS application, the microbial population was also profiled by NGS, as described in Results. At all timepoints, the five most prevalent phyla in both

trains were *Proteobacteria, Actinobacteria, Firmicutes, Bacteroidota*, and *Cyanobacteria*, echoing previous subway studies (Gohli et al., 2019; Leung et al., 2019; Vargas-Robles et al., 2020). Surface samples contained more diverse, less common phyla, like *Deinococcota,* and *Acidobacteria* while air filters samples were dominated by *Proteobacteria* and *Actinobacteria*. At the genus level, *Burkholderia–Caballeronia– Parabulkholderia* was the most abundant group in both air and surface samples (11% and 47%, respectively), representing a mix of environmental and potentially pathogenic species (Beukes et al., 2018), also detected in the human nasopharyngeal tract (Ferrari et al., 2022). PERMANOVA analysis confirmed distinct clustering of surface and air microbial communities, aligning with previous findings (Leung et al., 2019). The passenger microbial input was also well represented in train microbiomes as several human colonizers were detected, although at lower abundance compared with predominantly environmental strains. *Staphylococcus, Cutibacterium, Corynebacterium* and *Streptococcus* genera were indeed observed at levels of abundance between 0.8 and 2.2%, similarly to what reported in previous studies (Hernández et al., 2020; Vargas-Robles et al., 2020). However, although the predominant phyla and genera remained consistently ordered in abundance throughout the study, individual abundances varied considerably between bacterial groups in both trains, resulting in no statistically significant difference between the PCHS-treated and control trains. Similarly, the 28 taxa comprising the core train microbiome showed minimal changes, indicating that PCHS had little impact on the overall microbial signature. Only three genera, *Bacillus, Roseomonas,* and *Clostridium*, showed significant changes: *Bacillus* increased slightly in the PCHS train (0.2% to 0.4%, $p < 0.05$), while *Roseomonas* and *Clostridium* decreased (from 0.7% and 0.5% to 0.3% each, $p < 0.05$). This contrasts with CFU counts and microarray data showing reduced pathogen and AMR presence in the PCHS vs. control train. Nonetheless, *Bacillus* spp. did show a measurable increase in the PCHS-treated train compared to the control, although its relative abundance remained below 1%. In contrast to findings from hospital environments, where PCHS application significantly altered the whole microbiome (Caselli et al., 2016, 2019; Caselli et al., 2018; Vandini, Temmerman, et al., 2014), the subway microbiome showed minimal changes, likely due to its distinct basal composition. Indeed, unlike the hospital microbiome which is predominantly human derived (Dai et al., 2017; Lax et al., 2017; Mahnert et al., 2019), the train microbiome is mainly composed of environmental species, whose proportions are less affected by *Bacillus* additions. On the other hand, in hospital settings, human pathogens, with their high nutrient requirements, may be more readily suppressed by PCHS-*Bacillus* through competitive exclusion mechanisms. Interestingly, most of the human microbiome members identified in the train microbiome are typical of normal human skin and oral flora, consistent with previous findings (Human Microbiome Project Consortium, 2012; Leung et al., 2019). These results, alongside the

AMR data, highlight the need for active microbiological monitoring in mass transit settings, where dense crowds facilitate horizontal transmission between microorganisms and human hosts. Additionally, environmental microbes may contribute to the human skin microbiome and resistome (Kang et al., 2018), making it essential to manage microbial communities in high-occupancy environments like public transportation. In conclusion, this first phase of my PhD project provided the first detailed analysis of microbiome dynamics in a subway environment, comparing chemical sanitation to PBS methods, highlighting the effectiveness of PCHS in lowering pathogen contamination, AMR risk and pollution.

5.2. Shaping the microbiome of the subway BE (non-pandemic period)

The aim of this other phase of my PhD project was to evaluate the effects of PCHS used as a complete replacement for chemical disinfection. To this end, a study was conducted in collaboration with ATM of Milan, involving two underground trains on the M4 Line (registrations 4404 and 4435). The study was designed as a pre post study lasting a total of 4 months: the pre-PCHS phase (T0) and the PCHS phase (divided into three periods: T1, T2, T3). Initial results (T0) showed a high baseline microbial load, with a predominance of potentially pathogenic microorganisms such as *Staphylococcus* spp.. This situation corresponds to what is found in the literature in BE environments with a high density of human occupation, where skin-borne bacteria are among the most represented. Before the start of PCHS treatment, surfaces and air filters showed high levels of bacterial and fungal contamination. Following the introduction of PCHS, a significant reduction in baseline contamination was observed after just two weeks. Indeed, at T1, a greater than 70% decrease in pathogenic load on surfaces was observed, a decrease that further stabilized and increased at the following time point (T2). Pathogenic contamination was reduced by 92.6% on surfaces and 99% on air filters. Finally, after approximately four months (T3), an almost complete elimination of the pathogenic microbial load was observed, with values reaching near zero. These data demonstrate the true value of PCHS sanitization, capable of stably and long-lastingly decontaminating environments by promoting sustainable biological competition that hinders recolonization by pathogens. In parallel, resistome analyses using qPCR microarrays have shown that PCHS use is associated with a stable decrease in ARGs of up to 3 log (-99.9%), confirming previous studies conducted in healthcare settings. Interestingly, both trains at time T0 showed significant levels of several resistance genes against various antibiotic classes, including macrolides, methicillin, and β-lactams, both on surfaces and in the air. With the implementation of PCHS, a decrease in all ARGs present at T0 was observed, confirming and further supporting the use of PCHS as a means of counteracting the spread of AMR. The results obtained at T3 in the air, where a slight increase in the genes *OXA-50* (which confers resistance to β-lactams),

oprJ, *oprM*, *INP-5 group*, *FOX* and *NIH* were observed, can be traced back to the extremely dynamic nature of air and filters, elements continuously exposed to the external environment, capable of capturing even microbial DNA residues. Therefore, these isolated results should be interpreted as transient events that do not impact the overall reliability of the system, which instead consistently showed a significant decrease in most ARGs in all treated environments. To conclude, also in this other phase of my PhD project, the adoption of alternative sanitization methods based on the use of probiotics of the *Bacillus* genus has shown be effective in combating AMR and could ensure a sanitized environment capable of preserving the health of occupants. PCHS therefore emerges as a valid and sustainable solution, with potential benefits beyond the healthcare sector, extending to densely populated community environments. The integration of probiotic systems for environmental hygiene management could emerge, in the long term, as an innovative and reliable intervention to combat the worsening of antimicrobial resistance, while offering reduced environmental impact and greater protection of public health.

5.3. Shaping the microbiome e of the school BE: impact of combined interventions

Among high-traffic BEs, schools represent the one where humans spend most of their time during the educational phase of their lives. Of note, microbes have been persistently detected on surfaces and in the air of the school environment, where they have been associated with the onset of various diseases in this age group (Taubel et al., 2024, Etsy et al. 2018, Adams et al., 2021). Consequently, monitoring the school microbiome may be important to maintaining a healthy learning environment and preventing the onset of diseases in students and teaching staff. It is recognized that indoor microbiomes are affected by ventilation type, building features, and cleaning modalities. Inadequate airflow is in fact associated with health complications (Ferrari et al., 2023, Stabile et al., 2017), and plant introduction is considered a possible tool for improving air quality and cognitive performance in students (Bringslimark et al., 2007, Pegas et al., 2012). Moreover, bioburden control has so far been addressed by conventional chemical disinfection, which may increase chemical pollutants and induce the potential selection of drug-resistant microbes (Nabi et al.,2020, Kampf et al., 2018). In contrast, sustainable cleaning systems based on probiotic use (PCHS) have been reported as a promising alternative, having been shown to stably control bioburden without selecting AMR (Caselli et al., 2016, Caselli et al., 2018, Caselli et al, 2019, D'Accolti et al. 2019, D'Accolti et al. 2021 D'Accolti et al. 2023, Cason et al. 2021). In this last phase of my PhD project, it has been investigated the effects of different interventions (plant introduction, mechanical ventilation systems, and PCHS sanitation) on the school microbiome by performing a pre–post case–control study in an Italian high school. The results collected at the basal level in both enrolled classrooms (TR and CR)

showed the presence of several potential pathogens both on surfaces and in the air. The whole surface bioburden appeared consistent with that measured in non-sanitary environments (Park et al., 2021, Yang et al., 2022), with >12,000 CFU/m^2 before the entrance of students and around 20,000 CFU/m^2 after six hours of the continuous presence of occupants (students and teachers). Fungi and Staphylococci were the prevalent microbes, in line with what was previously observed. Fungi accounted for over 75% of the surface bioburden before lessons and *Staphylococcus* spp. represented >60% of the total bioburden measured after lessons. Of note, *Aspergillus* species were almost always detected as part of the resident fungal population, raising potential risks for the health of human occupants. In fact, some *Aspergillus* species can be harmful to humans, as they behave as significantly opportunistic pathogens, potentially causing multiple diseases, including invasive pulmonary infections and allergic diseases, especially in fragile subjects (Mousavi et al., 2016). These results are in line with those obtained in other studies, particularly in school areas with inadequate ventilation and high humidity (Salonen at al., 2015, Sadrizadeh et al., 2022, Song et al., 2024), and further support the fact that human-derived bacteria are common in spaces where people (such as students) congregate (Prussin et al., 2015, Gilbert et al., 2018). Of note, several genes conferring antibiotic resistance (ARGs) were identified in the microbial population persisting in the classroom environment, confirming that AMR is no longer confined to hospitals but is spreading significantly outside of sanitary settings. Prevalent ARGs included genes conferring resistance against different classes of antibiotics, including beta-lactams, aminoglycosides, and macrolides. Methicillin resistance, likely ascribable to the presence of methicillin-resistant *S. aureus* (MRSA) (Votintseva et al. 2014), was also detected. This finding further highlights the spread of MRSA in the general population in non-sanitary environments and is in line with previous studies performed on antibiotic-resistant microbes in schools (Stanforth et al., 2010, Małecka-Adamowicz et al., 2020, Wang et al. 2020) and in high-traffic community environments (D'Accolti et al. 2023). The introduction of *Golden pothos* and *Tillandsia kammii Rauh* plants, chosen essentially for their resilience and suitability for greening walls, caused an unexpected shift in the TR microbial population, with a significant increase in the staphylococcal component and a concomitant reduction in the fungal one. This may be attributed to the potential impact of plants on the classroom's microbial dynamics via changes in environmental factors able to slow fungal proliferation or through the release of bacterial species from the plant microbiome, in line with findings obtained in a previous study (Mahnert et al. 2015). More specifically, changes in physical parameters (such as temperature and relative humidity), as well as the release of volatile organic compounds (VOCs) and other plant-derived factors, may have created an environment favoring the growth of Staphylococci but not that of fungi, which typically thrive in more humid conditions (Portnoy et al., 2005). Also, specific plant-associated

bacteria may have outcompeted fungi and contributed to the observed increase in *Staphylococcus* spp. This hypothesis appears consistent with previous studies showing that plant-associated bacteria could effectively colonize indoor environments (Mahnert et al., 2015, Mahnert et al., 2018) and compete with fungi for nutrients and space. However, since comprehensive species identification of all the bacterial species was not performed, it is not currently possible to determine whether the increased bacteria were environmental Staphylococci or cocci of human origin, and more detailed analyses should be performed to further explore this aspect (including whole-genome sequencing, WGS) and clarify the sources of dynamics of these microbial populations. The installation of mechanical ventilation systems is instead recognized as a potential factor able to significantly impact the indoor microbiome, and consistent with this at T2, a significant reduction in contamination was observed in the TR compared to the CR. More specifically, a drop of 70% and 62% was observed in the total microbial population measured before and after lessons, confirming that mechanical ventilation can efficiently prevent over-contamination, likely by controlling the environmental factors that could favor the growth of pathogens, such as temperature and humidity (Niza et al., 2024). All the details of the ventilation systems adopted during the study are described in a companion article. Noticeably, during the first phase, room ventilation was obtained by opening windows and doors, resulting in a highly variable rate over time, also depending on external climatic conditions and the amount of time the windows were open. With the introduction of mechanical ventilation, the airflow rate was set at 440 m^3/h, equivalent to 24 m^3/h per person (17 students and 1 teacher—6.7 L/s per person), in accordance with the European national standards (UNI EN 16798-1), which require a minimum air exchange of 4 L/s (14.4 m^3/h) per person in schools. The records of CO_2, temperature, and relative humidity showed that relative humidity was significantly lower in the TR, where it did not exceed 45%, compared to the CR, where it constantly reached 65%. This parameter, together with the temperature (maintained at 21–23 ◦C), may have significantly impacted the growth of microorganisms inside the TR. Moreover, the accurate monitoring of CO_2 concentration (Ballerini et al., 2025) showed that CO_2 concentrations dropped significantly in the TR with mechanical ventilation (<1300 ppm-v) compared to natural ventilation (4500 ppm-v), with an average decrease in concentration of 62%, with potential improvement in psychological well-being and learning outcomes. At T3, further significant changes were observed, associated with the introduction of PCHS in substitution for conventional chemical cleaning, based on the use of denatured alcohol on furniture surfaces and chlorine on the floors. The results, in fact, evidenced a significant drop in surface pathogens of 83% before lessons and of 81% after lessons, compared with what was detected in the CR ($p < 0.001$). PCHS, by introducing beneficial *Bacillus* probiotics, has previously been recognized as an effective tool to stably control the colonization by pathogens in treated environments

(Vandini et al , 2014, Caselli et al., 2018, Caselli et al., 2019,Caselli et al., 2017, Caselli et al., 2016 D'Accolti et al., D'Accolti et al., 2019, D'Accolti et al. 2022, Urdaci et al., 2004) via active competition with the surrounding pathogens. Our data confirm the results previously obtained in other community spaces (D'Accolti et al., 2023), showing a significant stabilizing effect of PCHS both on the surface and in the air both before and after lessons, associated with the increase in *Bacillus* in the treated environment. In particular, the low number of pathogens detected after lessons highlights PCHS' ability to prevent recontamination of the treated environment, despite the continuous microbial spread associated with student occupancy. These data thus support the usefulness of PCHS in high-traffic non-sanitary settings to protect the environment long term from potentially harmful contamination (Caselli et al., 2016, Caselli et al., 2018, Caselli et al., 2019, D'Accolti et al. 2019, D'Accolti et al., 2022, D'Accolti et al., 2023). Moreover, considering that the abundance of indoor *Bacillus* spp. has been significantly associated with gut microbial diversity and the Gut Microbiome Health Index (Zhang et al., 2023), these data may also be of importance toward improving the general health of attending students. In addition, the resistome analysis showed that the application of PCHS was associated with an overall decrease in all the ARGs detected in the classroom environment of up to 3 Log10 in the previous study periods, confirming that it may be an important tool for tackling the spread of AMR in the community. Thus, probiotic cleaning was compatible with the presence of a mechanical ventilation system (which did not disturb the PCHS–*Bacillus* colonization) and could further contribute to the decrease in pathogens and AMR in a summation mode. The school microbiome data are in line with those reported in previous case studies, although a direct comparison is difficult because most published data derive from NGS analyses, whereas we quantified the microbial contaminants by CFU count. Despite these methodological differences, Staphylococci and other human-derived bacteria were reported as the main source of bacteria in classrooms, together with fungi mostly attributed to the outdoor environment (Lee et al., 2021). Of note, the bacterial/fungal fraction of the school indoor microbiome was associated with respiratory diseases (Park et al., 2025, Fu et al., 2024], whereas increased biodiversity in the school microbiome was instead suggested to protect students and school staff against these infections (Park et al., 2025). Various studies have shown that the presence of green plants is associated with an increased number of bacterial taxa (Dockx et al., 2022), and further research would be needed to analyze in detail the plant-related taxa composition of the indoor microbiome and to explore its contribution to health effects. Other studies have confirmed that indoor plants can enrich indoor microbial diversity (Dockx et al., 2021) and support the enhancement in commensal skin microbes and immune regulation (Soinen et al. 2022). Last, green plants were also associated with 10% lower CO_2 and more stable temperatures (Danielski et al., 2022). Ventilation was also reported to affect surface and airborne

bacteria and fungi possibly associated with respiratory symptoms in schoolrooms (Daisey et al., 2003), and to impact CO_2, temperature, and humidity parameters of the indoor air (Langiano et al., 2024), thus strengthening what we observed. No studies on PBS usage in the indoor school environment are available in the literature, but several studies were conducted in sanitary and non-sanitary areas, showing a significant decrease in potential surface and airborne pathogens (Vandini et al., 2014, Caselli et al., 2017 Caselli et al., 2018, Caselli et al., 2019, D'Accolti et al., 2022, D'Accolti et al., 2023). Major published school case studies are summarized in **Table 8** and emphasize the overall importance of understanding the interaction between the indoor microbiome and the environmental characteristics (plants, relative humidity, building confinement, and CO_2 concentration) in designing disease prevention strategies (Fu et. al., 2022). Our data show that different types of sustainable intervention can be compatible with each other and concur to obtain additive results, exceeding those achievable by applying separate interventions. A combined intervention including plants, mechanical ventilation, and probiotic cleaning could thus be highly effective at reducing the indoor bioburden and AMR, suggesting that an integrated approach may not only help to reduce all persistent pathogens but also have a direct impact on limiting the presence and spread of antibiotic resistant microorganisms in these environments, which is particularly important in public spaces, where exposure to resistant strains can pose a serious health risk to vulnerable populations such as students.

Setting	Study Aim	Analysis Methods	Primary Outcomes	References
Elementary schools (Korea)	Microbial monitoring	NGS (16S rRNA/ITS)	• Bacteria derived from humans • Fungi mostly attributed to the outdoor environment	Lee et al., 2021
Elementary schools (USA)	Impact of indoor microbiome on resp. infections	NGS (16S rRNA/ITS)	• Indoor microbiome with high biodiversity may provide better protection against respiratory infections	Park et al., 2025
Dormitory rooms (China)	Impact of indoor microbiome on rhinitis	NGS (16S rRNA)	• Indoor microbiome associated with rhinitis (specific taxa)	Fu et al., 2024
155 Households (Belgium)	Impact of plants on indoor microbiome	NGS (16S rRNA/ITS); qPCR	• Indoor plants associated with increased microbial biodiversity. • Bacterial/fungal richness increased with >3 plants	Dockx et al., 2022
176 Living rooms (Belgium)	Impact of outdoor green space on indoor microbiome	NGS (16S rRNA/ITS); qPCR	• Residential green associated with increased microbial biodiversity	Dockx et al., 2021
Offices (Finland)	Impact of green walls on skin microbiota and immunity	NGS (16S rRNA)	• Air-circulating green walls may enhance skin health and immune response.	Soininen et al., 2022
Secondary school (Sweden)	Impact of indoor plants on environment	Measurement of indoor physical parameters (sensors)	• Indoor plants associated with decreased CO_2 (−10%) and more stable temperature	Danielski et al., 2022
Several schools	Review of indoor air quality and health	Correlation of ventilation, CO_2, and CFU counts	• Bacterial/fungal pathogens in classroom air • Inadequate ventilation decreases air quality (CO_2 and bioburden).	Daisey et al., 2003
High school (Italy)	Environmental and microbial monitoring	Measurement of CO_2 and CFU counts in indoor air	• *Staphylococcus* spp. and other potential pathogens found in indoor air • Inadequate ventilation affects air quality (CO_2).	Langiano et al., 2024

Table 8. Major published studies on the indoor microbiome in community environments.

6. CONCLUSIONS

This PhD project on the use of PCHS as other sustainable strategies to improve indoor BE microbiome and health closely aligns with the One Health approach, which recognizes the interconnection of human, animal, and environmental health, and supports the need for sustainable, eco-friendly solutions that minimize negative impacts on the environmental microbiome while promoting public health. The One Health framework furthermore emphasizes the reduction of AMR, being currently one of the most significant challenges for the human health. Chemical disinfection, massively used so far to control the infectious risk, depletes microbial biodiversity and drives the spread of AMR.

Our results showed that PCHS, a patented PBS system set-up in collaboration with Copma S.c.p.a. as the industrial partner of my PhD., can instead provide an eco-sustainable and microbiome-friendly solution that can shape stably the microbial communities without disrupting beneficial microbes or fostering AMR. The collected data further showed that this approach can be generalizable, being applicable and effective not only in healthcare settings but also in non-sanitary environments, such as mass transportation spaces and classrooms. The versatility of PCHS highlights its adaptability for diverse settings, addressing human and environmental health needs through infection control practices that are safer for humans the natural ecosystem. In conclusion, PBS can contribute to a broader, sustainable health framework, by offering a preventive approach to infection control that safeguards public health, preserves ecological balance, and supports global efforts to combat AMR, advancing the critical objectives of the One Health vision. The research also revealed several key findings and paves the way for further investigations, including:

- Confined BEs can serve as reservoirs for human pathogens, thus long-lasting decontamination systems may be more effective in preventing infections compared to temporary decontamination;
- Deep sequencing techniques are continuously evolving; thus, a periodic reassessment of PCHS-*Bacillus* may be useful to further exclude the acquisition of any genes related to resistance or virulence, ensuring over time that probiotics maintain effectiveness and do not acquire potentially harmful features;
- Based on the versatility of PCHS and on the One Health indications, it would be important to extend the research to other fields where the PBS may be beneficial, such as livestock management and agriculture.

In parallel, this project included examining the impact of adopting other sustainable interventions (such as green plants and mechanical ventilation) in highly frequented indoor spaces, such as schools. The key findings showed that:

- green plants can substantially affect the classroom microbiome, although not significantly from a statistical point of view, suggesting that the inclusion of appropriate plants could apport benefits to the indoor BE;
- CO_2 concentration in the classroom dropped significantly with mechanical ventilation, with peak values reaching more than 4500 ppm(v) under natural ventilation and rarely exceeding 1500 ppm(v)with mechanical ventilation. The average concentration during occupancy decreased from around 2500 ppm(v) to levels close to 1000 ppm(v) and a reduction of 62%, considering mean values during the occupation period and the daily reduction reached -68% considering the maximum daily values (**Figure 54a, b**)

Future research might explore operating the machine at variable rates to adjust flow based on other pollutant dilution needs and assess the effects of slight positive pressure on radon, VOC, and particulate matter concentrations. Moreover, in future analyses, attention will be paid to the role of operative temperature. Renewable energy sources are also planned to be integrated, aiming to make the classroom nearly carbon-neutral. This study may be usefully included in multidisciplinary analyse addressed to sustainable regeneration of school spaces, focusing on improved habitability with objectives that include:

- Characterization of the environmental microbiome (microbial, bacterial, and fungal communities) and methods to support a balanced ecosystem, including compatible plant installations (green classrooms).
- Control of thermohygrometric, physical, chemical, and energy parameters to enhance occupant health and reduce the built environment's carbon footprint (decarbonization).
- Improvement in psychological well-being and learning outcomes by enhancing the aesthetic, functional, and material qualities of spaces, promoting interaction among people and between individuals and their environment (interior design);
- Development and prototyping of innovative educational spaces, using specific and quantitative knowledge of the various parameters that contribute to the livability of constructed spaces.

6.1. Scientific production

The results of the research projects developed during my PhD have been published in international peer-reviewed journals with impact factor, and presented in several national and international meetings:

Publications:

- Soffritti I, D'Accolti M, Cason C, Lanzoni L, Bisi M, Volta A, Campisciano G, Mazzacane S, Bini F, Mazziga E, Toscani P, Caselli E, Comar M. *Introduction of Probiotic-Based Sanitation in the Emergency Ward of a Children's Hospital During the COVID-19 Pandemic*. Infect Drug Resist. 2022 Mar 30;15:1399 1410. doi: 10.2147/IDR.S356740.
- D'Accolti M, Soffritti I, Bini F, Mazziga E, Arnoldo L, Volta A, Bisi M, Antonioli P, Laurenti P, Ricciardi W, Vincenti S, Mazzacane S, Caselli E. *Potential Use of a Combined Bacteriophage-Probiotic Sanitation System to Control Microbial Contamination and AMR in Healthcare Settings: A Pre-Post Intervention Study*. Int J Mol Sci. 2023 Mar 31;24(7):6535. doi: 10.3390/ijms24076535.
- D'Accolti M, Soffritti I, Bini F, Mazziga E, Cason C, Comar M, Volta A, Bisi M, Fumagalli D, Mazzacane S, Caselli E. *Shaping the subway microbiome through probiotic-based sanitation during the COVID-19 emergency: a pre-post case-control study*. Microbiome. 2023 Mar 30;11(1):64. doi: 10.1186/s40168-023-01512-2.
- Soffritti I, D'Accolti M, Bini F, Mazziga E, Volta A, Bisi M, Rossi S, Viroli F, Balzani M, Petitta M, Mazzacane S, Caselli E. *Characterization of the Pathogenic Potential of the Beach Sand Microbiome and Assessment of Quicklime as a Remediation Tool*. Microorganisms. 2023 Aug 7;11(8):2031. doi: 10.3390/microorganisms11082031.
- Bisi M. *Excessive presence of Nitrogen and Phosphorus in the Drini River and their impact on the Kune-Vain lagoon ecosystem: how anthropization can affect biodiversity*. September 2023. The Scientific Journal of the Observatory of Mediterranean Basin 8(1-2):94-98. doi:10.37199/o41008111.
- Soffritti I, D'Accolti M, Bini F, Mazziga E, Volta A, Bisi M, Mazzacane S, Caselli E. *Probiotic-Based Approaches for Sustainable Control of Infectious Risk in Mass Transport: Current Data and Future Perspectives*. Microb Biotechnol. 2025 Jun;18(6): e70177. doi: 10.1111/1751-7915.70177.
- D'Accolti M, Soffritti I, Mazziga E, Bini F, Bisi M, Volta A, Mazzacane S, Caselli E. *A Sustainable Combined Approach to Control the Microbial Bioburden in the School Environment*. Microorganisms. 2025 Mar 30;13(4):791. doi: 10.3390/microorganisms13040791.

- Bini F, Soffritti I, D'Accolti M, Mazziga E, Caballero JD, David S, Argimon S, Aanensen DM, Volta A, Bisi M, Mazzacane S, Caselli E. *Profiling the resistome and virulome of Bacillus strains used for probiotic-based sanitation: a multicenter WGS analysis*. BMC Genomics. 2025 Apr 18;26(1):382. doi: 10.1186/s12864-025-11582-1.
- Soffritti I, D'Accolti M, Bini F, Mazziga E, Volta A, Bisi M, Mazzacane S, Caselli E. *Probiotic-Based Approaches for Sustainable Control of Infectious Risk in Mass Transport: Current Data and Future Perspectives*. Microb Biotechnol. 2025 Jun;18(6): e70177. doi: 10.1111/1751-7915.70177.

Participation at national and international meetings

1. M. D'accolti, I. Soffritti, E. Mazziga, F. Bini, **M. Bisi**, A. Volta, S. Mazzacane, E. Caselli. A Sustainable Combined Approach to Control the Microbial Bioburden in the School Environment. Congresso SIM-Società Italiana di Microbiologia 2025 (Catania, 24-27 settembre 2025).
2. F. Bini, I. Soffritti, M. D'accolti, E. Mazziga, J. Diaz Caballero, S. David, S. Argimon, D. M. Aanensen, A. Volta, **M. Bisi** , S. Mazzacane, E. Caselli. Profiling the resistome and virulome of Bacillus strains used for probiotic-based sanitation: a multicenter WGS analysis. Congresso SIM-Società Italiana di Microbiologia 2025 (Catania, 24-27 settembre 2025).
3. E. Mazziga, M. D'Accolti, I. Soffritti, F. Bini, L. Arnoldo, A. Volta, **M. Bisi**, P. Antonioli, P. Laurenti, W. Ricciardi, S. Vincenti, S. Mazzacane, E. Caselli. Potential Use of a Combined Bacteriophage-Probiotic Sanitation System to Control Microbial Contamination and AMR in Healthcare Settings: A Pre-Post Intervention Study. Congresso SIM-Società Italiana di Microbiologia 2023 (Cagliari, 24-27 settembre 2023).
4. I. Soffritti, M. D'Accolti, F. Bini, E. Mazziga, C. Cason, M. Comar, A. Volta, **M. Bisi**, D. Fumagalli, S. Mazzacane, E. Caselli. Shaping the subway microbiome through probiotic-based sanitation during the COVID-19 emergency: a pre-post case-control study. Congresso WoM-World of Microbiome 2023 (Sofia, 26-28 ottobre 2023
5. M. D'Accolti, I. Soffritti, F. Bini, E. Mazziga, A. Volta**, M. Bisi**, S. Mazzacane, E. Caselli. Shaping the subway microbiome by a probiotic-based sanitation during the COVID-19 emergency. Congresso SIM-Società Italiana di Microbiologia 2023 (Cagliari, 24-27 settembre 2023).
6. F. Bini, I. Soffritti, M. D'Accolti, E. Mazziga, A. Volta, **M. Bisi**, S. Rossi, F. Viroli, M. Balzani, M. Petitta, S. Mazzacane, E. Caselli. Characterization of the Pathogenic Potential

of the Beach Sand Microbiome and Assessment of Quicklime as a Remediation Tool. CONGRESSO SIM-Società Italiana di Microbiologia 2023 (Cagliari, 24-27 settembre 2023).

7. M. D'Accolti, I. Soffritti, F. Bini, E. Mazziga, A. Volta**, M. Bisi**, S. Mazzacane, E. Caselli. Shaping the subway microbiome by a probiotic-based sanitation during the covid-19 emergency. CONGRESSO MicrobiotaMi (Milano 3-5 aprile 2023).
8. M. D'Accolti, I. Soffritti, F. Bini, E. Mazziga, A. Volta, **M. Bisi**, S. Mazzacane, E. Caselli. Shaping the subway microbiome by a probiotic-based sanitation during the COVID-19 emergency. CONFERENZA Young Minds at Work: Blending Biology and Bioinformatics (Online, 16 Dicembre 2022)
9. M. D'Accolti, I. Soffritti, F. Bini, E. Mazziga, A. Volta**, M. Bisi**, S. Mazzacane, E. Caselli. Shaping the subway microbiome through probiotic-based sanitation during the COVID-19 emergency. CONGRESSO SIM-Società Italiana di Microbiologia 2022 (Napoli, 18-21 settembre 2022).

7. BIBLIOGRAPHY

- Adams, R. I., Bateman, A. C., Bik, H. M., & Meadow, J. F. (2015). Microbiota of the indoor environment: a meta-analysis. *Microbiome*, *3*, 49.https://doi.org/10.1186/s40168-015-0108-3
- Adams, R. I., Bhangar, S., Dannemiller, K. C., Eisen, J. A., Fierer, N., Gilbert, J. A., Green, J. L., Marr, L. C., Miller, S. L., Siegel, J. A., Stephens, B., Waring, M. S., & Bibby, K. (2016). Ten questions concerning the microbiomes of buildings. *Building andEnvironment*, *109*, 224–234. https://doi.org/10.1016/j.buildenv.2016.09.001
- Adams, R.I.; Leppanen, H.; Karvonen, A.M.; Jacobs, J.; Borràs-Santos, A.; Valkonen, M.; Krop, E.; Haverinen-Shaughnessy, U.; Huttunen, K.; Zock, J.; et al. Microbial Exposures in Moisture-damaged Schools and Associations with Respiratory Symptoms in Students: A Multi-country Environmental Exposure Study. Indoor Air **2021**, 31, 1952–1966
- Adetitun, D., Suberu, R., Rajee, A. (2024). Inhibitory effects of some metal complexes on four culturable bacteria isolated from indoor air of 30 unilorin offices. https://doi.org/10.21203/rs.3.rs-3875081/v1
- Adetitun, D., Suberu, R., Rajee, A. (2024). Inhibitory effects of some metal complexes on four culturable bacteria isolated from indoor air of 30 unilorin offices. https://doi.org/10.21203/rs.3.rs-3875081/v1
- Ahmad N., Plorde J., & Drew W. L. (2013). Capitolo 22 - Patogenesi delle infezioni batteriche. In Ryan K. J. & Ray C. G. (Eds.), *Microbiologia Medica. Quinta edizione. Edizione italiana.* (pp. 398–406). EMS.
- Aires de Sousa, M., & Lencastre, H. (2004). Bridges from hospitals to the laboratory: genetic portraits of methicillin-resistant *Staphylococcus aureus* clones. *FEMS Immunology & Medical Microbiology*, *40*(2), 101–111. https://doi.org/10.1016/S0928-8244(03)00370-5
- Al-Ghalith, G. A., & Knights, D. (2015). Bygiene: The New Paradigm of Bidirectional Hygiene. *Yale J Biol Med*, *88*(4), 359–365. https://www.ncbi.nlm.nih.gov/pubmed/26604859
- Allen, J., MacNaughton, P., Satish, U., Santanam, S., Vallarino, J.,Spengler, J. (2016). Associations of cognitive function scores with carbon dioxide, ventilation, and volatile organic compound exposures in office workers: a controlled exposure study of green and conventional office environments. Environmental Health Perspectives, 124(6), 805-812. https://doi.org/10.1289/ehp.1510037
- Aly, M.M.; Abu Alsoud, N.M.; Elrobh, M.S.; Al Johani, S.M.; Balkhy, H.H. High Prevalence of the PER-1 Gene among Carbapenem-Resistant Acinetobacter Baumannii in Riyadh, Saudi Arabia. Eur. J. Clin. Microbiol. Infect. Dis. **2016**, 35, 1759–1766

- Ana, G., Fakunle, G., Ogunjobi, A. (2013). Indoor airborne microbial burden and risk of acute respiratory infections among children under five in ibadan, nigeria. Indoor and Built Environment, 24(3), 308-314. https://doi.org/10.1177/1420326x13499171
- Andualem, Z., Gizaw, Z., Bogale, L., Dagne, H. (2019). Indoor bacterial load and its correlation to physical indoor air quality parameters in public primary schools. Multidisciplinary Respiratory Medicine, 14(1). https://doi.org/10.1186/s40248-018-0167-y
- ANMDO. (2014). *L'Ospedale-Trimestrale di igiene, tecnologia, management degli ospedali e dei servizi sanitari territoriali*.
- Ashbolt, N. J. (2015). Microbial Contamination of Drinking Water and Human Health from Community Water Systems. *Current Environmental Health Reports*, *2*(1), 95–106. https://doi.org/10.1007/s40572-014-0037-5
- Baldelli, G., Aliano, M., Amagliani, G., Magnani, M., Brandi, G., Pennino, C., Schiavano, G. (2022). Airborne microorganism inactivation by a uv-c led and ionizer-based continuous sanitation air (csa) system in train environments. International Journal of Environmental Research and Public Health, 19(3), 1559. https://doi.org/10.3390/ijerph19031559
- Ballerini, V.; Coccagna, M.; Volta, A.; Droghetti, L.; Rossi di Schio, E.; Valdiserri, P.; Mazzacane, S. The Role of Mechanical Ventilation on Indoor Air Quality in Schools: An Experimental Comprehensive Analysis. Buildings **2025**, 15, 869
- Ballerini, V.; Lubowicka, B.; Valdiserri, P.; Krawczyk, D.A.; Sadowska, B.; Kłopotowski, M.; di Schio,(2023) E.R. The Energy Retrofit Impact in Public Buildings: A Numerical Cross-Check Supported by Real Consumption Data. Energies, 16, 7748
- Ballerini, V.; Valdiserri, P.; Krawczyk, D.A.; Sadowska, B.; Lubowicka, B.; di Schio, (2015) E.R. Design, comparison and application of artificial intelligence predictive models based on experimental data for estimating carbon dioxide concentration inside a building. Appl. Therm. Eng., 261, 125122
- Banupriya, B., Biswal, N., Srinivasaraghavan, R., Narayanan, P., & Mandal, J. (2015). Probiotic prophylaxis to prevent ventilator associated pneumonia (VAP) in children on mechanical ventilation: an open-label randomized controlled trial. *Intensive Care Med*, *41*(4), 677–685. https://doi.org/10.1007/s00134-015-3694-4
- Becker, K., Heilmann, C., & Peters, G. (2014). Coagulase-Negative Staphylococci. *Clinical Microbiology Reviews*, *27*(4), 870–926. https://doi.org/10.1128/CMR.00109-13
- Berg, G., Rybakova, D., Fischer, D., Cernava, T., Vergès, M. C. C., Charles, T., Chen, X., Cocolin, L., Eversole, K., Corral, G. H., Kazou, M., Kinkel, L., Lange, L., Lima, N., Loy, A., Macklin, J. A., Maguin, E., Mauchline, T., McClure, R., Schloter, M. (2020). Microbiome

definition re-visited: old concepts and new challenges. In *Microbiome* (Vol. 8, Issue 1). BioMed Central Ltd. https://doi.org/10.1186/s40168-020-00875-0

- Bini F, Soffritti I, D'Accolti M, Mazziga E, Caballero JD, David S, Argimon S, Aanensen DM, Volta A, Bisi M, Mazzacane S, Caselli E. *Profiling the resistome and virulome of Bacillus strains used for probiotic-based sanitation: a multicenter WGS analysis*. BMC Genomics. 2025 Apr 18;26(1):382. doi: 10.1186/s12864-025-11582-1
- Blubaugh, M., Razban, A., Chen, J. (2022). Demand-controlled ventilation energy savings for air handling unit. https://doi.org/10.46855/energy-proceedings-8665
- Boyce, J. M. (2016). Modern technologies for improving cleaning and disinfection of environmental surfaces in hospitals. *Antimicrob Resist Infect Control*, *5*, 10. https://doi.org/10.1186/s13756-016-0111-x
- Bragoszewska, E., & Biedroń, I. (2018). Indoor air quality and potential health risk impacts of exposure to antibiotic resistant bacteria in an office room in southern Poland. *International Journal of Environmental Research and Public Health*, *15*(11). https://doi.org/10.3390/ijerph15112604
- Bringslimark, T.; Hartig, T.; Patil, G.G. Psychological Benefits of Indoor Plants in Workplaces: Putting Experimental Results into Context. HortScience **2007**, 42, 581–587
- Brizio, A.; Conceic.o, T.; Pimentel, M.; Da Silva, G.; Duarte, A. High-Level Expression of IMP-5 Carbapenemase Owing to Point Mutation in the -35 Promoter Region of Class 1 Integron among Pseudomonas Aeruginosa Clinical Isolates. Int. J. Antimicrob. Agents **2006**, 27, 27–31.
- BS EN 16798-1:2019; Energy Performance of Buildings—Ventilation for Buildings—Part 1: Indoor Environmental Input Parameters for Design and Assessment of Energy Performance of Buildings Addressing Indoor Air Quality, Thermal Environment, Lighting and Acoustics. British Standards Institution: London, UK, 2019
- Caini S., Hajdu A., Kurcz A., & Böröcz K. (2013). *Hospital-acquired infections due to multidrug-resistant organisms in Hungary, 2005-2010*. Euro Surveillance. Available at: http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=20352
- Carling, P. C., Parry, M. F., Von Beheren, S. M., & Healthcare Environmental Hygiene Study, G. (2008). Identifying opportunities to enhance environmental cleaning in 23 acute care hospitals. *Infect Control Hosp Epidemiol*, *29*(1), 1–7. https: //doi.org/10.1086/524329
- Caselli, E. (2017). Hygiene: microbial strategies to reduce pathogens and drug resistance in clinical settings. *Microb Biotechnol*, *10*(5), 1079–1083. https://doi.org/10.1111/1751-7915.12755

- Caselli, E., Antonioli, P., & Mazzacane, S. (2016). Safety of probiotics used for hospital environmental sanitation. *J Hosp Infect*, *94*(2), 193–194. https://doi.org/10.1016/j.jhin.2016.06.021
- Caselli, E., Arnoldo, L., Rognoni, C., D'Accolti, M., Soffritti, I., Lanzoni, L., Bisi, M., Volta, A., Tarricone, R., Brusaferro, S., & Mazzacane, S. (2019). Impact of a probiotic-based hospital sanitation on antimicrobial resistance and HAI-associated antimicrobial consumption and costs: a multicenter study. *Infect Drug Resist*, *12*, 501–510. https://doi.org/10.2147/IDR.S194670
- Caselli, E., Brusaferro, S., Coccagna, M., Arnoldo, L., Berloco, F., Antonioli, P., Tarricone, R., Pelissero, G., Nola, S., La Fauci, V., Conte, A., Tognon, L., Villone, G., Trua, N., Mazzacane, S., & Group, S.-I. S. (2018). Reducing healthcare-associated infections incidence by a probiotic-based sanitation system: A multicentre, prospective, intervention study. *PLoS One*, *13*(7), e0199616. https://doi.org/10.1371/journal.pone.0199616
- Caselli, E., Brusaferro, S., Coccagna, M., Arnoldo, L., Berloco, F., Antonioli, P., Tarricone, R., Pelissero, G., Nola, S., La Fauci, V., Conte, A., Tognon, L., Villone, G., Trua, N., Mazzacane, S., & Group, S.-I. S. (2018). Reducing healthcare-associated infections incidence by a probiotic-based sanitation system: A multicentre, prospective, intervention study. *PLoS One*, *13*(7), e0199616. https://doi.org/10.1371/journal.pone.0199616
- Caselli, E., D'Accolti, M., Soffritti, I., Lanzoni, L., Bisi, M., Volta, A., Berloco, F., & Mazzacane, S. (2019). An Innovative Strategy for the Effective Reduction of MDR Pathogens from the Nosocomial Environment. *Adv Exp Med Biol*, *1214*, 79–91. https://doi.org/10.1007/5584_2019_399
- Caselli, E., D'Accolti, M., Vandini, A., Lanzoni, L., Camerada, M. T., Coccagna, M.,Branchini, A., Antonioli, P., Balboni, P. G., Di Luca, D., & Mazzacane, S. (2016). Impact of a Probiotic-Based Cleaning Intervention on the Microbiota Ecosystem of the Hospital Surfaces: Focus on the Resistome Remodulation. *PLoS One*, *11*(2), e0148857. https://doi.org/10.1371/journal.pone.0148857
- Casey, A. L., Adams, D., Karpanen, T. J., Lambert, P. A., Cookson, B. D., Nightingale, P., Miruszenko, L., Shillam, R., Christian, P., & Elliott, T. S. (2010). Role of copper in reducing hospital environment contamination. *J Hosp Infect*, *74*(1), 72–77. https://doi.org/10.1016/j.jhin.2009.08.018
- Cason, C., D'Accolti, M., Soffritti, I., Mazzacane, S., Comar, M., & Caselli, E. (2022). Nextgeneration sequencing and PCR technologies in monitoring the hospital microbiome and its drug resistance. *Front Microbiol*, *13*, 969863. https://doi.org/10.3389/fmicb.2022.969863

- Cason, C.; D'Accolti, M.; Campisciano, G.; Soffritti, I.; Ponis, G.; Mazzacane, S.; Maggiore, A.; Risso, F.M.; Comar, M.; Caselli, E. Microbial Contamination in Hospital Environment Has the Potential to Colonize Preterm Newborns' Nasal Cavities. Pathogens **2021**, 10, 615
- Cave, R., Cole, J., & Mkrtchyan, H. V. (2021). Surveillance and prevalence of antimicrobial resistant bacteria from public settings within urban built environments: Challenges and opportunities for hygiene and infection control. *Environment International*, *157*, 106836. https://doi.org/10.1016/j.envint.2021.106836
- Cave, R., Misra, R., Chen, J., Wang, S., & Mkrtchyan, H. V. (2021). Comparative Genomics Analysis Demonstrated a Link Between Staphylococci Isolated From Different Sources: A Possible Public Health Risk. *Frontiers in Microbiology*, *12*. https://doi.org/10.3389/fmicb.2021.576696
- Cesari, S.; Valdiserri, P.; Coccagna, M.; Mazzacane, S. (2020). The energy saving potential of wide windows in hospital patient rooms, optimizing the type of glazing and lighting control strategy under different climatic conditions. Energies, 13, 2116
- Chatzidiakou, L.; Archer, R.; Beale, V.; Bland, S.; Carter, H.; Castro-Faccetti, C.; Burridge, H.C. (2023) Schools' air quality monitoring for health and education: Methods and protocols of the SAMHE initiative and project. Dev. Built Environ, 16, 100266
- Chen, B., Han, J., Dai, H., & Jia, P. (2021). Biocide-tolerance and antibiotic-resistance in community environments and risk of direct transfers to humans: Unintended consequences of community-wide surface disinfecting during COVID-19? *Environmental Pollution*, *283*, 117074. https://doi.org/10.1016/j.envpol.2021.117074
- Chokshi, A., Sifri, Z., Cennimo, D., & Horng, H. (2019). Global contributors to antibiotic resistance. *Journal of Global Infectious Diseases*, *11*(1), 36–42. https://doi.org/10.4103/jgid.jgid_110_18
- Ciprandi, G., Scordamaglia, A., Venuti, D., Caria, M., & Canonica, G. W. (1986). In vitro effects of Bacillus subtilis on the immune response. *Chemioterapia*, *5*(6), 404–407. https://www.ncbi.nlm.nih.gov/pubmed/3100070
- Cîrstolovean, I., Horneț, M., Ancaș, A., Profire, M. (2018). Air exchange rate in air quality assurance – a case study. Journal of Applied Engineering Sciences, 8(1), 13-18. https://doi.org/10.2478/jaes-2018-0002
- Clancy, C. J., Buehrle, D. J., & Nguyen, M. H. (2020). PRO: The COVID-19 pandemic will result in increased antimicrobial resistance rates. *JAC Antimicrob Resist*, *2*(3), dlaa049. https://doi.org/10.1093/jacamr/dlaa049

- Collins, J. D., Noerrung, B., Budka, H., Andreoletti, O., Buncic, S., Griffin, J., Hald, T., Hope, J., Klein, G., Hald, T., McLauchlin, J., Müller-Graf, C., Koutsoumanis, K., Nguyen-The, C., Peixe, L., Prieto Maradona, M., Ricci, A., Sofos, J., Threlfall, J., … Vanopdenbosch, E. (2011). Scientific Opinion on the maintenance of the list of QPS biological agents intentionally added to food and feed (2011 update). *EFSA Journal*, *9*(12). https://doi.org/10.2903/j.efsa.2011.2497
- Contesini, F. J., Melo, R. R., & Sato, H. H. (2018). An overview of Bacillus proteases: from production to application. *Crit Rev Biotechnol*, *38*(3), 321–334. https://doi.org/10.1080/07388551.2017.1354354
- Cornejo-Juarez, P., Vilar-Compte, D., Perez-Jimenez, C., Namendys-Silva, S. A., Sandoval-Hernandez, S., & Volkow-Fernandez, P. (2015). The impact of hospital-acquired infections with multidrug-resistant bacteria in an oncology intensive care unit. *Int J Infect Dis*, *31*, 31–34. https://doi.org/10.1016/j.ijid.2014.12.022
- D'Accolti, M., Soffritti, I., Bini, F., Mazziga, E., Arnoldo, L., Volta, A., Bisi, M., Antonioli, P., Laurenti, P., Ricciardi, W., Vincenti, S., Mazzacane, S., & Caselli, E. (2023).Potential Use of a Combined Bacteriophage–Probiotic Sanitation System to Control Microbial Contamination and AMR in Healthcare Settings: A Pre-Post Intervention Study. *International Journal of Molecular Sciences*, *24*(7), 6535. https://doi.org/10.3390/ijms24076535
- D'Accolti, M., Soffritti, I., Bini, F., Mazziga, E., Mazzacane, S., & Caselli, E. (2022). Pathogen Control in the Built Environment: A Probiotic-Based System as a Remedy for the Spread of Antibiotic Resistance. *Microorganisms*, *10*(2). https://doi.org/10.3390/microorganisms10020225
- D'Accolti, M., Soffritti, I., Bonfante, F., Ricciardi, W., Mazzacane, S., & Caselli, E. (2021). Potential of an Eco-Sustainable Probiotic-Cleaning Formulation in Reducing Infectivity of Enveloped Viruses. *Viruses*, *13*(11). https://doi.org/10.3390/v13112227
- D'Accolti, M., Soffritti, I., Bonfante, F., Ricciardi, W., Mazzacane, S., & Caselli, E. (2021). Potential of an Eco-Sustainable Probiotic-Cleaning Formulation in Reducing Infectivity of Enveloped Viruses. *Viruses*, *13*(11). https://doi.org/10.3390/v13112227
- D'Accolti, M.; Soffritti, I.; Bini, F.; Mazziga, E.; Cason, C.; Comar, M.; Volta, A.; Bisi, M.; Fumagalli, D.; Mazzacane, S.; et al. (2023) Shaping the Subway Microbiome through Probiotic-Based Sanitation during the COVID-19 Emergency: A Pre–Post Case–Control Study. Microbiome **2023**, 11, 64
- D'Accolti M., Soffritti I., Mazzacane S., Caselli E. (2019) Fighting AMR in the Healthcare Environment: Microbiome-Based Sanitation Approaches and Monitoring Tools. Int J Mol Sci.

27;20(7):1535.

- Dai, D., Prussin, A. J., Marr, L. C., Vikesland, P. J., Edwards, M. A., & Pruden, A. (2017). Factors Shaping the Human Exposome in the Built Environment: Opportunities for Engineering Control. *Environmental Science & Technology*, *51*(14), 7759–7774. https://doi.org/10.1021/acs.est.7b01097

- Dai, D., Prussin, A. J., Marr, L. C., Vikesland, P. J., Edwards, M. A., & Pruden, A. (2017). Factors Shaping the Human Exposome in the Built Environment: Opportunities for Engineering Control. *Environmental Science & Technology*, *51*(14), 7759–7774. https://doi.org/10.1021/acs.est.7b01097

- Daisey, J.M.; Angell, W.J.; Apte, M.G. Indoor Air Quality, Ventilation and Health Symptoms in Schools: An Analysis of Existing Information. Indoor Air **2003**, 13, 53–64
- Dancer, S. J. (2014). Controlling hospital-acquired infection: focus on the role of the environment and new technologies for decontamination. *Clin Microbiol Rev*, *27*(4), 665–690. https://doi.org/10.1128/CMR.00020-14
- Danielski, I.; Svensson, A.;Weimer, K.; Lorentzen, L.;Warne, M. Effects of Green Plants on the Indoor Environment andWellbeing in Classrooms—A Case Study in a Swedish School. Sustainability **2022**, 14, 3777
- Davis, M. F., Iverson, S. A., Baron, P., Vasse, A., Silbergeld, E. K., Lautenbach, E., & Morris, D. O. (2012). Household transmission of meticillin-resistant Staphylococcus aureus and other staphylococci. *The Lancet Infectious Diseases*, *12*(9), 703–716.https://doi.org/10.1016/S1473-3099(12)70156-1
- Deshpande, A., Pasupuleti, V., Thota, P., Pant, C., Rolston, D. D., Sferra, T. J., Hernandez, A. V, & Donskey, C. J. (2013). Community-associated Clostridium difficile infection and antibiotics: a meta-analysis. *J Antimicrob Chemother*, *68*(9), 1951–1961. https://doi.org/10.1093/jac/dkt129
- DHHS. (1986). *Guidelines for Research Involving Recombinant DNA Molecules* (Office of the Federal Register, National Archives and Records Service, & General Services Administration, Eds.; 88th ed., Vol. 51).
- Dietze, B., Rath, A., Wendt, C., & Martiny, H. (2001). Survival of MRSA on sterile goods packaging. *Journal of Hospital Infection*, *49*(4), 255–261. https://doi.org/10.1053/jhin.2001.1094
- Dimitroulopoulou, S.; Dudzi´ nska, M.R.; Gunnarsen, L.; Hagerhed, L.; Maula, H.; Singh, R.; Toyinbo, O.; Haverinen-Shaughnessy. U (2023). Indoor Air Quality Guidelines from across

theWorld: An Appraisal Considering Energy Saving, Health, Productivity, and Comfort. Environ. Int, 178, 108127

- Dockx, Y.; Taubel, M.; Bijnens, E.M.; Witters, K.; Valkonen, M.; Jayaprakash, B.; Hogervorst, J. Nawrot, T.S.; Casas, L. Indoor Green Can Modify the Indoor Dust Microbial Communities. Indoor Air **2022**, 32, e13011
- Dockx, Y.; Taubel, M.; Bijnens, E.M.; Witters, K.; Valkonen, M.; Jayaprakash, B.; Hogervorst, J.; Nawrot, T.S.; Casas, L. Residential Green Space Can Shape the Indoor Microbial Environment. Environ. Res. **2021**, 201, 111543
- EFSA. (2005). *Opinion of the Scientific Committee on a request from EFSA related to a generic approach to the safety assessment by EFSA of microorganisms used in food/feed and the production of food/feed additives.* Available at: https://www.efsa.europa.eu/en/efsajournal/pub/226
- EFSA. (2019). *Statement on the update of the list of QPS-recommended biological agents intentionally added to food or feed as notified to EFSA 10: Suitability of taxonomic units notified to EFSA until March 2019.* Available at: https://doi.org/10.2903/j.efsa.2019.5753
- EFSA. (2022). Statement on the update of the list of QPS-recommended biological agents intentionally added to food or feed as notified to EFSA 15: suitability of taxonomic units notified to EFSA until September 2021. Available at: https://efsa.onlinelibrary.wiley.com/doi/epdf/10.2903/j.efsa.2022.7045
- EPA. (1991). *Indoor Air Facts No. 4 Sick Building Syndrome (revised)*. Available at: https://www.epa.gov/sites/default/files/201408/documents/sick_building_factsheet.pdf
- EPA. (1991). *Indoor Air Facts No. 4 Sick Building Syndrome (revised)*. Available at: https://www.epa.gov/sites/default/files/2014- 08/documents/sick_building_factsheet.pdf
- Esfehani, H.H.; Schauble, J.; Paul, E.; Bohne, D. Simulation and analysis of load shifting and energy saving potential of CO2-based demand-controlled ventilation in a sports training center. IOP Conf. Ser. Mater. Sci. Eng. **2019**, 609, 052042
- Esty, B.; Phipatanakul, W. School Exposure and Asthma. Ann. Allergy Asthma Immunol. **2018**, 120, 482–487
- FAO and WHO. (2002). *Guidelines for the Evaluation of Probiotics in Food*. Available at: https://isappscience.org/wp content/uploads/2019/04/probiotic_guidelines.pdf
- FDA. (1999). *Carbohydrase and protease enzyme preparations derived from Bacillus subtilis or Bacillus amyloliquefaciens. Affirmation of GRAS Status as direct food ingredients.* Available at: https://www.govinfo.gov/content/pkg/FR-1999-04-23/pdf/99-10011.pdf

- Ferrari, L., Favero, C., Solazzo, G., Mariani, J., Luganini, A., Ferraroni, M., Montomoli, E., Milani, G. P., & Bollati, V. (2022). Nasopharyngeal Bacterial Microbiota Composition and SARS-CoV-2 IgG Antibody Maintenance in Asymptomatic/Paucisymptomatic Subjects. *Frontiers in Cellular and Infection Microbiology*, *12*. https://doi.org/10.3389/fcimb.2022.882302
- Ferrari, S.; Blazquez, T.; Cardelli, R.; De Angelis, E.; Puglisi, G.; Escandon, R.; Suarez, R. Air Change Rates and Infection Risk in School Environments: Monitoring Naturally Ventilated Classrooms in a Northern Italian Urban Context. Heliyon **2023**, 9, e19120
- Fijan, S. (2014). Microorganisms with claimed probiotic properties: An overview of recent literature. In *International Journal of Environmental Research and Public Health* (Vol.11, Issue 5, pp. 4745–4767). MDPI. https://doi.org/10.3390/ijerph110504745
- Flannigan, B., Samson, R. A., & Miller, J. D. (2016). *Microorganisms in Home and Indoor Work Environments* (B. Flannigan, R. A. Samson, & J. D. Miller, Eds.). CRC Press. https://doi.org/10.1201/b10838
- Fu, X.; Ou, Z.; Sun, Y. Indoor Microbiome and Allergic Diseases: From Theoretical Advances to Prevention Strategies. Eco-Environ. Health **2022**, 1, 133–146
- Fu, X.; Shama, A.; Norback, D.; Chen, Q.; Xia, Y.; Zhang, X.; Sun, Y. Exploring the Role of Indoor Microbiome and Environmental Characteristics in Rhinitis Symptoms among University Students. Front. Microbiomes **2024**, 3, 1277177
- Getahun, H., Smith, I., Trivedi, K., Paulin, S., & Balkhy, H. H. (2020). Tackling antimicrobial resistance in the COVID-19 pandemic. *Bull World Health Organ*, *98*(7),442-442A. https://doi.org/10.2471/BLT.20.268573
- Gilbert, J. A., & Stephens, B. (2018). Microbiology of the built environment. *Nature Reviews Microbiology*, *16*(11), 661–670. https://doi.org/10.1038/s41579-018-0065-5
- Gilbert, J.A.; Stephens, B. Microbiology of the Built Environment. Nat. Rev. Microbiol. **2018**, 16, 661–670
- Gohli, J., Bøifot, K. O., Moen, L. V., Pastuszek, P., Skogan, G., Udekwu, K. I., & Dybwad, M. (2019). The subway microbiome: seasonal dynamics and direct comparison of air and surface bacterial communities. *Microbiome*, *7*(1), 160. https://doi.org/10.1186/s40168-019-0772-9
- Goodman, E. R., Platt, R., Bass, R., Onderdonk, A. B., Yokoe, D. S., & Huang, S. S. (2008). Impact of an environmental cleaning intervention on the presence of methicillinresistant Staphylococcus aureus and vancomycin-resistant enterococci on surfaces in intensive care unit rooms. *Infect Control Hosp Epidemiol*, *29*(7), 593–599. https://doi.org/10.1086/588566

- Gougoulias, C., Clark, J. M., & Shaw, L. J. (2014). The role of soil microbes in the global carbon cycle: Tracking the below-ground microbial processing of plant-derived carbon for manipulating carbon dynamics in agricultural systems. In *Journal of the Science of Food and Agriculture* (Vol. 94, Issue 12, pp. 2362–2371). John Wiley and Sons Ltd.https://doi.org/10.1002/jsfa.6577
- Guan, L.; Beig, M.; Wang, L.; Navidifar, T.; Moradi, S.; Motallebi Tabaei, F.; Teymouri, Z.; Abedi Moghadam, M.; Sedighi, M. Global Status of Antimicrobial Resistance in Clinical Enterococcus Faecalis Isolates: Systematic Review and Meta-Analysis. Ann. Clin. Microbiol. Antimicrob. **2024**, 23, 80
- Hawrelak, J. A., & Myers, S. P. (2004). The causes of intestinal dysbiosis: a review. *Altern Med Rev*, *9*(2), 180–197. https://www.ncbi.nlm.nih.gov/pubmed/15253677
- Hernández, A. M., Vargas-Robles, D., Alcaraz, L. D., & Peimbert, M. (2020). Station and train surface microbiomes of Mexico City's metro (subway/underground). *Scientific Reports*, *10*(1), 8798. https://doi.org/10.1038/s41598-020-65643-4
- Hong, H. A., Huang, J. M., Khaneja, R., Hiep, L. V., Urdaci, M. C., & Cutting, S. M. (2008). The safety of Bacillus subtilis and Bacillus indicus as food probiotics. *Journal of Applied Microbiology*, *105*(2), 510–520. https://doi.org/10.1111/j.1365- 2672.2008.03773.x
- Human Microbiome Project Consortium. (2012). Structure, function and diversity of the healthy human microbiome. *Nature*, *486*(7402), 207–214. https://doi.org/10.1038/nature11234
- ISS. (2020). Interim recommendations on cleaning and disinfection of non-healthcare settings during COVID-19 health emergency: Surfaces, indoor environments and clothing. In Istituto Superiore Sanità (Ed.), *ISS COVID-19 Working Group on Biocides, ISS ed.: Vol. 25/2020*.
- Jacksch, S., Zohra, H., Weide, M., Schnell, S., & Egert, M. (2021). Cultivation-Based Quantification and Identification of Bacteria at Two Hygienic Key Sides of Domestic Washing Machines. *Microorganisms*, *9*(5), 905. https://doi.org/10.3390/microorganisms9050905
- Jayaraman, S., Thangavel, G., Kurian, H., Mani, R., Mukkalil, R., & Chirakkal, H. (2013). Bacillus subtilis PB6 improves intestinal health of broiler chickens challenged with Clostridium perfringens-induced necrotic enteritis. *Poult Sci*, *92*(2), 370–374. https://doi.org/10.3382/ps.2012-02528
- Jeon, Y.-S., Chun, J., & Kim, B.-S. (2013). Identification of Household Bacterial Community and Analysis of Species Shared with Human Microbiome. *Current Microbiology*, *67*(5), 557–563. https://doi.org/10.1007/s00284-013-0401-y

- Jiang, D., Gong, X., Xu, Z., Yuan, K., & Bu, Z. (2023). Prediction models of bioaerosols inside office buildings: a field study investigation. Building Services Engineering Research and Technology, 44(5), 577-600. https://doi.org/10.1177/01436244231189138
- Jin, P., Kang, Z., Yuan, P., Du, G., & Chen, J. (2016). Production of specific-molecularweight hyaluronan by metabolically engineered Bacillus subtilis 168. *Metab Eng*, *35*, 21–30. https://doi.org/10.1016/j.ymben.2016.01.008
- Jovel, J., Patterson, J., Wang, W., Hotte, N., O'Keefe, S., Mitchel, T., Perry, T., Kao, D., Mason, A. L., Madsen, K. L., & Wong, G. K. S. (2016). Characterization of the gut microbiome using 16S or shotgun metagenomics. *Frontiers in Microbiology*, *7*(APR). https://doi.org/10.3389/fmicb.2016.00459
- Kampf, G. (2018). Biocidal Agents Used for Disinfection Can Enhance Antibiotic Resistance in Gram-Negative Species. *Antibiotics (Basel)*, *7*(4). https://doi.org/10.3390/antibiotics7040110
- Kang, K., Ni, Y., Li, J., Imamovic, L., Sarkar, C., Kobler, M. D., Heshiki, Y., Zheng, T.,Kumari, S., Wong, J. C. Y., Archna, A., Wong, C. W. M., Dingle, C., Denizen, S., Baker, D. M., Sommer, M. O. A., Webster, C. J., & Panagiotou, G. (2018). The Environmental Exposures and Inner- and Intercity Traffic Flows of the Metro System May Contribute to the Skin Microbiome and Resistome. *Cell Reports*, *24*(5), 1190-1202.e5. https://doi.org/10.1016/j.celrep.2018.06.109
- Kelly, A. M., Mathema, B., & Larson, E. L. (2017). Carbapenem-resistant Enterobacteriaceae in the community: a scoping review. *International Journal of Antimicrobial Agents*, *50*(2), 127–134. https://doi.org/10.1016/j.ijantimicag.2017.03.012
- Kembel, S., Meadow, J., O'Connor, T., Mhuireach, G., Northcutt, D., Kline, J., Green, J. (2014). Architectural design drives the biogeography of indoor bacterial communities. Plos One, 9(1), e87093. https://doi.org/10.1371/journal.pone.0087093
- Khovalyg, D.; Kazanci, O.B.; Halvorsen, H.; Gundlach, I.; Bahnfleth, W.P.; Toftum, J.; Olesen, B.W., 2020 Critical review of standards for indoor thermal environment and air quality. Energy Build, 213, 109819.
- Kramer, A., Schwebke, I., & Kampf, G. (2006). How long do nosocomial pathogens persist on inanimate surfaces? A systematic review. In *BMC Infectious Diseases* (Vol. 6). https://doi.org/10.1186/1471-2334-6-130
- Krawczyk, D.A.; Rodero, A.; Gładyszewska-Fiedoruk, K.; Gajewski, A. (2016). CO_2 Concentration in Naturally Ventilated Classrooms Located in Different Climates—Measurements and Simulations. Energy Build, 129, 491–498

- Lai, C. C., Chen, S. Y., Ko, W. C., & Hsueh, P. R. (2021). Increased antimicrobial resistance during the COVID-19 pandemic. *Int J Antimicrob Agents*, *57*(4), 106324. https://doi.org/10.1016/j.ijantimicag.2021.106324
- Landry, K., Ascension, N., Chatue, I., Hortense, G., Etoa, F. (2018). Assessment of indoor microbial quality of library's premise: case of central library of the university of yaound& #233; i. Open Journal of Preventive Medicine, 08(04), 109-120. https://doi.org/10.4236/ojpm.2018.84011
- Langiano, E.; Ferrara, M.; Falese, L.; Lanni, L.; Diotaiuti, P.; Di Libero, T.; De Vito, E. Assessment of Indoor Air Quality in School Facilities: An Educational Experience of Pathways for Transversal Skills and Orientation (PCTO). Sustainability **2024**, 16, 6612
- Lansdown, A. B. (2006). Silver in health care: antimicrobial effects and safety in use. *Curr Probl Dermatol*, *33*, 17–34. https://doi.org/10.1159/000093928
- Lawley, T. D., Clare, S., Deakin, L. J., Goulding, D., Yen, J. L., Raisen, C., Brandt, C., Lovell, J., Cooke, F., Clark, T. G., & Dougan, G. (2010). Use of purified Clostridium difficile spores to facilitate evaluation of health care disinfection regimens. *Appl Environ Microbiol*, *76*(20), 6895–6900. https://doi.org/10.1128/AEM.00718-10
- Lax, S., Sangwan, N., Smith, D., Larsen, P., Handley, K. M., Richardson, M., Guyton, K., Krezalek, M., Shogan, B. D., Defazio, J., Flemming, I., Shakhsheer, B., Weber, S., Landon, E., Garcia-Houchins, S., Siegel, J., Alverdy, J., Knight, R., Stephens, B., & Gilbert, J. A. (2017). Bacterial colonization and succession in a newly opened hospital. *Science Translational Medicine*, *9*(391). https://doi.org/10.1126/scitranslmed.aah6500
- Lax, S., Smith, D. P., Hampton-Marcell, J., Owens, S. M., Handley, K. M., Scott, N. M., Gibbons, S. M., Larsen, P., Shogan, B. D., Weiss, S., Metcalf, J. L., Ursell, L. K., Vázquez-Baeza, Y., Van Treuren, W., Hasan, N. A., Gibson, M. K., Colwell, R., Dantas, G., Knight, R., & Gilbert, J. A. (2014). Longitudinal analysis of microbial interaction between humans and the indoor environment. *Science*, *345*(6200), 1048–1052. https://doi.org/10.1126/science.1254529
- Lederberg, J., & Mccray, A. T. (n.d.). *COMMENTARY 'Ome Sweet 'Omics-A Genealogical Treasury of Words*. www.-ics.com
- Lee, B.G.; Yang, J.I.; Kim, E.; Geum, S.W.; Park, J.; Yeo, M. Investigation of Bacterial and Fungal Communities in Indoor and Outdoor Air of Elementary School Classrooms by 16S RRNA Gene and ITS Region Sequencing. Indoor Air **2021**, 31, 1553–1562

- Leung, M. H. Y., Tong, X., & Lee, P. K. H. (2019). Indoor Microbiome and Airborne Pathogens. In *Comprehensive Biotechnology* (pp. 96–106). Elsevier. https://doi.org/10.1016/B978-0-444-64046-8.00477-8
- Leung, M., Tong, X., Bøifot, K., Bezdan, D., Butler, D., Danko, D., Lee, P. (2021). Characterization of the public transit air microbiome and resistome reveals geographical specificity. Microbiome, 9(1). https://doi.org/10.1186/s40168-021-01044-7
- Leyva Salas, M., Mounier, J., Valence, F., Coton, M., Thierry, A., & Coton, E. (2017). Antifungal microbial agents for food biopreservation—a review. In *Microorganisms* (Vol. 5, Issue 3). MDPI AG. https://doi.org/10.3390/microorganisms5030037
- Li, J., Cao, J., Zhu, Y. G., Chen, Q. L., Shen, F., Wu, Y., Xu, S., Fan, H., Da, G., Huang, R. J., Wang, J., De Jesus, A. L., Morawska, L., Chan, C. K., Peccia, J., & Yao, M. (2018). Global Survey of Antibiotic Resistance Genes in Air. *Environmental Science andTechnology*, *52*(19), 10975–10984. https://doi.org/10.1021/acs.est.8b02204
- Li, S., Yang, Z., Hu, D., Cao, L., & He, Q. (2021). Understanding building-occupantmicrobiome interactions toward healthy built environments: A review. *Frontiers of Environmental Science & Engineering*, *15*(4), 65. https://doi.org/10.1007/s11783-020-1357-3
- Liu, Y., Yang, B., Lin, Z. (2022). A pilot study of occupant centric control stratum ventilation based on computer vision. E3s Web of Conferences, 356, 01029. https://doi.org/10.1051/e3sconf/202235601029
- Loreti, L.; Valdiserri, P.; Garai,. M (2016). Dynamic simulation on energy performance of a school. Energy Procedia, 101, 1026–1033
- Lowther, S.D.; Dimitroulopoulou, S.; Foxall, K.; Shrubsole, C.; Cheek, E.; Gadeberg, B.; Sepai, O. (2021) Low Level Carbon Dioxide Indoors—A Pollution Indicator or a Pollutant? A Health-Based Perspective. Environments, 8, 125
- Lutz, J. K., van Balen, J., Crawford, J. Mac, Wilkins, J. R., Lee, J., Nava-Hoet, R. C., & Hoet, A. E. (2014). Methicillin-resistant Staphylococcus aureus in public transportation vehicles (buses): Another piece to the epidemiologic puzzle. *American Journal of Infection Control*, *42*(12), 1285–1290. https://doi.org/10.1016/j.ajic.2014.08.016
- Madsen, A. M., Moslehi-Jenabian, S., Islam, M. Z., Frankel, M., Spilak, M., & Frederiksen, M. W. (2018). Concentrations of Staphylococcus species in indoor air as associated with other bacteria, season, relative humidity, air change rate, and S. aureus-positive occupants. *Environmental Research*, *160*, 282–291. https://doi.org/10.1016/j.envres.2017.10.001
- Mahnert, A., Moissl-Eichinger, C., Zojer, M., Bogumil, D., Mizrahi, I., Rattei, T., Martinez,

J. L., & Berg, G. (2019). Man-made microbial resistances in built environments. *Nature Communications*, *10*(1). https://doi.org/10.1038/s41467-019-08864-0

- Mahnert, A.; Haratani, M.; Schmuck, M.; Berg, G. Enriching Beneficial Microbial Diversity of Indoor Plants and Their Surrounding Built Environment with Biostimulants. Front. Microbiol. **2018**, 9, 2985
- Mahnert, A.; Moissl-Eichinger, C.; Berg, G. Microbiome Interplay: Plants Alter Microbial Abundance and Diversity within the Built Environment. Front. Microbiol. **2015**, 6, 887
- Małecka-Adamowicz, M.; Koim-Puchowska, B.; Dembowska, E.A. Diversity of Bioaerosols in Selected Rooms of Two Schools and Antibiotic Resistance of Isolated Staphylococcal Strains (Bydgoszcz, Poland): A Case Study. Atmosphere **2020**, 11, 1105
- Manshadi, F. D., Karpiscak, M., & Gerba, C. P. (2013). Enteric bacterial contamination and survival on produce during irrigation with dairy wastewater in the field. *Journal of Water Reuse and Desalination*, *3*(2), 102–110. https://doi.org/10.2166/wrd.2013.161
- Marchesi, J. R., & Ravel, J. (2015). The vocabulary of microbiome research: a proposal.*Microbiome*, *3*(1). https://doi.org/10.1186/s40168-015-0094-5
- Mazza, P. (1994). The use of Bacillus subtilis as an antidiarrhoeal microorganism. *Bollettino Chimico Farmaceutico*, *133*(1), 3–18.
- McDonnell, G., & Russell, A. D. (1999). Antiseptics and Disinfectants: Activity, Action, and Resistance. *Clinical Microbiology Reviews*, *12*(1), 147–179. https://doi.org/10.1128/CMR.12.1.147
- Meadow, J., Altrichter, A., Kembel, S., Kline, J., Mhuireach, G., Moriyama, M., Bohannan, B. (2013). Indoor airborne bacterial communities are influenced by ventilation, occupancy, and outdoor air source. Indoor Air, 24(1), 41-48. https://doi.org/10.1111/ina.12047
- Meletis, G. (2016). Carbapenem resistance: overview of the problem and future perspectives. *Therapeutic Advances in Infectious Disease*, *3*(1), 15–21. https://doi.org/10.1177/2049936115621709
- Microbiomes of the Built Environment; National Academies Press: Washington, DC, USA, 2017; ISBN 978-0-309-44980-9.
- Moran, N. A. (2007). *Symbiosis as an adaptive process and source of phenotypic complexity*.www.nasonline.org/adaptationandcomplexdesign.
- Morrill, H. J., Pogue, J. M., Kaye, K. S., & LaPlante, K. L. (2015). Treatment Options for Carbapenem-Resistant Enterobacteriaceae Infections. *Open Forum Infectious Diseases*, *2*(2). https://doi.org/10.1093/ofid/ofv050

- Mousavi, B.; Hedayati, M.T.; Hedayati, N.; Ilkit, M.; Syedmousavi, S. Aspergillus Species in Indoor Environments and Their Possible Occupational and Public Health Hazards. Curr. Med. Mycol. **2016**, 2, 36–42
- Nabi, G., Wang, Y., Hao, Y., Khan, S., Wu, Y., & Li, D. (2020). Massive use of disinfectants against COVID-19 poses potential risks to urban wildlife. *Environ Res*, *188*, 109916. https://doi.org/10.1016/j.envres.2020.109916
- National Oceanic and Atmospheric Administration (NOAA). Climate Change: Atmospheric Carbon Dioxide. Available online: https://www.climate.gov/news-features/understanding-climate/climate-change-atmospheric-carbon-dioxide
- Nevalainen, A., Täubel, M., & Hyvärinen, A. (2015). Indoor fungi: companions and contaminants. *Indoor Air*, *25*(2), 125–156. https://doi.org/10.1111/ina.12182
- Niza, I.L.; Bueno, A.M.; Gameiro da Silva, M.; Broday, E.E. Air Quality and Ventilation: Exploring Solutions for Healthy and Sustainable Urban Environments in Times of Climate Change. Results Eng. **2024**, 24, 103157
- Norbäck, D., Hashim, J., Hashim, Z., Sooria, V., Ismail, S., Wieslander, G. (2017). Ocular symptoms and tear film break up time (but) among junior high school students in penang, malaysia – associations with fungal dna in school dust. International Journal of Hygiene and Environmental Health, 220(4), 697-703. https://doi.org/10.1016/j.ijheh.2017.01.016
- Nowrotek, M., Jałowiecki, Ł., Harnisz, M., & Płaza, G. A. (2019). Culturomics and metagenomics: In understanding of environmental resistome. In *Frontiers of Environmental Science and Engineering* (Vol. 13, Issue 3). Higher Education Press.https://doi.org/10.1007/s11783-019-1121-8
- Noyce, J. O., Michels, H., & Keevil, C. W. (2006). Potential use of copper surfaces to reduce survival of epidemic meticillin-resistant Staphylococcus aureus in the healthcare environment. *J Hosp Infect*, *63*(3), 289–297. https://doi.org/10.1016/j.jhin.2005.12.008
- Oie, S., & Kamiya, A. (1996). Survival of methicillin-resistant Staphylococcus aureus (MRSA) on naturally contaminated dry mops. *Journal of Hospital Infection*, *34*(2), 145–149. https://doi.org/10.1016/S0195-6701(96)90140-1
- One Health High-Level Expert Panel (OHHLEP), Adisasmito, W. B., Almuhairi, S.,Behravesh, C. B., Bilivogui, P., Bukachi, S. A., Casas, N., Cediel Becerra, N., Charron, D. F., Chaudhary, A., Ciacci Zanella, J. R., Cunningham, A. A., Dar, O., Debnath, N., Dungu, B., Farag, E., Gao, G. F., Hayman, D. T. S., Khaitsa, M., … Zhou, L. (2022). One Health: A new definition for a sustainable and healthy future. *PLOS Pathogens*, *18*(6), e1010537. https://doi.org/10.1371/journal.ppat.1010537

- Park, J.-H.; Lemons, A.R.; Croston, T.L.; Roseman, J.; Green, B.J.; Cox-Ganser, J.M. More Diverse School Microbiota May Provide Better Protection against Respiratory Infections for School Staff. Build. Environ. **2025**, 271, 112657
- Park, J.-H.; Lemons, A.R.; Roseman, J.; Green, B.J.; Cox-Ganser, J.M. Bacterial Community Assemblages in Classroom Floor Dust of 50 Public Schools in a Large City: Characterization Using 16S RRNA Sequences and Associations with Environmental Factors. Microbiome **2021**, 9, 15
- Patel, R. M., & Underwood, M. A. (2018). Probiotics and necrotizing enterocolitis. *Semin Pediatr Surg*, *27*(1), 39–46. https://doi.org/10.1053/j.sempedsurg.2017.11.008
- Pegas, P.N.; Alves, C.A.; Nunes, T.; Bate-Epey, E.F.; Evtyugina, M.; Pio, C.A. Could Houseplants Improve Indoor Air Quality in Schools? J. Toxicol. Environ. Health A **2012**, 75, 1371–1380
- Piñar, G., Piombino-Mascali, D., Maixner, F., Zink, A.,Sterflinger, K. (2013). Microbial survey of the mummies from the capuchin catacombs of palermo, italy: biodeterioration risk and contamination of the indoor air. Fems Microbiology Ecology, 86(2), 341-356. https://doi.org/10.1111/1574-6941.12165
- Portnoy, J.M.; Kwak, K.; Dowling, P.; VanOsdol, T.; Barnes, C. Health Effects of Indoor Fungi. Ann. Allergy Asthma Immunol. **2005**, 94, 313–320
- Prussin, A. J., & Marr, L. C. (2015). Sources of airborne microorganisms in the built-environment. *Microbiome*, *3*, 78.https://doi.org/10.1186/s40168-015-0144-z
- Prussin, A., Garcia, E., Marr, L. (2015). Total concentrations of virus and bacteria in indoor and outdoor air. Environmental Science & Technology Letters, 2(4), 84-88. https://doi.org/10.1021/acs.estlett.5b00050
- Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., & Glöckner, F. O. (2012). The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Research*, *41*(D1), D590–D596. https://doi.org/10.1093/nar/gks1219
- Rai S., Singh D.K., and Kumar A. (2021). "Microbial, Environmental and Anthropogenic Factors Influencing the Indoor Microbiome of the Built Environment." Journal of Basic Microbiology 61: 267–292
- Rayes, N., Seehofer, D., Hansen, S., Boucsein, K., M??ller, A. R., Serke, S., Bengmark, S., & Neuhaus, P. (2002). Early enteral supply of lactobacillus and fiber versus selective bowel decontamination: a controlled trial in liver transplant recipients. *Transplantation*, *74*(1), 123–128. https://doi.org/10.1097/00007890-200207150-00021

- Rocha, C., Silva, R., Monzón, A., Alfonzo, J. (2012). Characterization of indoor air bioaerosols in an electrical headquarter building. Indoor and Built Environment, 22(6), 910-919. https://doi.org/10.1177/1420326x12462911
- Rutala, W. A., & Weber, D. J. (2014). Selection of the ideal disinfectant. *Infect Control Hosp Epidemiol*, *35*(7), 855–865. https://doi.org/10.1086/676877
- Sadrizadeh, S.; Yao, R.; Yuan, F.; Awbi, H.; Bahnfleth, W.; Bi, Y.; Cao, G.; Croitoru, C.; de Dear, R.; Haghighat, F.; et al. Indoor Air Quality and Health in Schools: A Critical Review for Developing the Roadmap for the Future School Environment. J. Build. Eng. **2022**, 57, 104908
- Salonen, H.; Duchaine, C.; Mazaheri, M.; Clifford, S.; Lappalainen, S.; Reijula, K.; Morawska, L. Airborne Viable Fungi in School Environments in Different Climatic Regions—A Review. Atmos. Environ. **2015**, 104, 186–194
- Sanchez-Fernandez, A.; Coll-Aliaga, E.; Lerma-Arce, V.; Lorenzo-Saez, E. (2023). Evaluation of Different Natural Ventilation Strategies by Monitoring the Indoor Air Quality Using CO_2 Sensors. Int. J. Environ. Res. Public Health, 20, 6757
- Satish, U.; Mendell, M.J.; Shekhar, K.; Hotchi, T.; Sullivan, D.; Streufert, S.; Fisk, W.J. (2012) Is CO_2 an Indoor Pollutant? Direct Effects of Low-to-Moderate CO_2 Concentrations on Human Decision-Making Performance. Environ. Health Perspect, 120, 1671–1677
- Schibuola, L.; Tambani, C. (2020). Indoor Environmental Quality Classification of School Environments by Monitoring PM and CO_2 Concentration Levels. Atmos. Pollut. Res, 11, 332–342
- Schütze, A., Baur, T., Leidinger, M., Reimringer, W., Jung, R., Conrad, T.,Sauerwald, T. (2017). Highly sensitive and selective voc sensor systems based on semiconductor gas sensors: how to? Environments, 4(1), 20. https://doi.org/10.3390/environments4010020
- Sender, R., Fuchs, S., & Milo, R. (2016). Revised Estimates for the Number of Human and Bacteria Cells in the Body. *PLoS Biology*, *14*(8). https://doi.org/10.1371/journal.pbio.1002533
- Settimo, G.; Indinnimeo, L.; Inglessis, M.; De Felice, M.; Morlino, R.; di Coste, A.; Carriera, F.; Di Fiore, C.; Avino, P. (2024) CO_2 Levels in Classrooms: What Actions to Take to Improve the Quality of Environments and Spaces. Sustainability, 16, 8619
- Sha, H., Zhang, X., Qi, D. (2021). Optimal control of high-rise building mechanical ventilation system for achieving low risk of covid-19 transmission and ventilative cooling. Sustainable Cities and Society, 74, 103256. https://doi.org/10.1016/j.scs.2021.103256
- Shin, S. K., Kim, J., Ha, S. M., Oh, H. S., Chun, J., Sohn, J., & Yi, H. (2015). Metagenomic

insights into the bioaerosols in the indoor and outdoor environments of childcare facilities. *PLoS ONE*, *10*(5). https://doi.org/10.1371/journal.pone.0126960

- Smith, S. M., Eng, R. H., & Padberg, F. T. Jr. (1996). Survival of nosocomial pathogenic bacteria at ambient temperature. *Journal of Medicine*, *27*(5–6), 293–302. Available at: https://pubmed.ncbi.nlm.nih.gov/9151198
- Soffritti, I., D'Accolti, M., Fabbri, C., Passaro, A., Manfredini, R., Zuliani, G., Libanore, M., Franchi, M., Contini, C., & Caselli, E. (2021). Oral Microbiome Dysbiosis Is Associated With Symptoms Severity and Local Immune/Inflammatory Response in COVID-19 Patients: A Cross-Sectional Study. *Frontiers in Microbiology*, *12*. https://doi.org/10.3389/fmicb.2021.687513
- Soffritti, I., D'Accolti, M., Cason, C., Lanzoni, L., Bisi, M., Volta, A., Campisciano, G., Mazzacane, S., Bini, F., Mazziga, E., Toscani, P., Caselli, E., & Comar, M. (2022). Introduction of Probiotic-Based Sanitation in the Emergency Ward of a Children's Hospital During the COVID-19 Pandemic. *Infect Drug Resist*, *15*, 1399–1410. https://doi.org/10.2147/IDR.S356740
- Soininen, L.; Roslund, M.I.; Nurminen, N.; Puhakka, R.; Laitinen, O.H.; Hyoty, H.; Sinkkonen, A.; Cerrone, D.; Gronroos, M.; Hui, N.; et al. Indoor GreenWall Affects Health-Associated Commensal Skin Microbiota and Enhances Immune Regulation: A Randomized Trial among Urban OfficeWorkers. Sci. Rep. **2022**, 12, 6518
- Sommacal, H. M., Bersch, V. P., Vitola, S. P., & Osvaldt, A. B. (2015). Perioperative Synbiotics Decrease Postoperative Complications in Periampullary Neoplasms: A Randomized, Double-Blind Clinical Trial. *Nutrition and Cancer*, *67*(3), 457–462. https://doi.org/10.1080/01635581.2015.1004734
- Song, Z.; Chen, L.; Sun, S.; Yang, G.; Yu, G. Unveiling the Airborne Microbial Menace: Novel Insights into Pathogenic Bacteria and Fungi in Bioaerosols from Nursery Schools to Universities. Sci. Total Environ. **2024**, 929, 172694
- Squellati, R. (2018). Evidence-Based Practice in the Treatment for Antibiotic-Associated Diarrhea in the Intensive Care Unit. *Crit Care Nurs Clin North Am*, *30*(1), 87–99. https://doi.org/10.1016/j.cnc.2017.10.008
- Stabile, L.; Dell'Isola, M.; Russi, A.; Massimo, A.; Buonanno, G. The Effect of Natural Ventilation Strategy on Indoor Air Quality in Schools. Sci. Total Environ. **2017**, 595, 894–902

- Stanforth, B.; Krause, A.; Starkey, C.; Ryan, T.J. Prevalence of Community-Associated Methicillin-Resistant Staphylococcus Aureus in High School Wrestling Environments. J. Environ. Health **2010**, 72, 12–16
- Stephens, B. (2016). What Have We Learned about the Microbiomes of Indoor Environments? *MSystems*, *1*(4). https://doi.org/10.1128/mSystems.00083-16
- Stonner, C.; Edtbauer, A.; Williams, J. Real-world volatile organic compound emission rates from seated adults and children for use in indoor air studies. Indoor Air **2018**, 28, 164–172.
- Szczurek, A.; Maciejewska, M.; Pietrucha, (2015) T. CO2 and Volatile Organic Compounds as Indicators of IAQ. Available online https://www.aivc.org/resource/co2-and-volatile-organic-compounds-indicators-iaq
- Taubel, M.; Castagnoli, E.; Salthammer, T.; Morawska, L.; Salonen, H. The Impact of Cleaning on the Microbiomes of Indoor Surfaces. Indoor Environ. **2024**, 1, 100021
- Teo, Y.-L., & Tan, H.-M. (2006). *Effect of Bacillus subtilis PB6 (CloSTAT) on Broilers Infected with a Pathogenic Strain of Escherichia coli 1 DESCRIPTION OF PROBLEM*
- Toleman, M.A.; Rolston, K.; Jones, R.N.; Walsh, T.R. VIM-7, an Evolutionarily Distinct Metallo-β-Lactamase Gene in a Pseudomonas Aeruginosa Isolate from the United States. Antimicrob. Agents Chemother. **2004**, 48, 329–332
- Tong, S. Y. C., Davis, J. S., Eichenberger, E., Holland, T. L., & Fowler, V. G. (2015). Staphylococcus aureus Infections: Epidemiology, Pathophysiology, Clinical Manifestations, and Management. *Clinical Microbiology Reviews*, *28*(3), 603–661. https://doi.org/10.1128/CMR.00134-14
- Trabelsi, D., & Mhamdi, R. (2013). Microbial inoculants and their impact on soil microbial communities: A review. *BioMed Research International*, *2013*. https://doi.org/10.1155/2013/863240
- Trinh, P., Zaneveld, J. R., Safranek, S., & Rabinowitz, P. M. (2018). One Health Relationships Between Human, Animal, and Environmental Microbiomes: A Mini- Review. In *Frontiers in Public Health* (Vol. 6). Frontiers Media S.A. https://doi.org/10.3389/fpubh.2018.00235
- Tzouvelekis, L.S.; Tzelepi, E.; Mentis, A.F. Nucleotide Sequence of a Plasmid-Mediated Cephalosporinase Gene (BlaLAT-1) Found in Klebsiella Pneumoniae. Antimicrob. Agents Chemother. **1994**, 38, 2207–2209
- Urdaci, M.C.; Pinchuk, I. Antimicrobial Activity of Bacillus Probiotics. In Bacterial Spore Formers—Probiotics and Emerging Applications; 2004; pp. 1–12. Available online: https://www.cabidigitallibrary.org/doi/full/10.5555/20043171459

- van Bruggen, A. H. C., Goss, E. M., Havelaar, A., van Diepeningen, A. D., Finckh, M. R., & Morris, J. G. (2019). One Health - Cycling of diverse microbial communities as a connecting force for soil, plant, animal, human and ecosystem health. In *Science of the Total Environment* (Vol. 664, pp. 927–937). Elsevier B.V. https://doi.org/10.1016/j.scitotenv.2019.02.091
- Vandini, A., Antonioli, P., Lanzoni, L., Camerada, M. T., Balboni, P. G., Coccagna, M., & Mazzacane, S. (2014). *Proposta di nuovi indicatori di igiene ambientale*. Available at: https://cias-ferrara.it/onewebmedia/pubblicazioni/Ospedale_2014_03_p64.pdf
- Vandini, A., Temmerman, R., Frabetti, A., Caselli, E., Antonioli, P., Balboni, P. G., Platano, D., Branchini, A., & Mazzacane, S. (2014). Hard surface biocontrol in hospitals using microbial-based cleaning products. *PLoS One*, *9*(9), e108598. https://doi.org/10.1371/journal.pone.0108598
- Vangay, P., Ward, T., Gerber, J. S., & Knights, D. (2015). Antibiotics, pediatric dysbiosis, and disease. *Cell Host Microbe*, *17*(5), 553–564. https://doi.org/10.1016/j.chom.2015.04.006
- Vargas-Robles, D., Gonzalez-Cedillo, C., Hernandez, A. M., Alcaraz, L. D., & Peimbert, M. (2020). Passenger-surface microbiome interactions in the subway of Mexico City. *PLOS ONE*, *15*(8), e0237272. https://doi.org/10.1371/journal.pone.0237272
- Vary, P. S., Biedendieck, R., Fuerch, T., Meinhardt, F., Rohde, M., Deckwer, W. D., & Jahn, D. (2007). Bacillus megaterium--from simple soil bacterium to industrial protein production host. *Appl Microbiol Biotechnol*, *76*(5), 957–967. https://doi.org/10.1007/s00253-007-1089-3
- Vaseeharan, B., & Ramasamy, P. (2003). Control of pathogenic Vibrio spp. by Bacillus subtilis BT23, a possible probiotic treatment for black tiger shrimp Penaeus monodon. *Lett Appl Microbiol*, *36*(2), 83–87. https://doi.org/10.1046/j.1472-765x.2003.01255.x
- Volterra, V. Fluctuations in the Abundance of a Species considered Mathematically. Nature **1926**, 118, 558–560
- Votintseva, A.A.; Fung, R.; Miller, R.R.; Knox, K.; Godwin, H.; Wyllie, D.H.; Bowden, R.; Crook, D.W.; Walker, A.S. Prevalence of Staphylococcus Aureus Protein A (Spa) Mutants in the Community and Hospitals in Oxfordshire. BMC Microbiol. **2014**, 14, 63
- Wand, M. E., Bock, L. J., Bonney, L. C., & Sutton, J. M. (2017). Mechanisms of Increased Resistance to Chlorhexidine and Cross-Resistance to Colistin following Exposure of Klebsiella pneumoniae Clinical Isolates to Chlorhexidine. *Antimicrob AgentsChemother*, *61*(1). https://doi.org/10.1128/AAC.01162-16
- Wang, B., Yao, M., Lv, L., Ling, Z., & Li, L. (2017). The Human Microbiota in Health and Disease. *Engineering*, *3*(1), 71–82. https://doi.org/10.1016/J.ENG.2017.01.008

- Wang, J., Engvall, K., Smedje, G., Nilsson, H., & Norbäck, D. (2017). Current wheeze, asthma, respiratory infections, and rhinitis among adults in relation to inspection data and indoor measurements in single-family houses in sweden-the betsi study. Indoor Air, 27(4), 725-736. https://doi.org/10.1111/ina.12363
- Wang, Y.; Lin, J.; Zhang, T.; He, S.; Li, Y.; Zhang, W.; Ye, X.; Yao, Z. Environmental Contamination Prevalence, Antimicrobial Resistance and Molecular Characteristics of Methicillin-Resistant Staphylococcus Aureus and Staphylococcus Epidermidis Isolated from Secondary Schools in Guangzhou, China. Int. J. Environ. Res. Public Health **2020**, 17, 623
- Westers, L., Westers, H., & Quax, W. J. (2004). Bacillus subtilis as cell factory for pharmaceutical proteins: A biotechnological approach to optimize the host organism. In *Biochimica et Biophysica Acta - Molecular Cell Research* (Vol. 1694, Issues 1-3 SPEC.ISS., pp. 299–310). https://doi.org/10.1016/j.bbamcr.2004.02.011
- Whipps J., Lewis K., & Cooke R. (1988). Mycoparasitism and plant disease control. In *Fungi in Biological Control Systems* (pp. 161–187)
- WHO. (2011). *Report on the Burden of Endemic Health Care-Associated Infection Worldwide*. Available at: https://iris.who.int/bitstream/handle/10665/80135/9789241501507
- WHO. (2020). *World Malaria Report 2020*. Available at: https:// www.who.int/publications-detail-redirect/9789240015791
- WHO. (2021). *Healthcare-associated infections FACT SHEET*. Available at: https://www.who.int/gpsc/country_work/ gpsc_ccisc_fact_sheet_en.pdf
- WHO. (2022). *Global Antimicrobial Resistance and Use Surveillance System (GLASS) Report 2022*. Available at: https://iris.who.int/bitstream/handle/10665/364996/9789240062702
- WHO. (2024). *WHO Bacterial Priority Pathogens List, 2024*. Available at: https://iris.who.int/bitstream/handle/10665/376776/9789240093461
- Wu-Chen, R. A., Feng, J., Elhadidy, M., Nambiar, R. B., Liao, X., Yue, M., & Ding, T. (2023). Long-term exposure to food-grade disinfectants causes cross-resistance to antibiotics in Salmonella enterica serovar Typhimurium strains with different antibiograms and sequence types. *Antimicrobial Resistance & Infection Control*, *12*(1). https://doi.org/10.1186/s13756-023-01333-w
- Xu, Z., Mkrtchyan, H. V., & Cutler, R. R. (2015). Antibiotic resistance and mecA characterization of coagulase-negative staphylococci isolated from three hotels in London, UK. *Frontiers in Microbiology*, *6*.https://doi.org/10.3389/fmicb.2015.00947
- Xu, Z., Shah, H. N., Misra, R., Chen, J., Zhang, W., Liu, Y., Cutler, R. R., & Mkrtchyan, H.V. (2018). The prevalence, antibiotic resistance and mecA characterization of coagulase negative

staphylococci recovered from non-healthcare settings in London, UK. *Antimicrobial Resistance and Infection Control*, *7*(1). https://doi.org/10.1186/s13756-018-0367-4

- Yang, J.I.L.; Lee, B.G.; Park, J.-H.; Yeo, M.-K. Airborne Fungal and Bacterial Microbiome in Classrooms of Elementary Schools during the COVID-19 Pandemic Period: Effects of School Disinfection and Other Environmental Factors. Indoor Air **2022**, 32, e13107
- Zampolli J., De Giani A., Rossi M., Finazzi M., and Di Gennaro P. (2024) "Who Inhabits the Built Environment? A Microbiological Point of View on the Principal Bacteria Colonizing Our Urban Areas." Frontiers in Microbiology 15: 1380953
- Zhang, H., Tang, W., Chen, Y., & Yin, W. (2020). Disinfection threatens aquatic ecosystems. *Science*, *368*(6487), 146–147. https://doi.org/10.1126/science.abb8905
- Zhang, M.; Tang, H.; Chen, Y.; Chen, Z.; Xu, Y.; Fu, X.; Sun, Y.; Zhao, Z. Impact of Environmental Characteristics on Children's Gut Microbiota—A Pilot Study in Assessing the Role of Indoor Microbiome and Metabolites. Environ. Res. **2023**, 234, 116114
- Zilber-Rosenberg, I., & Rosenberg, E. (2008). Role of microorganisms in the evolution of animals and plants: The hologenome theory of evolution. In *FEMS Microbiology Reviews* (Vol. 32, Issue 5, pp. 723–735). https://doi.org/10.1111/j.1574-6976.2008.00123.x
- Zweers, J. C., Barák, I., Becher, D., Driessen, A. J. M., Hecker, M., Kontinen, V. P., Saller, M. J., Vavrová, L., & van Dijl, J. M. (2008). Towards the development of Bacillus subtilis as a cell factory for membrane proteins and protein complexes. In *Microbial Cell Factories* (Vol. 7). https://doi.org/10.1186/1475-2859-7-10

8. ACKNOWLEDGEMENTS

I would like to thank a lot Professor Elisabetta Caselli for her great availability and expertise which have been an extraordinary guide for me in completing the great adventure of the doctoral path.
I would also like to thank Professor Theo Zaffagnini for the precision and great passion he has shown me throughout this long journey of academic growth.
Deep thanks also go to all the professors and members of IDAUP, for the great opportunity for professional growth that has given me excellent tools which will certainly be extremely useful to me.
A special thanks also goes to Professor Sante Mazzacane, who has encouraged me assiduously in these 4 years.
I would also like to thank all my colleagues and companions in this doctoral course with whom I have shared an important part of my life.
I also extend my deepest thanks to my lab colleagues: Antonella Volta, Maria D'Accolti, Irene Soffritti, Francesca Bini, Eleonora Mazziga, and Gaia Caramazza. With them, I have shared the joys and adversities we have overcome together over these 10 years of scientific research.
I also want to thank my family for their support and the love I feel for them: my mother, Rita and Edoardo, my uncles: Chiara, Mauro, Teresa, Michele, Virna, and Mario, my cousins, Irene and Francesco and my ‘sister’ Alice. I love you all.
I also thank my dearest friends, always available to listen to me and lighten my mood with wholesome fun: Toni and Eleonora, Alfredo, Ilaria, Mauro, Andrea, Cristiano, Daniela and Momò, Preva, Juan and many others for which at least three more pages would not be enough to mention them all.
Finally, I want to thank all the people who are no longer with us but who are surely watching over me from above, and I know they are proud of me: my father Mauro, my grandmother Giovanna, my grandfather Franco, my grandmother Maria, my grandmother Altema, and my grandfather Walter. I miss you so much...
Lastly, this isn't just a thank you, it's a dedication: I dedicate this thesis to my mother, Rita, who has photographed my entire life (she knows what I mean). I love you, Mom.