UNIVERSITÀ DEGLI STUDI DI TRIESTE

XXXIII CICLO DEL DOTTORATO DI RICERCA IN

SCIENZE DELLA RIPRODUZIONE E DELLO SVILUPPO

NEW GENERATION TECHNIQUES FOR THE CHARACTERIZATION OF THE ENVIRONMENTAL MICROBIOME AND RESISTOME IN MATERNAL AND CHILD HOSPITAL

Settore scientifico-disciplinare: MED/17

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ABSTRACT

Healthcare associated infections (HAIs) are the most frequent adverse event threatening patients' safety worldwide, with high social and health impact. Contaminated surfaces are a reservoir for many pathogens and can act as an important source for transmission of infections. Moreover, the acquisition of resistance genes by bacteria has made the HAIs an increasing problem, with peculiarities also in the paediatric field. The monitoring of hospital surfaces is generally addressed by microbial cultural isolation, with some performance limitations, highlighting the need to evaluate the introduction of new generation technologies.

The aim of this study was to implement the standard procedures for hospital environmental monitoring using new generation technologies (NGS, qPCR array) to better understand the bacterial colonization and the resistome profile of surfaces, and its impact on preterm newborns colonization.

The results from this study showed that NGS and qPCR array were more effective tools in characterizing the hospital environmental microbiota, highlighting with highest sensitivity the presence of bacteria such as *Staphylococcus* spp., and detecting potentially pathogenic microorganisms like *Cutibacterium* spp., *Streptococcus* spp., and *Corynebacterium* spp., and the presence in hospital wards of numerous genes of antimicrobial resistance, undetected by traditional methods.

Looking at the bacterial composition of nasal swabs of preterm newborns during a time course analysis, starting from the time of birth and during the permanence in the Neonatal Intensive Care Unit (NICU), we showed the presence of resident bacterial species found in the departments (Delivery Room and NICU respectively). The most frequent environmental bacteria detected on newborns at time of birth were *Cutibacterium* spp. and *Staphylococcus* spp., while during the permanence in the NICU was reported an increased colonization by bacteria from hospital surfaces including *Staphylococcus* spp., *Streptococcus* spp. and

Escherichia/ Shigella, as well as the appearance and increase of resistance genes, including beta-lactam, quinolone and macrolide resistance genes, detected also on the hospital surfaces.

Data from these studies revealed a characteristic cluster of paediatric environmental bacteria, validating the use of a more rapid and less expensive multiplex assay allowing the simultaneous identification of microorganisms and antimicrobial resistance genes associated to HAIs. This test has been used as standard procedure for environmental contamination monitoring in the Infection Prevention and Control system.

During COVID-19 emergency, using the same detection methodology, the impact of the new strategy of cleaning (increasing in the frequency of cleaning interventions with chlorine-based detergents) on environmental microbiome and subject's colonization was analysed. SARS-CoV-2 surface infection was additionally evaluated, and moreover, in order to evaluate the influence of air-route transmission of bacteria and viruses, a system for microbiological monitoring of the air was activate. The results reported a moderate increase in bacterial colonization (*Staphylococcus* spp*., Streptococcus* spp*., Candida* spp*., Enterobactericaeae, Acinetobacter* spp*., Klebsiella* spp. and *E. coli*) and the presence on the surfaces of antimicrobial resistance genes, in particular Carbapenemase, pointing out the need to evaluate the introduction of alternative sanitizing methods.

Starting from the Emergency Room of IRCCS Burlo Garofolo, Trieste, for the first time in the paediatric field, was introduced as part of infection prevention and control strategies the Probiotic Cleaning Hygiene System (PCHS), an eco-sustainable probiotic-based cleaning system able to stably abate surface pathogens and their impact on human colonization, without selecting antibiotic-resistant species.

Keywords: HAIs, NGS, Microbiome, resistome, Environment.

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1 INTRODUCTION

1.1 Healthcare associated infections (HAIs)

Healthcare associated infections (HAIs), formerly called nosocomial infections, are defined by the Centres for Disease Control (CDC) as infections acquired in a hospital or health service unit that first appear 48 hours or more after hospitalization or within 30 days of discharge following patient care. Are pathologies for which there was no evidence of manifestation or incubation at the time of the patient's admission to healthcare facilities (Garner et al., 1988).

HAIs represent one of the most frequent and serious complications that can occur in healthcare facilities, it is estimated that 5 to 15% of patients worldwide acquire a care-related infection (Allegranzi et al., 2011). In Europe is estimated that approximately 4,100,000 patients contract an HAI each year, and that these are the direct cause of at least 37,000 deaths (European Centre for Disease Prevention and Control., 2013). In Italy the overall frequency of HAIs is approximately of 10.5% in hospitalized patients, 5% in patients living in nursing homes and 1% in homecare patients (Messineo and Marsella), the mortality rate can reach 20-30% for infections such as pneumonia (Tablan et al., 2004).

1.2 Risk of acquiring HAIs

Infection is the result of a complex interrelationship between a potential host and an infectious agent. A potential host for acquiring HAIs is represented not only by patients, but also by healthcare personnel or visitors. The risk of acquiring a hospital infection is linked

to the characteristics of the infectious agent, such as intrinsic virulence, resistance of antimicrobial agents or amount of infective material (Siegel et al., 2007b), but above all it is linked to the characteristics of the individual who contracts it. Some people exposed to infectious agents in fact never develop symptomatic disease while others become severely ill and can even die: in susceptible hosts microorganisms commonly part of the human microbiome can give rise to opportunistic infections (Siegel et al., 2007a). Among the predisposing factors there are the age of the subject, the long of hospital stay (Gravel et al., 2007; Al-Rawajfah et al., 2013), the compromise of the immune system, other concomitant diseases or infections, and consequently surgical interventions or invasive procedures such as intravascular and urinary catheters (Humphreys et al., 2008; Deptuła et al., 2015), intubation (Phu et al., 2016) or mechanical ventilation. The complexity of the conditions to be treated increases the likelihood that invasive procedures and long-term treatments will have to be performed. There are also highly complex cases, such as transplants or oncohematological diseases, in which there is a deficit in the immune response induced by immunosuppressive drugs or caused by the underlying disease. The combination of all these factors increases the risk of infections (Siegel et al., 2007a).

The problem of HAIs is exacerbated by the spread of antibiotic resistant bacterial strains. The development of Antimicrobial Resistance (AMR) is a natural phenomenon caused by mutations in bacterial genes, or exchange of genetic resistant elements horizontally between bacteria. Bacteria can acquire multiple resistance mechanisms and hence become resistant to several antimicrobial agents. The use of antimicrobial agents, often empirically, and the transmission of AMR microorganisms between humans, animals and the environment are the main causes of the spread of resistant pathogens. Antimicrobial use exerts ecological pressure on bacteria and contributes to the emergence and selection of AMR, furthermore poor infection prevention and control strategies favour the further spread of these microorganisms, for which, to date, in what is defined the post-antibiotic era, effective

treatments are increasingly limited (Surveillance of antimicrobial resistance in Europe 2017, 2018). The infections by Multi Drug Resistant (MDR) bacteria are increasingly common in the hospital setting, and are associated with the severity of HAIs. (Caini et al., 2013; Cornejo-Juárez et al., 2015). The microorganisms most responsible for HAIs are *Escherichia coli, Staphylococcus aureus, Enterococcus* spp., *Pseudomonas aeruginosa, Klebsiella* spp*.,* coagulase-negative *staphylococci*, *Clostridium difficile*, *Enterobacter* spp*., Proteus* spp*., Acinetobacter* spp. and *Candida* spp. (**Figure 1**). The European Antimicrobial Resistance Surveillance Network (EARS-Net) reported for *Escherichia coli* and *K. pneumoniae* a common production of extended-spectrum beta-lactamase (ESBL), as well as the frequent combined resistance to several antimicrobial groups, including cephalosporin. *Pseudomonas* spp*., Acinetobacter* spp. and, to a less extent, *K. Pneumoniae* and *E. coli*. had the highest carbapenem resistance percentages. *S. pneumoniae* exhibited more frequent resistance to macrolides, while for *E. faecium* there was an increasing trend of resistance to vancomycin. As regard *S. aureus*, a decline in the percentage of methicillin-resistant (MRSA) isolates was reported, however MRSA remains an important pathogen in Europe, as its levels are still high in several countries, and combined resistance to other antimicrobial groups is common (Surveillance of antimicrobial resistance in Europe 2017, 2018).

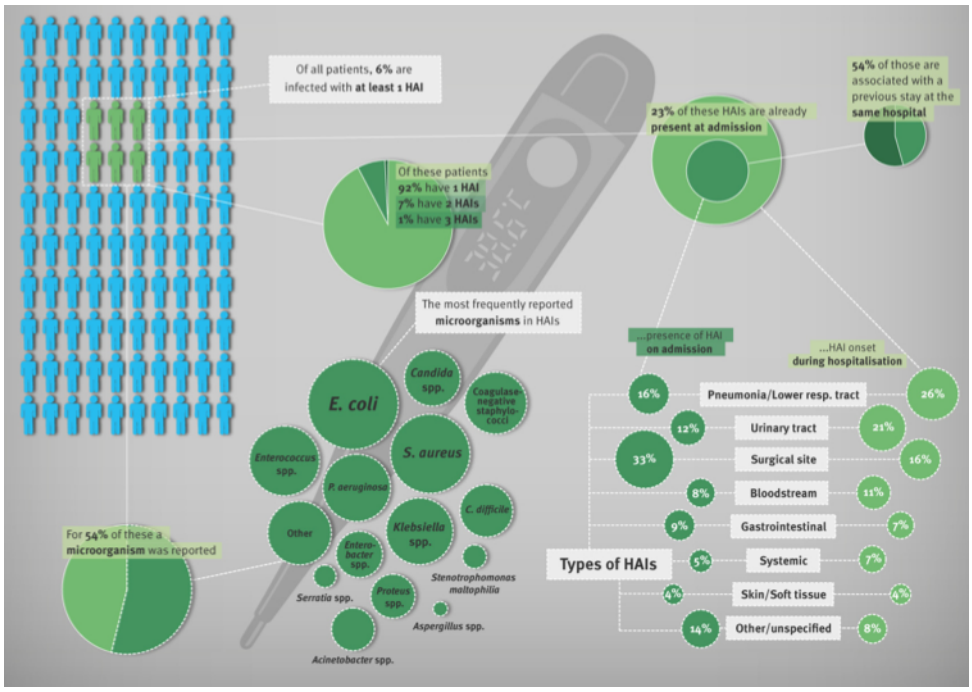

Figure 1. Healthcare-associated infections in European hospitals.
Source: https://www.ecdc.europa.eu/en/publications-data/healthcare-associated-infections-european-hospitals. Las access 30 October 2020.

1.3 Hospital-acquired infections in the Neonatal Intensive Care Unit (NICU)

Due to their characteristics preterm infants hospitalized in Neonatal Intensive Care Units (NICUs) are a group at high risk of contracting HAIs and they exhibit the highest sepsis-related morbidity and mortality among paediatric patients (Martinot et al., 1997).

The immune system of newborns is still developing, so they have inefficient mucosal and cutaneous barriers, and are exposed to variety of therapeutic interventions, such as the use of invasive devices and broad-spectrum antimicrobials. The birth weight is also linked to the severity of the pathologies, and is inversely proportional to the risk of contracting infections (Saiman, 2002).

The overlap of different conditions with neonatal sepsis and the difficulty in laboratory diagnosis translate into a tendency to over treatment of neonatal sepsis. Antibiotics are the most commonly used drugs in the NICUs (Hsieh et al., 2014), preterm infants with very low

birth weight (VLBW) are often treated empirically with antibiotics for prolonged periods, even in the absence of a confirmed infection, (Nash et al., 2002; Clark et al., 2006b; Hsieh et al., 2014; Blaser, 2016). Antibiotic exposures after birth are associated with multiple subsequent poor outcomes, prolonged hospitalization, and multidrug-resistant bacterial infections, making the risk/benefit balance of these approaches uncertain (Maragakis et al., 2008, de Man et al., 2000; Cotten et al., 2006; Alexander et al., 2011; Kuppala et al., 2011; Hoskin-Parr et al., 2013; Bailey et al., 2014, 7; Cantey et al., 2015), furthermore the association between inadequate use of antibiotics and increase HAIs caused by AMR bacteria in neonatal units have been reported.(Silva et al., 2018).

Late Onset Sepsis (LOS), defined as a bloodstream infection occurring in neonates at or after 72 hours of age, is considered one of the main HAIs among preterm infants, with high levels of morbidity and mortality (Greenberg et al., 2017) (el Manouni el Hassani et al., 2019), and serious sequelae such as bronchopulmonary dysplasia necrotizing enterocolitis, and neurodevelopmental impairment (Tsai et al., 2014). Gram-positive organisms are the predominant cause of LOS in the NICUs (48% to 70% of cases), however also gram-negative organisms and fungi can be important causative agents (Sass and Karlowicz, 2018).

A large proportion of all LOS is represented by central line-associated bloodstream infections (CLABSIs) (Cantey and Milstone, 2015). Coagulase-negative *staphylococci* (CONS) and *E. coli* are the microorganisms most commonly causative of CLABSI among VLBW (Bizzarro et al., 2005). Also fungi are involved, *Candida* spp. has been noting with increasing frequency and is the third most common causative agent of LOS in VLBW infants (Tan et al., 2014) with a higher incidence in NICU wards than in other paediatric or adult populations (Kung et al., 2016). Healthcare-associated pneumonia is the second most common of nosocomial infections affecting NICU patients, and one of the most difficult to diagnose (Sass and Karlowicz, 2018).The risk of those pathologies increases with the low

weight of the newborn, the gestational age and the prolonging of the medical practice (Kawanishi et al., 2014)(Tan et al., 2014) (Pople et al., 1992).

1.4 Routes of transmission

Infections can be spread to a susceptible host through various routes (**Figure 2** (Otter et al., 2011b)): through contact, which can be direct or indirect, through droplets, or via airborne transmission. (Siegel et al., 2007a) Direct contact occurs when the transfer of bacteria is between an infected subject and another in the absence of an intermediary, be it a person or an object. Indirect contact, on the other hand, occurs in the presence of an intermediary, this makes it difficult to establish the exact circumstance in which the contact took place, however it has been seen that the contaminated hands of the health personnel are important contributors to transmission.

Transmission via droplets larger than 5 μm takes place when these are translocated directly from the respiratory tract of an infected patient to the mucosal surfaces of the recipient, transmission via droplets can also occur indirectly for example when, once generated, they can disseminate microorganisms in the environment. Finally is defined airborne transmission when airborne droplet nuclei or small particles in the respirable size range containing infectious agents remain infective over time and distance (Siegel et al., 2007a).

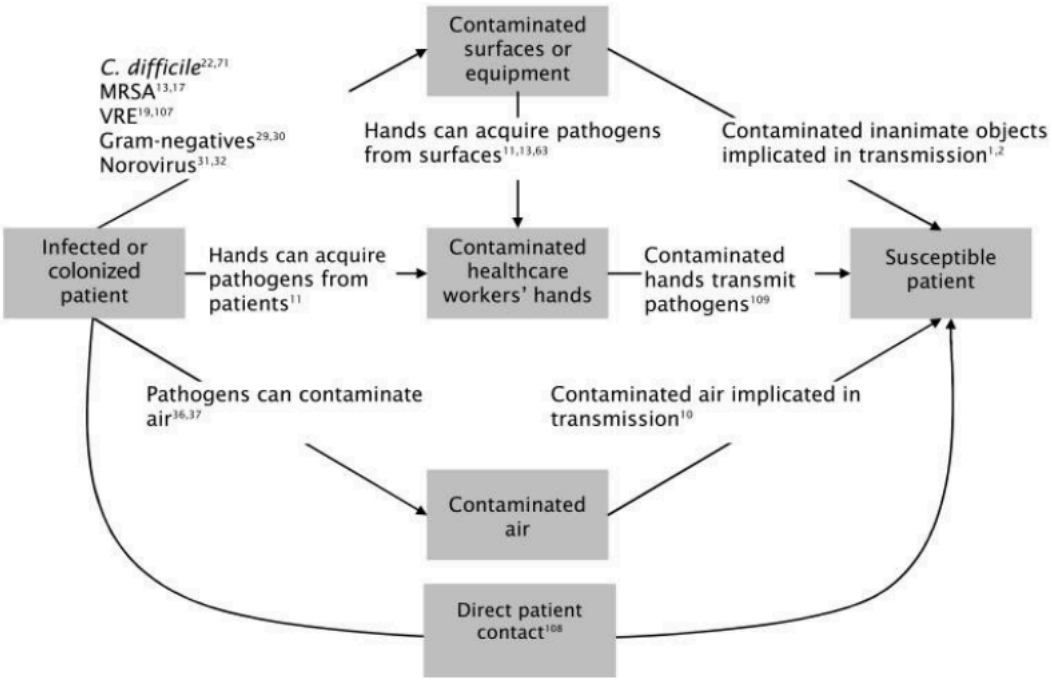

Figure 2. Generic transmission routes. MRSA, methicillin-resistant Staphylococcus aureus; VRE, vancomycin-resistant enterococci (Otter et al., 2011a).

1.5 The environment as a source of transmission of pathogens

The role of the environment in the transmission of HAIs has long been the subject of debate (Dancer, 2009a), to date is recognized that the environment has a weight in the transmission of pathogens not only bacteria but also fungi and viruses (Dancer, 2014).

Microorganisms coming from patients, but also from asymptomatic healthcare workers, as well as from visitors, colonize hospital surfaces thus constituting a possible source of infection (Riggs et al., 2007). The risk for a patient of acquiring microorganisms, even resistant strains, has been seen to increase if hospitalized in a room previously occupied by an infected patient (Huang et al., 2006; Drees et al., 2008; Datta et al., 2011). MRSA for instance is shed from patients into the near patient-environment and is acquired on gloved hands of healthcare workers even without a direct contact with the colonized patient, (Boyce

et al., 1997) The same has been observed for other pathogens such as VRE (Huang et al., 2006), *C. difficile* (Samore et al., 1996) and *Acinetobacter* (Getchell-White et al., 1989). Microorganisms can also persist on inanimate surfaces for a long time, resisting even desiccation, and therefore increasing the risk of colonization for the patient (Kramer et al., 2006). The survival times on surfaces observed for the main pathogens responsible for HAIs are reported in **Table 1** (Dancer, 2014). The surfaces most touched by the hands are the most contaminated and their contact by health workers constitutes the main route of transmission of pathogens from environment to patient, in addition to the direct contact of the patient himself with the colonized surface (Otter et al., 2011b).

Organism	Survival time	Infectious dose
Methicillin-resistant *Staphylococcus aureus*	7 days–>7 mo	4 CFU
Acinetobacter	3 days–>5 mo	250 CFU
Clostridium difficile	>5 mo	5 spores
Vancomycin-resistant *Enterococcus*	5 days–>4 mo	$<10^3$ CFU
Escherichia coli	2 h–16 mo	10^2-10^5 CFU
Klebsiella	2 h–>30 mo	10^2 CFU
Norovirus	8 h–7 days	<20 virions

Table 1. Survival times and infectious doses retrieved or extrapolated from published studies (Dancer, 2014)

The abatement of the microbial load consisting of pathogens from hospital surfaces, accompanied by specific campaigns to raise awareness for the importance of hand hygiene, was seen to be associated with a reduction in the acquisition of HAIs (Rampling et al., 2001; Martínez et al., 2003; Caselli et al., 2018). It therefore appears clear that it is not sufficient

that the surfaces are simply clean, but that there must be sanitization processes, deeply monitored. Visual assessment is insufficient for defining cleanliness, nor will it accurately predict the infection risk for patients (Dancer, 2009b).

To date, in hospitals and healthcare facilities, surface disinfection takes place mainly with chemical detergents, however it has been seen that they do not always eradicate pathogens efficiently. *C. difficile* spores, VRE, MRSA, and *Acinetobacter* species are detected on hospital surfaces even after disinfection with bleach, and also following intensification of cleaning interventions resulting from the reporting of outbreaks (Otter et al., 2011b).

Chemical disinfectants have an immediate action but are unable to prevent recontamination phenomena responsible for the persistence of pathogens on surfaces (Rutala and Weber, 2014). Furthermore it has been seen that chemical disinfectants can favour the selection of resistant strains not only towards the disinfectants themselves but also towards antibiotics (Webber et al., 2015; Wand et al., 2016). Nonetheless, chemical detergents have high environmental impact and may damage equipment, especially metals.

Microbial monitoring of the inanimate environment is considered a tool for prevention and control of infections, it can be used to monitor hygiene standards and also to examine for the presence of nosocomial pathogens which may be the source of an outbreak (Galvin et al., 2012). The monitoring of hospital surfaces usually take place with use of adenosine triphosphate (ATP) bioluminescence assay, designed to detect the presence of organic material on surfaces. Despite advantages such as speed and reduced cost, this type of detection however shows important limitations: first of all, it does not distinguish the type of material detected, it fails to detect very low bacterial loads and can give results distorted by the presence of chemical residues on surfaces. This type of monitoring is therefore ineffective for a thorough characterization of the microbial composition of the hospital surfaces that is required to prevent the onset of HAIs (Nante et al., 2017).

Other techniques usually employed for the microbiological monitoring of surfaces are the culture-based methods (Dancer, 2014; D'Accolti et al., 2019). Compared to ATP bioluminescence assays they allow to detect the presence of viable bacteria or fungi and their load. However, this method also has limitations due to the need to keep microorganisms in culture, often with long incubation times, using specific selective media that limit research to known and cultivable organisms. (D'Accolti et al., 2019).

Advances in molecular biology resulted in the development of tools for a rapid identification and enumeration of microorganisms in samples such as qPCR, overcoming the limitations encountered with traditional methods (Galvin et al., 2012; D'Accolti et al., 2019). The molecular methods are in fact rapid, highly specific and sensitive, and allow the identification of microorganisms even if they cannot be cultivated, however only the advent of modern technologies based on DNA sequencing has allowed the identification of bacteria without deciding a priori which microorganisms to research, therefore improving the microbiome investigation and allowing definition of complex populations in deep detail (Comar et al., 2019).

1.6 Innovative strategies for surface sanitation: The Probiotic Cleaning Hygiene System (PCHS)

Recently, the approach towards the hospital environment has become more and more similar to that towards the human body, where often a balance of microbial flora, composed of non-pathogenic microorganisms, can be effective in preventing infections. (Al-Ghalith and Knights, 2015; Pettigrew et al., 2016). Considering this aspect, an innovative strategy has been developed to counter the spread of HAIs. It is the Probiotic Cleaning Hygiene System (PCHS) (Copma, Ferrara, Italy), an alternative detergent to conventional chemical cleaning with low environmental impact, for the sanitization of hospital surfaces, containing probiotic

microorganisms. This detergent contains the species of *Lactobacilli B. subtilis, B. pumilus, B. megaterium* in vegetative and spore form, which, acting through the competitive exclusion mechanism, are able to stably and for long time break down the bacterial load represented by pathogenic microorganisms from the surfaces. (Caselli et al., 2016b, 2018, 2019). It has also been shown that the *Lactobacilli* present in the detergent do not colonize the patient and do not acquire resistance genes (Caselli et al., 2016a). A multicentre study performed in 6 Italian hospitals has shown how, following the introduction of the PCHS system, there has been a significant reduction in HAI, resulting from the remodelling of the microbiome of hospital surfaces **Figure 3**. In fact, a significant abatement of pathogenic microorganisms was observed such as: *Staphylococcus* spp*., Enterobacteriaceae* spp*., Acinetobacter* spp*., Candida* spp*., Pseudomonas* spp*., Clostridium* spp. (Caselli et al., 2018) Also with regard to the presence of resistance genes on surfaces, a clear decrease was found in the structures in which the PCHS was introduced.(Caselli et al., 2016b). Finally, also considering the economic aspect, the PCHS has led to numerous advantages, mainly due to the reduction of costs resulting from the treatment of HAIs (Tarricone et al., 2020).

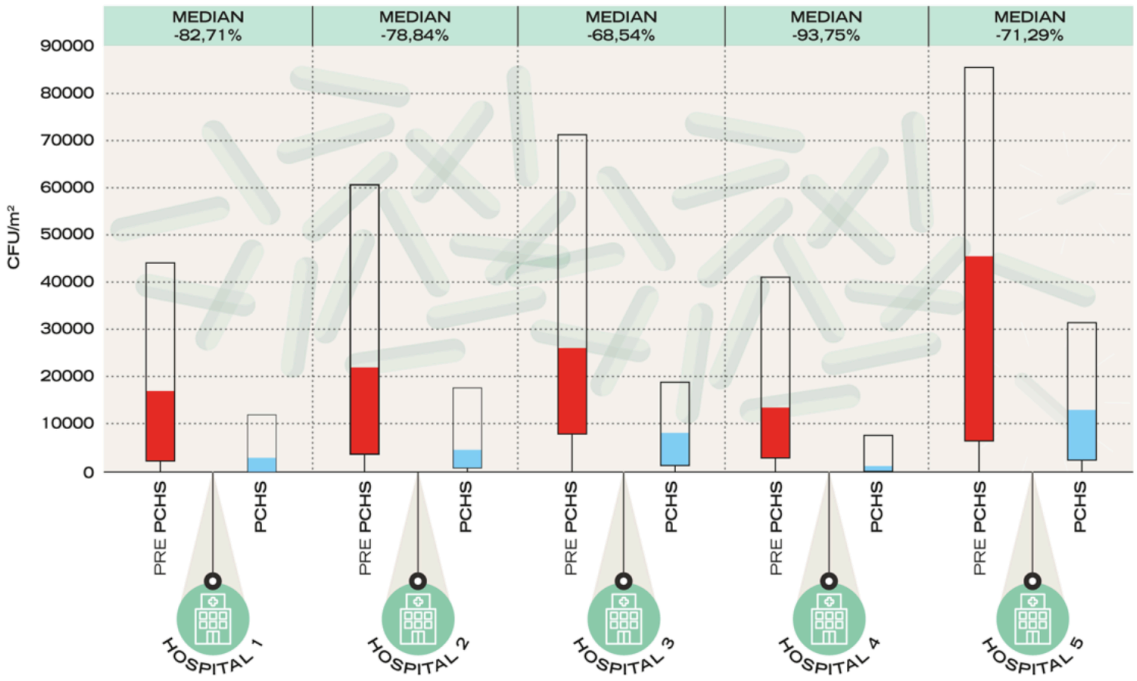

Figure 3. Surface Contamination. Pathogenic load on hospital surfaces, expressed in CFU/m2: *Staphylococcal* spp., *Enterobacteriaceae* spp., *Acinetobacter* spp., *Candida* spp., *Pseudomonas* spp., *Clostridium* spp. Results expressed as percentage reduction (%) in the PCHS® phase compared to pre-PCHS® phase (PCHS® vs pre-PCHS®). Image from: https://pchs.it/en/our-researches/system-reliability/ Las access 30 October 2020.

2 AIM

Healthcare-associated infections (HAI) are a public health problem due to their high morbidity and mortality rates and subsequent economic consequences (Allegranzi et al., 2011; Weber et al., 2013). Hospital surfaces are frequently contaminated with important healthcare-associated pathogens, playing a pivotal role in the transmission of infections in nosocomial settings (Weber et al., 2013), some microorganisms can persist on dry inanimate surfaces even for more than a week, and some spores can survive for months (Kramer et al., 2006). To date, the characterization of the microbiome of hospital surfaces usually takes place with low specificity and sensitivity culture methods, often with long response times (Dancer, 2009a). Considering this, the primary objective of this study is to introduce new highly sensitive molecular methods, for the characterization of the environmental microbiome, evaluating their characteristics, in comparison with the traditional cultural and molecular methods usually employed.

We intend to introduce and evaluate the use of these innovative methods for monitoring the environmental microbiological composition in terms of presence of pathogens, bacterial load and presence and quantification of antimicrobial resistance genes, since that the emergence and spread of multi-drug resistant organisms threatens healthcare systems worldwide, making HAIs an increasingly difficult problem to manage (Caini et al., 2013; Cornejo-Juárez et al., 2015).

The secondary objective is to use the methods developed to evaluate if environmental microbiome composition may have a role in preterm infant's bacterial colonization, representing a group at high risk for contracting HAIs (Plano, 2010; Resende et al., 2015).

The tertiary objective is to introduce the molecular methods as part of the hospital infection prevention and control system in order to activate an active surveillance system that allows

to have a complete picture of the microbiological composition of the hospital wards, in order to be able to act promptly, possibly improving disinfection strategies where is needed, and being able to provide a rapid response even in the event of an outbreak.

Finally, one of the purposes is to evaluate the introduction of new strategies for the sanitization of surfaces, using a system that, thanks to the principle of competitive exclusion, is able to favour the colonization of surfaces by non-pathogenic microorganisms instead of those potentially dangerous and to minimize patient colonization.

3 METHODS

3.1 Introduction of new generation technologies for the characterization of environmental microbiome.

3.1.1 Study design

The initial step was to analyse the environmental microbiome of hospital surfaces of selected wards of the Institute for Maternal and Child Health "IRCCS Burlo Garofolo" (Trieste, Italy), using new generation molecular methods, comparing them with standard molecular and cultural techniques regularly employed for environmental monitoring of hospital surfaces.

A total of 108 environmental critical points were considered from 8 wards of the hospital: Paediatric Clinic (PC), Paediatric Surgery (PS), Paediatric Oncology (PO), Neonatal Intensive and Sub-Intensive Care Unit (NICU and NICUs), children's Intensive Care Unit (ICU), Surgical Rooms (SR: Orthopaedics, Gynaecology and Oculistics) and Delivery Room (DR).

Different hard surfaces were sampled for each wards, considering their characteristics and basing on previous protocols (Vandini et al., 2014; Caselli et al., 2016b): floor; bed footboard, operating bed or incubator for inpatients wards, ICU, SR and NICU, respectively; sink or operating table for inpatients rooms or SR/DR, respectively, floor for the all ward types. For each department, up to three rooms were considered, where possible with adjacent bathroom, chosen at random, in each of the two sampling campaigns carried out.

Samples were taken seven hours after the last cleaning intervention, time representative of the level of contamination in a 24-hour period, as shown by previous studies (Vandini et al., 2014; Caselli et al., 2016b, 2018).

At the same time the characterization of the microbiome was performed with Ion Torrent next generation high-throughput sequencing of the 16s rRNA in comparison with cultural microbiological contact plates, a custom molecular qPCR panel kit and a Microbial DNA qPCR Array for Antibiotic Resistance Genes for the characterization of the resistome on surfaces. Depending on the type of analysis, molecular or cultural, the same points were collected following two different methodologies. The study was conducted after obtaining approval from the Institutional Scientific Board of the Institute for Maternal and Child Health "IRCCS Burlo Garofolo" (Trieste, Italy), in collaboration with the University of Ferrara.

3.1.2 Cultural analyses

For microbiological analyses, 108 critical points were collected in duplicate (216 total samples) by Replicate Organism Detection and Counting (RODAC) contact plates (**Figure 4**). The following microorganisms, with specific culture media, were considered:

- Total bacteria (TSA medium, Merck Millipore, Milan, Italy)
- *Staphylococcus* spp. (Baird Parker medium, Merck Millipore, Milan, Italy)
- *Enterobacteriaceae* spp. (MacConkey medium, Merck Millipore, Milan, Italy)
- *Acinetobacter* spp. (Herella medium, Lickson, Milan, Italy)
- *Pseudomonas* spp. (Cetrimide medium, Incofar, Modena, Italy)
- *Clostridium difficile* (Clostridium difficile selective medium, Lickson, Milan, Italy)
- *Enterococcus* spp. (BEA medium, Incofar, Modena, Italy)
- Mycetes (Sabouraud medium, Merck Millipore, Milan, Italy).

Plates for general or selective growth of bacteria were incubated at 30°C for 24–48 h (respectively for general and selective media), whereas plates for the specific growth of mycetes were incubated at 25°C for 72 h. Colony forming units (CFU) were enumerated. Plates containing ≥ 200 CFUs were considered to have 200 CFUs, following the guideline INAIL-2017 (Giovinazzo, 2017).

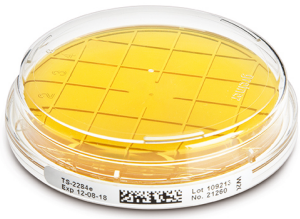

Figure 4: RODAC Plates (Merck Millipore, Milan, Italy), image from: https://www.sigmaaldrich.com/technical-documents/articles/microbiology/contact-plates-and-swabs.html Last access: 30 October 2020.

3.1.3 Molecular analyses

For molecular analyses, 324 total environmental samples representing total microbial population were collected by sterile rayon swabs rubbed in four different directions in an area delimited by a sterile 10x10 cm disposable plastic template (Copan, Brescia, Italy) corresponding to 100 cm^2 (**Figure 5**), as previously described (Caselli et al., 2016b).

Swabs were pre-moistened in sterile Tryptic Soy Broth (TSB) for subsequent qPCR Array for the Antibiotic Resistance Genes, or in duplicate, in saline solution, for the downstream Real Time and NGS analyses. After rubbing, swabs were put in 5 mL TSB or 0.4 mL saline respectively, immediately refrigerated and transported to the laboratory.

Figure 5: 10x10 cm disposable plastic template (Copan, Brescia, Italy), image from: https://www.copanusa.com/wp-content/uploads/2019/08/SRK_4page-brochure-web.pdf Last access: 30 October 2020.

3.1.4 qPCR Array for Antibiotic Resistance Genes

Samples collected in TSB were incubated at 37°C for 24 h for a controlled bacterial amplification; afterwards microbial cells were pelletized (14,000 × g for 5 min) and stored until use at −20°C as already described (Caselli et al., 2016b). Microbial DNA was extracted from microbial pellets by the QIAmp UCP Pathogen Mini Kit (Qiagen, Hilden, Germany), and analysed by the Microbial DNA qPCR Array for Antibiotic Resistance Genes (Antibiotic Resistance Genes, catalog no. BAID-1901ZRA; Qiagen), using 1 µg of extracted DNA per plate (10 ng/well/reaction). as previously described (Vandini et al., 2014; Caselli et al., 2016b).

This method allows the identification and relative profiling of 84 genes coding for antimicrobial resistance, belonging to aminoglycoside, β-lactam, erythromycin, fluoroquinolone, macrolide-lincosamide-streptogramin B, tetracycline, vancomycin, and

multidrug resistance classifications, as listed in (**Table 2**), alongside the bacterial specie *Staphylococcus aureus*, the positive controls Pan Bacteria 1, 3, the Positive PCR Control PPC, and negative controls.

Position	Species (NCBI Tax ID)/Gene	NCBI Tax ID	Antibiotic classification / Gene Description	May detect (species) / Also detect (antibiotic resistance genes) / Associated species (virulence factor genes)	Sensitivity	Assay Catalog #
A01	AAC(6)-Ib-cr		Fluoroquinolone resistance		100	BPAR00366A
A02	aacC1		Aminoglycoside-resistance		50	BPAR00367A
A03	aacC2		Aminoglycoside-resistance		30	BPAR00368A
A04	aacC4		Aminoglycoside-resistance		20	BPAR00369A
A05	aadA1		Aminoglycoside-resistance		200	BPAR00370A
A06	aphA6		Aminoglycoside-resistance		40	BPAR00373A
A07	BES-1		Class A beta-lactamase		20	BPAR00375A
A08	BIC-1		Class A beta-lactamase		100	BPAR00376A
A09	CTX-M-1 Group		Class A beta-lactamase	Detects CTX-M-1 type (37 variants)	50	BPAR00377A
A10	CTX-M-8 Group		Class A beta-lactamase	Detects CTX-M-8 type (3 variants)	40	BPAR00378A
A11	CTX-M-9 Group		Class A beta-lactamase	Detects CTX-M-9 type (40 variants)	30	BPAR00379A
A12	GES		Class A beta-lactamase	GES,IBC	20	BPAR00380A
B01	IMI & NMC-A		Class A beta-lactamase	NMC-A,IMI-2,IMI-3	30	BPAR00381A
B02	KPC		Class A beta-lactamase	KPC-1,KPC-2,KPC-3,KPC-4, KPC-5,KPC-6,KPC-7,KPC-8, KPC-9,KPC-10,KPC-11	40	BPAR00382A
B03	Per-1 group		Class A beta-lactamase	Per-1,Per-3,Per-4,Per-5	30	BPAR00383A
B04	Per-2 group		Class A beta-lactamase	Per-2,Per-6	50	BPAR00384A
B05	SFC-1		Class A beta-lactamase		50	BPAR00385A
B06	SFO-1		Class A beta-lactamase		20	BPAR00386A
B07	SHV		Class A beta-lactamase		200	BPAR00387A
B08	SHV(156D)		Class A beta-lactamase		100	BPAR00388A
B09	SHV(156G)		Class A beta-lactamase		50	BPAR00389A
B10	SHV(238G240E)		Class A beta-lactamase		30	BPAR00390A
B11	SHV(238G240K)		Class A beta-lactamase		40	BPAR00391A
B12	SHV(238S240E)		Class A beta-lactamase		50	BPAR00392A
C01	SHV(238S240K)		Class A beta-lactamase		30	BPAR00393A
C02	SME		Class A beta-lactamase	SME-1,SME-2,SME-3	30	BPAR00394A
C03	TLA-1		Class A beta-lactamase		50	BPAR00395A
C04	VEB		Class A beta-lactamase	VEB-1,VEB-2,VEB-3,VEB-4, VEB-5,VEB-6,VEB-7	20	BPAR00396A
C05	ccrA		Class B beta-lactamase		30	BPAR00397A
C06	IMP-1 group		Class B beta-lactamase	IMP-1,IMP-3,IMP-4,IMP-6, IMP-10,IMP-25,IMP-26	50	BPAR00398A
C07	IMP-12 group		Class B beta-lactamase	IMP-12,IMP-14,IMP-16, IMP-18	200	BPAR00399A
C08	IMP-2 group		Class B beta-lactamase	IMP-2,IMP-8,IMP-11,IMP-19, IMP-20,IMP-21,IMP-24	30	BPAR00400A
C09	IMP-5 group		Class B beta-lactamase	IMP-5,IMP-7,IMP-9,IMP-13, IMP-15,IMP-22	200	BPAR00401A
			Class B			

Position	Species (NCBI Tax ID)/Gene	NCBI Tax ID	Antibiotic classification / Gene Description	May detect (species) / Also detect (antibiotic resistance genes) / Associated species (virulence factor genes)	Sensitivity	Assay Catalog #
C10	NDM		beta-lactamase	NDM-1,NDM-2	50	BPAR00402A
C11	VIM-1 group		Class B beta-lactamase	VIM-1,VIM-2,VIM-3,VIM-4, VIM-5,VIM-6,VIM-8,VIM-9, VIM-10,VIM-11,VIM-12, VIM-14,VIM-15,VIM-16, VIM-17,VIM-18,VIM-19, VIM-20,VIM-23,VIM-24, VIM-25,VIM-26	50	BPAR00403A
C12	VIM-13		Class B beta-lactamase	VIM-13 (28 variants)	20	BPAR00404A
D01	VIM-7		Class B beta-lactamase		40	BPAR00405A
D02	ACC-1 group		Class C beta-lactamase	ACC-1,ACC-2,ACC-4	100	BPAR00406A
D03	ACC-3		Class C beta-lactamase		30	BPAR00407A
D04	ACT 5/7 group		Class C beta-lactamase	ACT-5,ACT-7	30	BPAR00408A
D05	ACT-1 group		Class C beta-lactamase	ACT-1,ACT-2,ACT-3,ACT-4, ACT-6	100	BPAR00409A
D06	CFE-1		Class C beta-lactamase		50	BPAR00410A
D07	CMY-10 Group		Class C beta-lactamase	CMY-1,CMY-8,CMY-9, CMY-10,CMY-19	30	BPAR00411A
D08	DHA		Class C beta-lactamase	DHA-1,DHA-2,DHA-3, DHA-5,DHA-6,DHA-7	20	BPAR00412A
D09	FOX		Class C beta-lactamase	FOX-1,FOX-2,FOX-3,FOX-4, FOX-5,FOX-6,FOX-7	100	BPAR00413A
D10	LAT		Class C beta-lactamase	LAT-1,LAT-3,LAT-4,CMY-2 group	100	BPAR00414A
D11	MIR		Class C beta-lactamase	MIR-1,MIR-2,MIR-3,MIR-4, MIR-5	30	BPAR00415A
D12	MOX		Class C beta-lactamase	MOX-1,MOX-2,MOX-3, MOX-4,MOX-5,MOX-6, MOX-7	30	BPAR00416A
E01	OXA-10 Group		Class D beta-lactamase	OXA-10,OXA-11,OXA-14, OXA-16,OXA-17,OXA-19, OXA-28,OXA-35,OXA-142, OXA-145,OXA-147	20	BPAR00417A
E02	OXA-18		Class D beta-lactamase		30	BPAR00418A
E03	OXA-2 Group		Class D beta-lactamase	OXA-2,OXA-15,OXA-32, OXA-34,OXA-141,OXA-161	40	BPAR00419A
E04	OXA-23 Group		Class D beta-lactamase	OXA-23,OXA-27,OXA-49, OXA-73,OXA-133,OXA-146, OXA-165,OXA-166, OXA-167,OXA-168, OXA-169,OXA-170,OXA-171	50	BPAR00420A
E05	OXA-24 Group		Class D beta-lactamase	OXA-24,OXA-25,OXA-26, OXA-40,OXA-72,OXA-139, OXA-160	20	BPAR00421A
E06	OXA-45		Class D beta-lactamase		100	BPAR00422A
E07	OXA-48 Group		Class D beta-lactamase	OXA-48,OXA-162,OXA-163, OXA-181	50	BPAR00423A
E08	OXA-50 Group		Class D beta-lactamase	OXA-50 group (50 variants)	20	BPAR00424A
E09	OXA-51 Group		Class D beta-lactamase	OXA-51 group (65 variants)	100	BPAR00425A
E10	OXA-54		Class D beta-lactamase		20	BPAR00426A
E11	OXA-55		Class D beta-lactamase	OXA-55,OXA-SH	30	BPAR00427A
E12	OXA-58 Group		Class D beta-lactamase	OXA-58,OXA-96,OXA-97, OXA-164	20	BPAR00428A
F01	OXA-60		Class D beta-lactamase	OXA-60,OXA-60a,OXA-60b, OXA-60c	30	BPAR00429A
F02	ereB		Erythromycin resistance		20	BPAR00431A
			Fluoroquinolone			

Position	Species (NCBI Tax ID)/Gene	NCBI Tax ID	Antibiotic classification / Gene Description	May detect (species) / Also detect (antibiotic resistance genes) / Associated species (virulence factor genes)	Sensitivity	Assay Catalog #
F03	QepA		resistance	QepA1,QepA2	50	BPAR00432A
F04	QnrA		Fluoroquinolone resistance	QnrA1,QnrA2,QnrA3,QnrA4, QnrA5,QnrA6,QnrA7	40	BPAR00433A
F05	QnrB-1 group		Fluoroquinolone resistance	QnrB1,QnrB2,QnrB3,QnrB6, QnrB7,QnrB9,QnrB13, QnrB14,QnrB15,QnrB16, QnrB17,QnrB18,QnrB20, QnrB23,QnrB24,QnrB29, QnrB30	20	BPAR00434A
F06	QnrB-31 group		Fluoroquinolone resistance	QnrB31,QnrB32	20	BPAR00435A
F07	QnrB-4 group		Fluoroquinolone resistance	QnrB4,QnrB11,QnrB12, QnrB22	30	BPAR00436A
F08	QnrB-5 group		Fluoroquinolone resistance	QnrB5,QnrB10,QnrB19	40	BPAR00437A
F09	QnrB-8 group		Fluoroquinolone resistance	QnrB8,QnrB21,QnrB25, QnrB27,QnrB28	20	BPAR00438A
F10	QnrC		Fluoroquinolone resistance		30	BPAR00439A
F11	QnrD		Fluoroquinolone resistance		40	BPAR00440A
F12	QnrS		Fluoroquinolone resistance	QnrS1,QnrS2,QnrS3,QnrS4	40	BPAR00441A
G01	ermA		Macrolide Lincosamide Streptogramin_b		100	BPAR00442A
G02	ermB		Macrolide Lincosamide Streptogramin_b		20	BPAR00443A
G03	ermC		Macrolide Lincosamide Streptogramin_b		100	BPAR00444A
G04	mefA		Macrolide Lincosamide Streptogramin_b		100	BPAR00445A
G05	msrA		Macrolide Lincosamide Streptogramin_b		100	BPAR00446A
G06	oprj		Multidrug resistance efflux pump		50	BPAR00447A
G07	oprm		Multidrug resistance efflux pump		20	BPAR00448A
G08	tetA		Tetracycline efflux pump		40	BPAR00449A
G09	tetB		Tetracycline efflux pump		30	BPAR00450A
G10	vanB		Vancomycin resistance		100	BPAR00451A
G11	vanC		Vancomycin resistance		30	BPAR00452A
G12	Staphylococcus aureus	1280		Staphylococcus epidermidis(1282)	100	BPID00314A
H01	mecA		Beta-lactam resistance		40	BPAR00374A
H02	lukF		Panton-Valentine leukocidin chain F precursor	Staphylococcus aureus	20	BPVF00517A
H03	spa		Immunoglobulin G binding protein A precursor	Staphylococcus aureus	200	BPVF00518A
H04	Pan Bacteria 1					BPCL00360A
H05	Pan Bacteria 1					BPCL00360A
H06	Pan Bacteria 1					BPCL00360A
H07	Pan Bacteria 3					BPCL00362A
H08	Pan Bacteria 3					BPCL00362A
H09	Pan Bacteria 3					BPCL00362A
H10	PPC					BPCL00365A
H11	PPC					BPCL00365A
H12	PPC					BPCL00365A

Table 2. List of the resistance genes detected by DNA qPCR Array for Antibiotic Resistance Genes (Antibiotic Resistance Genes, catalog no. BAID-1901ZRA; Qiagen) Source: https://www.qiagen.com/it/products/discovery-and-translational-research/pcr-qpcr-dpcr/qpcr-assays-and-instruments/microbial-dna-qpcr-assays-and-panels/microbial-dna-qpcr-arrays/?clear=true#orderinginformation. Last access: 30 October 2020.

3.1.5 qPCR microarray

Samples collected in saline solution were immediately frozen at −80 °C. After being thawed, samples were vortexed to detached microbes from the swabs and total DNA was extracted from 300 µL of each sample in a final elution volume of 100 µL, by the commercial kit Exgene Cell SV Kit (Gene All, Tema Ricerca, Bologna, Italy), following the manufacturer's instructions.

Samples were analysed by a customized qPCR microarray (BAID-00047RA Qiagen, Hilden, Germany), using 120 ng of DNA per sample (corresponding to 7 ng of template DNA per well/reaction).

The presence of the following microbes was assessed simultaneously: *S. aureus, S. epidermidis, E. faecalis, E. faecium, E. coli, K. pneumonia/Enterobacter, A. baumannii, P. mirabilis, P. aeruginosa, C. perfringens, C. difficile, A, fumigatus and C. albicans*, as previously reported (D'accolti et al., 2019)

3.1.6 NGS analyses

For NGS analyses, samples collected in saline solution were immediately frozen at −80 °C. After being thawed, samples were vortexed to detached microbes from the swabs, then total DNA was extracted from 300 µL of each sample in a final elution volume of 50 µL by the automatic extractor Maxwell CSC DNA Blood Kit (Promega, Madison, WI, USA), according to manufacturer's instruction.

The characterization of the bacterial composition of the samples was performed by sequencing the region V3 of the 16S rRNA gene. The steps leading up to the sequencing are summarized in **Figure 6**.

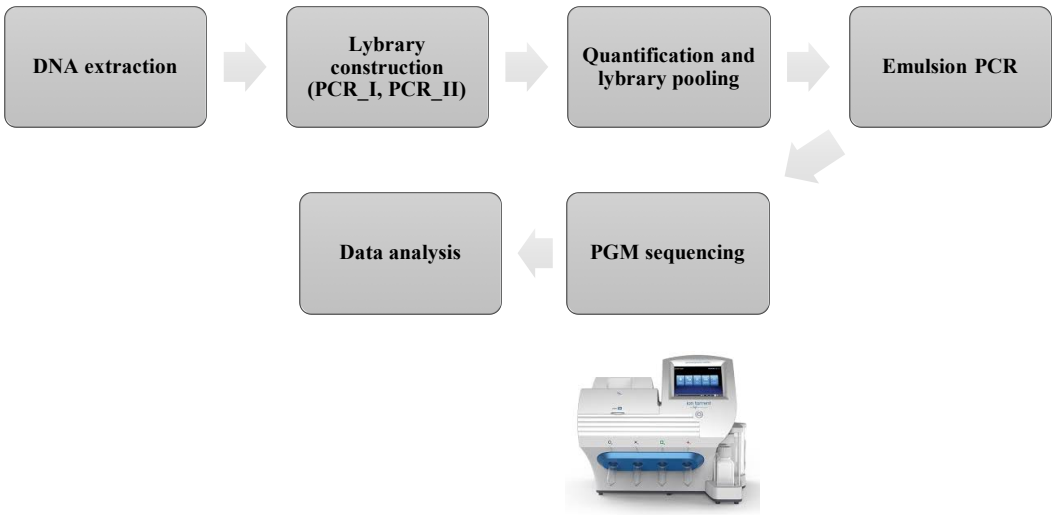

Figure 6. Schematic representation of the NGS analyses.

A qPCR targeting the V1–V3 region 16S rRNA gene (500 bp) was performed by employing the U534R primer and the degenerated primer 27FYM (PCR_I). A nested PCR was subsequently carried out with the primers B338F_P1-adaptor and U534R_A-adaptor_barcode, targeting the V3 region (200 bp) of the 16S rRNA gene (PCR_II), with a different barcode for each sample linked to the reverse primer (Sundquist et al., 2007) . The PCR reactions were performed using EvaGreen® dye (Fisher Molecular Biology, Waltham, MA, USA), the Kapa 2G HiFi Hotstart ready mix 2X (Kapa Biosystems, Wilmington, MA, USA), 0.5 µM of each primer and 400 ng/µL of Bovine Serum Albumin (BSA), in a final volume of 10 µL. Primers sequences and thermal conditions are reported in **Table 3 and 4**.

Negative controls including no template and no bacterial DNA were processed with clinical samples, starting from the pre-analytic phase of samples manipulation. A total absence of amplification signal at the end of PCR runs (I and II step of PCR) was successfully obtained.

The correct size of the amplicons (560 bp for PCR_I and 260 bp for the PCR_II) was assessed on a 2% agarose gel. The amount of dsDNA of each sample after PCR_II was quantified with a Qubit® 2.0 Fluorimeter (Invitrogen, Carlsbad, California, USA) using the Qubit® dsDNA BR Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA), and an equal amount of each sample (100 ng) was mixed into a single batch to generate a pooled library at a final concentration of 100 pM, according to manufacturer's instructions.

Template preparation was performed by emulsion PCR using The Ion OneTouch™ 2 System (Life Technologies, Gran Island, New York, USA), with the Ion PGM Hi-Q View OT2 200 kit (Life Technologies, New York, USA) and a subsequent quality control was carried out on Qubit® 2.0 Fluorimeter. Sequencing was performed with the Ion PGM™ System technology by using the Ion PGM Hi-Q View sequencing kit (Life Technologies, New York, USA). The high-throughput sequencing data were processed using the Quantitative Insights Into Microbial Ecology (QIIME 1.9.1.) software (Caporaso et al., 2010), (available at: https://www.nature.com/articles/nmeth.f.303 Last access 30 0ctober 2020). High quality sequences (Q > 25) were demultiplexed and filtered by quality using split_libraries_fastq.py with default parameters, retaining sequences with a minimum length of 150 bp. Sequences with homopolymer length >8 or ambiguous bases were removed. Operational Taxonomic Units (OTUs) were picked at 97% similarity and clustered against the reference taxonomy database SILVA V.132 (Quast et al., 2013).

Primers	**Sequence**
U534R	5′-ATTACCGCGGCTGCTGG-3′
27FYM	5′-AGRGTTYGATYMTGGCTCAG-3′
B338F_P1-adaptor	5′-ACTCCTACGGGAGGCAGC-3′
U534R_A_barcode	5′-ATTACCGCGGCTGCTGG-3′

Table 3. Library construction for NGS: primers

Cycles	**Temperatures**	**Time**
1	95°C	5 mi.
23 (PCR_I) 13 (PCR_II)	95°C	30 sec.
	59°C (PCR_I) 57° C (PCR_II)	30 sec.
	72°C	45 sec.
1	72°C	10 min.
1	4°C	∞

Table 4. Library construction for NGS: thermal conditions

3.1.7 Statistical Analyses

Statistical analyses were performed using parametric Student's t-test, assuming as statistically significant a P value <0.05. Bonferroni correction for multiple comparisons was applied for analysis of microarray data, considering significant a Pc value <0.05. Differences in the microbial community composition between samples grouped by ward and different surfaces obtained by NGS were statistically analysed by QIIME 1.9.1.

Beta diversity was assessed by weighted and unweighted UniFrac distance matrices (Lozupone et al., 2006) and presented with principal coordinates analysis (PCoA). Analyses of Similarities (ANOSIM) was performed to compare the composition of microbial community among groups of samples.

3.2 Role of environmental microbial contamination on preterm newborns colonization

3.2.1 Study design

We next explored the role of environmental microbiome on preterm infant's colonization, by the analysis of nasal swabs from preterm infants at the time of birth and during the permanence in Neonatal Intensive Care Unit (NICU). A total of 55 nasal swabs were collected from 30 very low birth weight (VLBW) newborns admitted to NICU of IRCCS Burlo Garofolo, from November 2018 to January 2019, independently from their clinical conditions. The microbiological composition of nasal swabs was compared with that of environmental swabs collected from the delivery room (DR) at time of birth, and with that from the NICU, during the hospitalization period.

The study time-course of sample collection included: 30 nasal swabs collected at the time of birth (group N0), 18 after 9 days (group N9), and 7 after 13 days of permanence in the ward (group N13) depending on the health status of the newborns.

At the same time a total of 24 samples from critical points, including the floor, footboard and sink, were gathered from four randomized rooms in the NICU, while 6 samples were collected from floor, footboard and hospital trolley of one room of DR following a standard protocol (**methods section 4.3**) (Caselli et al., 2016b).

Samples were analysed by NGS, by real time quantitative qPCR microarray approach, and by Microbial DNA qPCR Array for Antibiotic Resistance Genes

Furthermore, to evaluate the contribution of vaginal bacteria in the colonization of nasal microbiome of newborns, we analysed by NGS the microbial composition of 20 vaginal swabs from pregnant women before giving birth in delivery room. For this part of the study, conducted in collaboration with University of Ferrara, since the surveillance was part of the

hospital infection control, and no patient personal information was collected, approval of ethics committees was not considered necessary.

3.2.2 Swabs collection

Anterior nasal swabs were collected by nurses and put in a sterile medium, using eSwabs and liquid amies transportation medium (Copan, Brescia, Italy).

Environmental Samples was collected in duplicate as described in **methods section 3.1.3** by sterile rayon swabs premoistened in saline solution.

Vaginal swabs were collected using a 200 mm polyethylene Cervix brush device (Mitra et al., 2017) (Rovers Medical Devices B.V, The Netherlands) under speculum examination, by a 360° rotation of the brush. Samples were then suspended in 1.5 ml of TE buffer. All the swabs were immediately transported to the laboratory under refrigerated conditions and stored at −80 °C until analysis.

3.2.3 NGS analyses

NGS analyses was performed as described in **methods section 3.1.6**, on nasal, environmental and vaginal swabs. All samples were defrosted and vortexed prior analyses, DNA was extracted from 300 µL of sample and eluted in a final volume of 100 µL as regard nasal and vaginal swabs, and of 50 µL as regard environmental ones.

3.2.4 Molecular microbiological analyses

Environmental samples were thawed and vortexed prior analyses to detach cells from swabs, total DNA was extracted by a commercial kit (Gene All, Tema Ricerca, Italy), following the manufacturer's instruction. Total DNA was extracted from nasal swabs as described for NGS analyses (previous section). Characterization of the microbial contamination of nasal swabs was performed by a customized array (Qiagen, Hilden, Germany). Total bacterial and mycetes load was evaluated by respectively a A pan-bacterial (panB) and pan-mycetes (panM) quantitative real-time PCR (qPCR). Characterization of the microbial contamination was performed by a customized real-time microarray assessing simultaneously the presence of the following bacterial and mycetes species: *Acinetobacter baumanii, Aspergillus fumigatus, Candida albicans, Citrobacter freundii, Clostridium difficile, Clostridium perfrigens, Enterobacter cloacae/Klebsiella oxytoca, Enterococcus faecalis, Enterococcus faecium, Escherichia coli, Klebsiella pneumoniae, Proteus vulgaris* and *mirabilis, Pseudomonas aeruginosa, Staphylococcus aureus* and *epidermidis, Streptococcus agalactiae, anginosus, prneumoniae, pyogenes, infantis/oralis* and *salivarius*, a positive amplification control (PPC) and a negative control were also included. The use of a real-time microarray, together with the normalization based on panB/panM results, allowed relative quantification of each parameter. The microbial environmental contamination level was performed by the same method, except for *Streptococci* (Qiagen, Hilden, Germany).

On nasal and environmental swabs, the antimicrobial resistance (AMR) genes of the contaminant population were characterized by a microarray detecting and quantifying simultaneously 84 different AMR genes (Qiagen, Hilden, Germany), as described in **methods section 3.1.4**. (Vandini et al., 2014; Caselli et al., 2016b, 2018, 2019).

3.2.5 Statistical Analyses

Statistical analyses were performed with QIIME 1.9.1. Beta diversity (between sample-diversity comparison) was assessed with weighted and unweighted UniFrac distance matrices(Lozupone et al., 2006) and presented with principal coordinates analysis (PCoA). Analyses of Similarities (ANOSIM) and Kruskal-Wallis tests were performed to compare the community composition in the group considered.

For microarray results, statistical analyses were performed using parametric (Student's *t* test) and non-parametric (Mann-Whitney) tests, assuming as statistically significant a *P* value at least <0.05. Bonferroni correction for multiple comparisons was applied for the analysis of microarray data (a *Pc* value <0.05 was considered significant).

3.3 Molecular monitoring of environmental surfaces during the COVID-19 era

3.3.1 Study design

The molecular methods have become part of the environmental surveillance system of IRCCS Burlo Garofolo, for the periodic active monitoring of hospital surfaces and in case of outbreak. The identification of a recurrent cluster of microorganisms contaminating the environment and patients validate the introduction of a new faster and less expensive molecular method of analyses, based on PCR and subsequent hybridization, which allows the simultaneous identification of 36 microorganisms, and 20 antimicrobial resistance genes mainly associated with health care-related infections.

This molecular analysis system was also in use during the COVID-19 pandemic and made it possible to make a preliminary comparison of the microbiological composition of hospital surfaces before and during the COVID-19 era, in which there was an intensification of sanitization interventions in the wards. During the COVID-19 era the presence of genetic material of SARS-CoV-2 was also assessed on selected surfaces. furthermore, a system of air monitoring was introduced.

A total of 58 critical environmental points was collected in two sampling campaign, the first in February 2020, and the second in June 2020, and tested by PCR and hybridization for the characterization of the microbiome and by a bioluminometric assay.

The following wards were considered: Paediatric Clinic (PC), Paediatric Surgery (PS), Paediatric Oncology (PO), Neonatal Intensive and Sub-Intensive Care Unit (NICU and NICUs), children's Intensive Care Unit (ICU), Surgical Rooms (SR), Delivery Room (DR) and Intensive Care Unit (ICU), including the same surfaces randomly selected as described in **methods section 3.1.1**, and including door handles, side tables and touch monitors.

12 environmental critical points were collected in the COVID-19 era in duplicate for the detection of viral RNA, specifically floors, bed footboards and sinks of PC, PO and NICUs

Air monitoring for microorganism, resistance genes and presence of SARS-CoV-2 was performed during the COVID-19 pandemic in 5 rooms of PO, SR and NICU.

3.3.2 Sampling

Environmental samples were collected by sterile swabs moistened in saline solution, as described in previous paragraph **method section 3.1.3**.

Samples of circulating air were collected using the system Airport MD8 Air Sampler (Sartorius, Göttingen, Germany) (**Figure 7**) following the manufacturer's instructions. Samples were collected onto an 80mm gelatine filter operating an air speed of 50/l min for 20 min (total sample size of 1000 l), after sampling, the gelatine membrane was immediately dissolved in 5 ml of saline solution in a 15 ml tube and transported to the laboratory. Prior nucleic acids extractions samples were spinned at 3000 × g and incubated in a heating block for 10 min at 37°C to dissolve the gelatine.

Figure 7: disposable and gelatine membranes employed for air sampling, (Sartorius, Göttingen, Germany) https://www.sartorius.com/shop/ww/en/usd/products-industrial-microbiology-gelatine-membrane-filters/c/M_Gelatine_Membrane_Consumables#. Last access 20 October 2020.

Total DNA was extracted from 300 µL of environmental swabs and from 500 µL of air samples in a final elution volume of 50 µL by the automatic extractor Maxwell CSC DNA Blood Kit (Promega, Madison, WI, USA), while RNA was extracted from 300 µL of environmental swabs and from 500 µL of air samples in a final elution volume of 50 µL by the automatic extractor Maxwell RSC Viral TNA Kit (Promega, Madison, WI, USA),

according to manufacturer's instruction.

The concentration of extracted DNA was measured by spectrophotometric reading using Nanodrop instrument (Thermo Scientific, Milan, Italy), (optical density at of 260nm and 280nm). DNA samples of DNA were stored at -20°C, while RNA was stored at -80°C until use.

3.3.3 Microbial characterization through multiplex PCR and reverse hybridization.

The microbial profile of surfaces and air samples was assessed using a multiplex PCR followed by reverse dot blot automatic hybridization into a macroarray CHIP based on DNA-Flow Technology (Hybri- Spot) (Vitro Master Dignostica, Sevilla, Spain).

The assay is based on two parallel multiplex PCR amplifications of each samples, optimized using 100 ng of DNA for each reaction, with biotinylated primers followed by an automatic reverse hybridization in membrane containing specific probes for detecting the most important microorganism and resistance genes associated with HAIs. Positive signals are visualized via a colorimetric immunoenzymatic reaction in a chip membrane by the Hybri-Spot platform (HS12) (Vitro Master Dignostica, Sevilla, Spain). The colorimetric reaction is captured as image by a camera and analysed by the Hybri-Soft software reporting the pattern of positive signals as exemplified in **Figure 8**. The assay can simultaneously detect the presence of the following bacteria/fungi:

Coagulase-Negative Staphylococci

Staphylococcus aureus

Streptococcus spp.

Streptococcus pneumoniae

Streptococcus agalactiae

Streptococcus pyogenes

Listeria monocytogenes

Enterococcus spp.

Pseudomonas aeruginosa

Acinetobacter baumannii

Neisseria meningitidis

Stenotrophomonas maltophilia

Escherichia coli

Klebsiella pneumoniae

Serratia marcescens

Enterobacteriaceae species

Proteus/Morganella spp.

Candida albicans

Candida spp.

Regarding the antibiotic resistance markers, the kit detects one gene for methicillin resistance (mecA), two genes for vancomycin resistance (vanA and vanB), two for β-lactam antibiotic resistance (blaSHV and extended-spectrum blaCTX- M), and fifteen genes for carbapenems resistance (kpc, sme, nmc/imi, ges, vim, gim, spm, ndm, sim, imp3, 15, 19_like, oxa23_like, oxa24_like, oxa48_like, oxa51_like, oxa58_like).

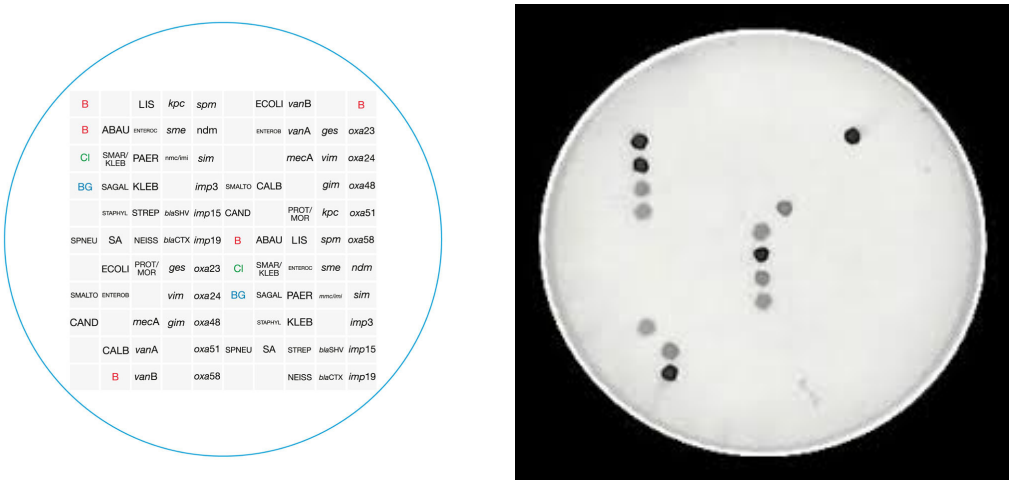

Figure 8. Hybridization probes and example of dot pattern obtained by reverse hybridization obtained with the Hybri-Spot platform (Vitro Master Dignostica, Sevilla, Spain). https://www.vitro.bio/Nac/Catalogo/ProductDetails?productNo=VIT-HS12A Last access 20 October 2020.

3.3.4 Real Time SARS-CoV-2 Detection

The presence of SARS-CoV-2 on samples was investigated using the commercial kit Neoplex™ COVID-19 Detection (Genematrix, Yong-in, South Korea) following the manufacturer's instructions. The kit can detect simultaneously the presence of the RdRp and N genes of SARS-CoV-2, starting from 5 µl of extracted RNA.

3.3.5 Bioluminometric assay

The effectiveness of cleaning procedures was simultaneously assessed on environmental critical points by the system 3M Clean-Trace^{TM} (3M, Saint Paul, USA) following the manufacturer's instructions. Briefly, a humidified rayon swab was rubbed on the surface to be sampled, was then inserted into a tube containing a solution with luciferin / luciferase.

Light emitted in the presence of ATP was detected by a luminometer, which reports a value expressed in relative light units (RLU), proportional to the amount of ATP present in the sample.

3.3.6 Statistical Analyses

Statistical analyses were performed on samples grouped in the pre COVID-19 vs COVID-19 era, using non parametric Fisher-Yates exact test, assuming as statistically significant a *P* value at least <0.05.

4 RESULTS AND DISCUSSION

4.1 Introduction of new generation technologies for the characterization of environmental microbiome.

With the aim to introduce new generation technologies to implement protocols of hospital environmental surveillance, and to characterize the resistance genes profile, Next Generation Sequencing (NGS) analysis on environmental samples was set up using the Ion Torrent platform, and compared with traditional cultural and molecular methods. A total of 108 environmental critical points were selected from 8 wards of the maternal and child hospital IRCCS Burlo Garofolo (Trieste), including Pediatric Clinic (PC), Pediatric Surgery (PS), Pediatric Oncology (PO), Neonatal Intensive and sub-Intensive Care Unit (NICU and sNICU), children Intensive Care Unit (ICU), Surgical Rooms (SR: Orthopedics, Gynecology and Oculistics) and Delivery Room (DR), in two consecutive sampling campaigns, surface microbiota and its resistome were analyzed as follows:

- Conventional cultural sampling (RODAC plates).
- Ion Torrent next generation high-throughput sequencing (NGS) of the 16s rRNA for bacterial microbiota characterization.
- Custom molecular qPCR panel kit for the detection of selected bacteria associated to HAIs.
- Microbial DNA qPCR Array for Antibiotic Resistance Genes) for the resistome characterization.

The results obtained are publishes in a scientific journal and briefly summarized below.

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Introduction of NGS in Environmental Surveillance for Healthcare-Associated Infection Control

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Abstract: The hospital environment significantly contributes to the onset of healthcare associated infections (HAIs), representing the most frequent and severe complications related to health care. The monitoring of hospital surfaces is generally addressed by microbial cultural isolation, with some performance limitations. Hence there is need to implement environmental surveillance systems using more effective methods. This study aimed to evaluate next-generation sequencing (NGS) technologies for hospital environment microbiome characterization, in comparison with conventional and molecular methods, in an Italian pediatric hospital. Environmental samples included critical surfaces of randomized rooms, surgical rooms, intensive care units and delivery rooms. The resistome of the contaminating population was also evaluated. NGS, compared to other methods, detected with higher sensitivity the environmental bacteria, and was the only method able to detect even unsearched bacteria. By contrast, however, it did not detect mycetes, nor it could distinguish viable from dead bacteria. Microbiological and PCR methods could identify and quantify mycetes, in addition to bacteria, and PCR could define the population resistome. These data suggest that NGS could be an effective method for hospital environment monitoring, especially if flanked by PCR for species identification and resistome characterization, providing a potential tool for the control of HAI transmission.

Keywords: healthcare-associated infections; antimicrobial resistance; contamination; molecular methods; next generation sequencing

In this study, for the first time in the paediatric field, the hospital environmental microbiome was characterized by comparing different methodologies. To date, the analysis of the environmental microbiome takes place mainly with low sensitivity and specificity culture methods, with long response times linked to the need to keep microorganisms in culture (Dancer, 2009a; D'Accolti et al., 2019). Given the role that the environmental microbiome plays in the transmission of pathogens (Otter et al., 2011b, 2013), the introduction of new methods with higher performance characteristics appears essential for a thorough characterization of the hospital environmental microbiome in the active monitoring of HAIs. The comparison between traditional cultural methods with molecular methods (NGS and qPCR array) showed that the latter are more sensitive in the characterization of microbial communities. In particular, the high throughput sequencing of 16s rRNA made it possible to detect all bacterial microbes present in samples, without establishing a priori which ones to search for, providing a semi-quantitative representation of the bacterial genera. This allowed to characterize the environmental microbiome in depth, also detecting poorly represented species. One of the limitations of this method, basically linked to the region of the bacterial genome selected for sequencing, was the ability to detect only bacterial species and not mycetes, detected by qPCR. Although, NGS can also be used for the characterization of mycome, by sequencing the 18S rRNA fungal gene (Banos et al., 2018).

The results obtained by RODAC plates and qPCR array evidenced a different level of contamination in different wards of the hospital and surfaces and PC turned out to be the most contaminated ward, with a greater presence of microorganisms on floor and sink.

Staphylococcus spp. was the most frequently detected microorganism on surfaces with all the methods employed and the amount of Staphylococci was proportional to total contamination. However, other species of bacteria and mycetes were also easily detectable:

qPCR, compared to bacterial cultures, allowed the identification and quantification of a higher number of potentially pathogenic microorganisms such as *Enterococcus* spp*., Enterobacteriaceae (K. pneumonia/Enterobacter, E. coli, A. baumannii, P. mirabilis), P. aeruginosa, C, perfringens/difficile, C. albicans* and *A. fumigatus*. NGS, in addition to confirming this data, evidenced also the presence of a multiplicity of microorganisms, to note pointing out the presence of *Pseudomonas* spp. and *Acinetobacter* spp. in 75% and 65.7% of samples, respectively, while the other methods detected these microorganisms only in 7.5% (RODAC plates) and in 24.1% (qPCR assays) of samples, a noteworthy difference considering the potential of such pathogens to be associated with difficult-to treat HAIs, especially in ICU. Moreover, NGS allowed the identification of bacterial generally belonging to the human skin and mucosa, particularly *Cutibacterium* spp. in 94.4% of the samples, *Streptococcus* spp. in 82.4% and *Corynebacterium* spp. in 75.0% of the samples. NGS was also the only technique that was able to highlight the presence the environmental, non-anthropic component of the hospital bacterial contamination, detecting bacteria with low pathogenicity like *Paracoccus* spp. and *Rothia* spp. in the majority of critical points considered.

The results related to the characterization of the resistome highlighted the presence in the departments of numerous genes of antimicrobial resistance, which may consistently belong to the microorganisms detected on the surfaces. These was R genes such as methicillin-resistance gene, likely linked to the presence of *Staphylococcus spp.*, followed by genes conferring resistance against macrolides and β-lactams/carbapenems IMP-5 group and NDM gene. The analyses carried out made it possible to identify differences in the microbiological composition of the surfaces, with peculiarities linked to specific departments due to both the bacterial species and the resistance genes present. The presence of potentially pathogenic microorganisms such as *Staphylococcus spp.* was also detected in the departments for which a higher level of sanitation is ideally required, such as the Surgical Rooms. This reinforces

the significance of an active and routine monitoring of hospital surfaces to perform a microbial hygiene evaluation (Galvin et al., 2012; Crofts et al., 2017), in order to predict the risk of colonization of patients and allowing the infection prevention and control department to act in an effective and timely manner on the sanitization strategies, also preventing the spread of outbreaks (Dancer, 2009a). Overall the NGS analysis proved to be an excellent tool for in-depth characterization of the environmental microbiome with performance greater than cultural methods currently in use. NGS data, together with qPCR resistome data and implementable with mycome and virulome analysis, proved to be a promising instruments to be included in the routine methods for hospital environmental screening to improve IPC strategies.

4.2 Impact of environmental microbiome on preterm newborn's colonization

4.2.1 Microbiome characterization by NGS

In order to explore the role of environmental microbiome on colonization of fragile patients a longitudinal study was carried out analysing the bacterial composition of nasal swabs of low-weight preterm newborns at birth (group N0) and after entering to the NICU (group N9 after 9 days, and group N13 after 13 days of permanence in the ward). The microbiome of the newborns was compared with that of the environmental surfaces of the wards of provenience: delivery room (DR) at time of delivery and Neonatal Intensive Care Unit (NICU) during the period of hospitalization. The analysis was performed by the molecular methods settled up in the first part of the project, which proved to be more efficient (NGS and qPCR). Furthermore, to evaluate the contribution of vaginal bacteria in the colonization of the nasal microbiome of newborns, 20 vaginal swabs from women before giving birth in the DR were analysed by NGS.

A first analysis of the results was made by observing longitudinally the variation of the bacterial composition of the nasal microbiome of newborns, both considering the prevalence of microorganism in grouped samples, and considering the values of relative abundance obtained with NGS.

As reported in **Figure 9**. the main bacterial genera identified were: *Corynebacterium* spp., *Staphylococcus* spp., *Streptococcus* spp., *Escherichia-Shigella* spp., *Acinetobacter* spp.

Pseudomonas spp. *Klebsiella* spp. *Enterobacter* spp., *Lactobacillus* spp., *Cutibacterium* spp., *Stenotrophomonas* spp. *Haemophilus* spp., *Gemella* spp., and *Rothia* spp.

In **panel A** are reported the percentages of positive patients on the total for each of the examined groups. *Cutibacterium* spp., and *Pseudomonas* spp. had higher percentages in infants at the time of birth (N0), while most of the bacterial genera detected had higher positivity values in the group of newborns with longer permanence in NICU (N13), such as *Corynebacterium* spp., *Staphylococcus* spp., *Streptococcus* spp., *Escherichia-Shigella* spp., *Klebsiella* spp. *and Enterobacter* spp.

Considering instead the average of the relative abundances for each genus within the three groups, reported in **Figure 9B**, *Corynebacterium* spp., *Staphylococcus* spp., *and Escherichia-Shigella* spp. demonstrated an increase of relative abundance from the group N0 to the group N13: *Corynebacterium* spp. (N0:2%; N9: 5%; N13: 11%), *Staphylococcus* spp. (N0:6%; N9: 37%; N13: 44%), and *Escherichia-Shigella* spp. (N0:7%; N9: 0%; N13: 29%). The observed differences were statistically assessed by Kruskal–Wallis test, showing significance for *Staphylococcus* spp. (P= 0.047) and *Escherichia-Shigella* spp. (p= 0.047). To note, *Staphylococcus* spp. and *Escherichia-Shigella* spp. had an increasing trend both for the percentage of positive patients for each group and in the relative abundance values.

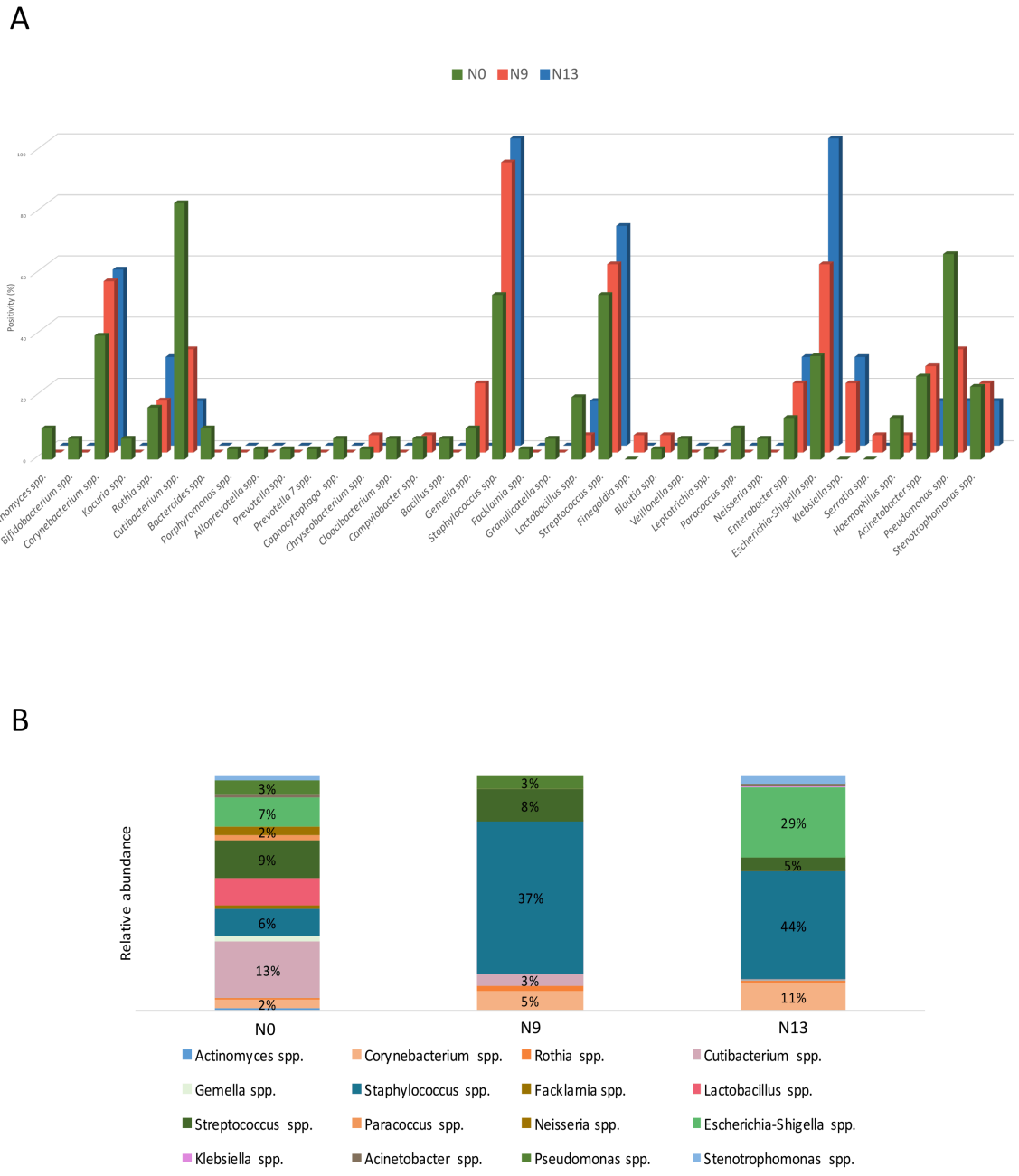

Figure 9. The main bacterial communities of nasal swabs from newborns at time of birth and after two follow-ups. Results were obtained by NGS. **(A)** Percentage of positive patients for each genus on the total for each group; **(B)** Mean of bacterial relative abundances for each group. N0: samples collected at time of birth. N9: samples collected after 9 days of permanence in NICU. N13: sample collected after 13 days of permanence in NICU.

The comparison between the results obtained with NGS of nasal swabs and of environment was primarily analysed using unweighted and weighted UniFrac distance matrices in order to test the significant differences between the groups of samples. For the comparison between the group N0 and DR (Figure **10A**), the ANOSIM statistical test attributed a significant difference to the grouping for the unweighted UniFrac ($p = 0.001$ R=0,9), but not for the weighted UniFrac (p=0,8 R=-0,12). For the N9 and NICU groups (**Figure 10B**) there was a significant difference to the grouping both for the unweighted ($p = 0.001$, R:0,67) and weighted ($p = 0.001$, R:0,27), as well as for groups N13 and NICU (**Figure 10C**) (unweighted UniFrac $p = 0.001$ R:0,82; weighted UniFrac $p = 0.008$ R:0,36). This preliminary comparison between groups revealed a great difference in terms of bacterial composition between the N0 and DR groups, and some overlaps of the relative abundances. Instead more overlaps were shown between the N9 vs NICU and N13 vs NICU groups, both in terms of bacterial composition and relative abundances.

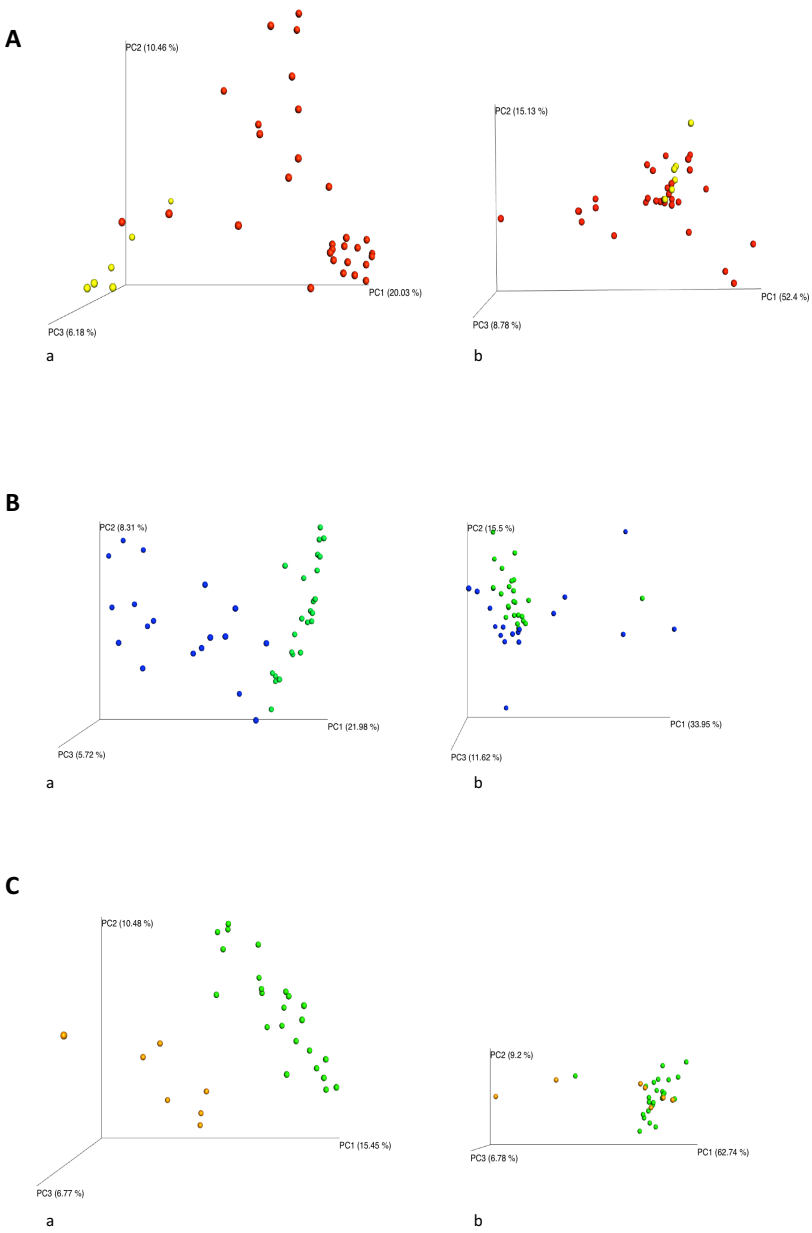

Figure 10. Emperor PCoA plot generated from the jackknifed_beta_diversity.py script of QIIME. Unweighted (a) and weighted (b) UniFrac-based PCoA, each dot represents a sample. **(A)** N0 vs DR. N0 (red), DR (yellow). N0: nasal swabs collected at time of birth. DR: environmental samples from the Delivery Room. **(B)** N9 vs NICU. N9 (blue), NICU (green). N9: nasal swabs collected collected after 9 days of permanence in NICU. NICU: environmental samples from the ward. **(C)** N13 vs NICU. N13 (orange), NICU (green). N13: nasal swabs collected collected after 13 days of permanence in NICU.

Considering how these overlaps translate in terms of bacterial composition, in **Figure 11A** is represented the comparison between the relative abundance of bacterial genera found in DR and the nasal swabs of newborns of group N0. The bacterial genera found with highest relative abundance in the group N was *Cutibacterium* spp., found with a relative abundance of 13%, in the 83% of the babies. It was detected in all the surfaces of DR, at higher values in the medical trolley (23%). *Staphylococcus* spp., as well, was present in all the surfaces of DR mainly on the floors, and showed a relative abundance of 6% in nasal swabs, this genus was present in the 53% of the patients of the group N0. Among the other bacterial genera that showed higher relative abundance values in the N0 group and also detected in the environment or DR we found *Lactobacillus* spp. and *Corynebacterium* spp., both with a relative abundance of 4% in the beds' footboards. In the newborns, the first showed values of relative abundance of 6%, the second of 2%. To note, *Streptococcus* spp. on the surfaces of DR showed relative abundance values of less than 2%, while was one of the most represented genera in the N0 group.

Following the variation in the composition of the microbiome of newborns over time, in comparison with the environmental one, the results related to the group of hospitalized children (N9 and N13) and the NICU environment are represented in **Figure 11B.**

The bacterial genus most represented in the nasal swabs of both the N9 and N13 groups was *Staphylococcus* spp., with an average of relative abundance of 37% and 44% respectively, it was the most detected bacterium also in all surfaces of the NICU with values of abundance between 9 and 10%.

Escherichia-Shigella spp. was detected in 29% of the N13 group, and in the NICU was found mainly on the floor (4%). *Streptococcus* spp. had similar values in noses (N9: 8%, N13: 5%) and surfaces (floor and sink: 5%, footboard 8%). *Corynebacterium* spp., present in all three

surfaces of the NICU in percentages less than 3%, had an average relative abundance of 5% in the N9 group and 11% in the N13 group.

Among the other bacterial genera observed both in nasal swabs and NICU environment were *Pseudomonas* spp. (S: 3%, footboard: 7%, floor: 2%, sink 5%) and *Stenotrophomonas* spp. (N13: 4%, footboard: 3%, sink: 1%).

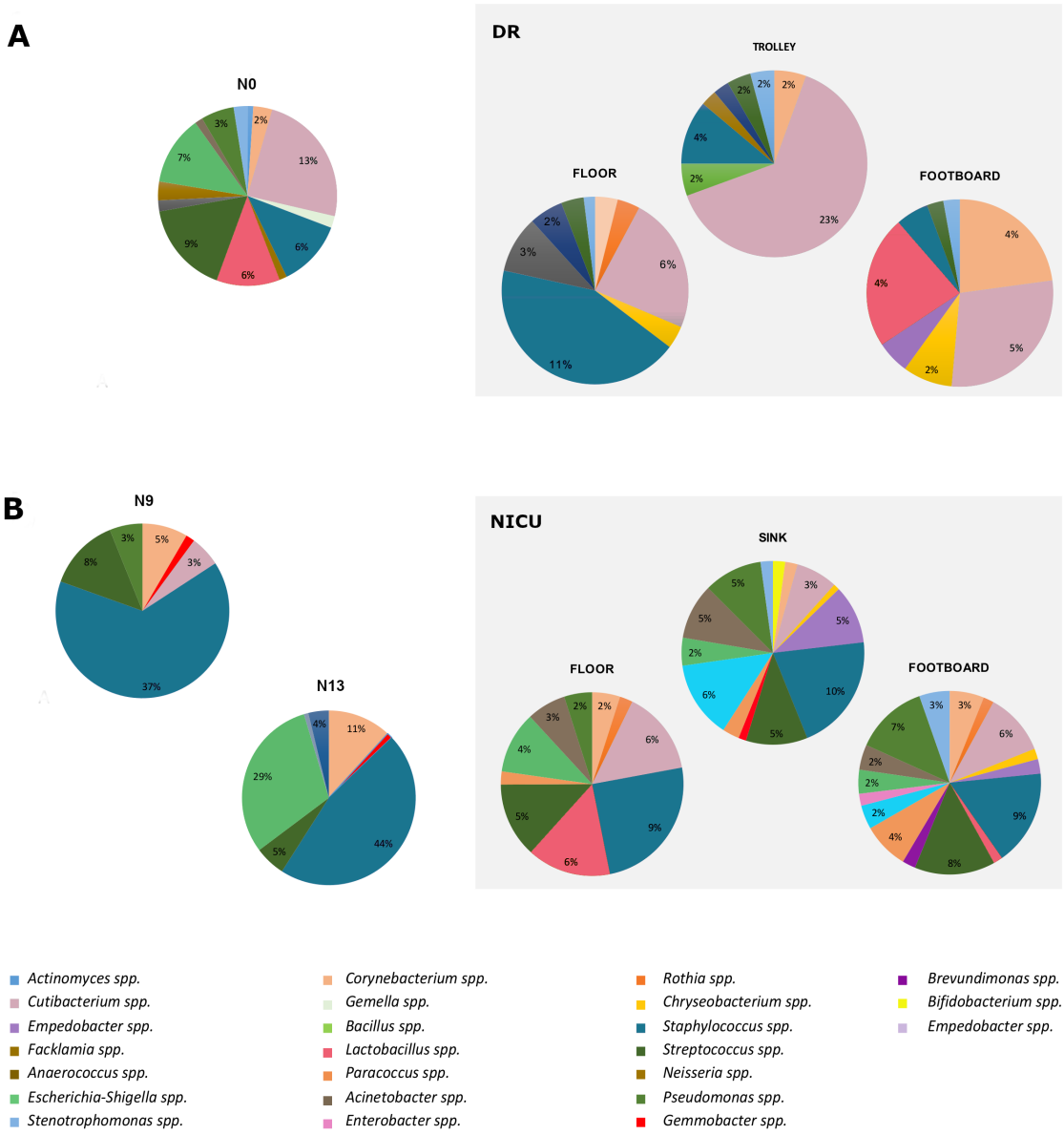

Figure 11. The predominant bacterial communities of nasal swabs from newborns in comparison with the environmental ones. Results were obtained by NGS. Data are expressed as mean relative abundance values. **(A)** N0 vs DR. N0: nasal swabs collected at time of birth; DR: environmental samples of delivery room divided into type of surface (floor, footboard and trolley). **(B)** N9 and N13 vs NICU. N9: samples collected after 9 days of permanence in NICU. N13: sample collected after 13 days of permanence in NICU; NICU: environmental samples of NICU divided into type of surface (floor, footboard and sink).

As regard the microbial composition of vaginal swabs of pregnant women during labour, results obtained by NGS are represented in **Figure 12**, reporting the mean of values of relative abundance of the predominant bacterial communities detected. The main genus observed was represented by *Lactobacillus* spp. (with a relative abundance of 86%), considered the hallmark of health in the female reproductive tract, followed by *Prevotella* spp. and *streptococcus* spp. These bacterial genera were detected also in the nasal cavities of the babies, in particular *Lactobacillus* spp. was present in the 20% of the newborns of the group N0 with a relative abundance of 6%, higher than the other groups considered. *Prevotella* spp. was detected only in 3% of N0 group, and *Streptococcus* spp. was detected with higher relative abundance in the N0 group than N9 and N13. (9% in the 53% of the babies of group N0).

The presence of bacteria of maternal origin in newborns is recognized, the bacteria of the mother, in particular those of the vaginal canal, colonize the newborn at the time of delivery (Ferretti et al., 2018; Freitas et al., 2018; Fettweis et al., 2019) and in this study they were found in variable proportions in particular in the N0 group, along with bacteria found on the surfaces of DR such as *Cutibacterium* spp., *Staphylococcus* spp. *Corynebacterium* spp. *Acinetobacter* spp. *Pseudomonas* spp. *Stenotrophomonas* spp. and *Rothia* spp. showing a dynamic profile associated with the time of hospitalization.

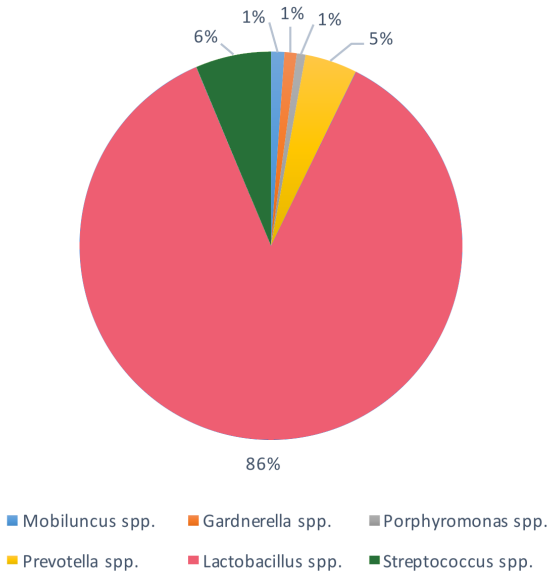

Figure 12. The predominant bacterial communities of vaginal swabs from pregnant women. Results were obtained by NGS. Data are represented as mean of relative abundances.

4.2.2 qPCR Array for microbiome and resistome characterization

The results obtained by NGS were confirmed by the qPCR microarray analyses, allowing the identification and quantification of specific bacteria and fungi associated to HAIs, up to species level. Again, also with this method, a progressive increase of positivity of bacteria on nasal swabs, and a progressive overlap with environmental species, associated with prolonging of hospitalization, was observed.

Within the genus *Staphylococcus,* the main species detected on nasal swabs were *aureus* (N0: 3%, N9: 33%, N13: 43%) and *epidermidis* (N0: 27%, N9: 89%, N13: 100%). *S. epidermidis* was the major contaminant of the environment of DR and NICU (2200 genome copies per 100 cm^2), while *S. aureus* showed a lower bacterial load (153.3 copies/100 cm^2). Within the genus *Streptococcus,* the most frequently detected species were *pneumoniae, infantis, oralis* and *salivarius,* with higher identification rates in the N13 group. Among other bacterial species that showed an increase of positivity over time, and the highest environmental contamination values compared to other microorganisms, *Klebsiella*

pneumoniae (N0, 0%; N9, 44%; N13, 85%), and *K. oxytoca/Enterobacter cloacae* (N0, 23%; N9, 67%; N13, 86%) showed a contamination rate on the environment of 1833 genome copies per 100 cm^2, while *Pseudomonas aeruginosa* (N0, 7%; N9, 38%; N13, 43%) was present on the surfaces with 186.7 copies/100 cm^2. Other microorganisms increasing in N9 and N13 compared to group N0 included *Enterococcus faecalis* (N0, 20%; N9, 55%; N13, 85%), *Escherichia-Shigella* (N0, 20%; N9, 38%; N13, 71%), and, although to a less extent, *Acinetobacter baumannii* (N0, 13%; N9, 11%; N13, 28%).

Comparative quantitation between newborns groups evidenced significant increases over time (N13 *vs* N0) of *S. epidermidis*, *K. pneumoniae/oxytoca, Escherichia-Shigella* (about 3 logs; $p< 0.01$), *E. faecalis*, *S. aureus*, *P. aeruginosa*, *S. pneumoniae/infantis/oralis/salivarius* and *C. albicans* (between 1 and 2 logs, $p<0.01$).

Not detectable by high-throughput sequencing of 16S rRNA gene, qPCR array allowed the identification of the fungus *Candida albicans* in group N9 (6%) and N13 (43%), but not in group N0. In general, lower contamination levels were found in the surfaces of the DR compared to the NICU, ascribing essentially to *Staphylococci* (S. *epidermidis*, 310 copies/100 cm^2; *S. aureus* 3.3 copies/100 cm^2) and to *E. faecium* (13.4 copies/100 cm^2), mainly on floors and sinks.

For a more in-depth characterization of the environmental and nasal microbiome, a qPCR array was performed for the simultaneous detection of 84 antimicrobial resistance genes. Notably, as shown in **Figure 13**, no resistance genes were detected in babies of the N0 group, at the time of birth, although, as shown above, numerous bacterial genera were detected. With the stay in the NICU ward, on the other hand, a progressive onset and increase of R genes was highlighted, many of which were also present in the environmental surfaces of the wards **Figure 13A**, including beta-lactam resistance genes (SHV), quinolone resistance genes (QnrS), and macrolide resistance genes (ermA, ermB, ermC, mefA, msrA). This

progressive increase of resistance genes, including environmental ones, in patients with prolonged hospitalization, reflects was been observed for microbial taxa.

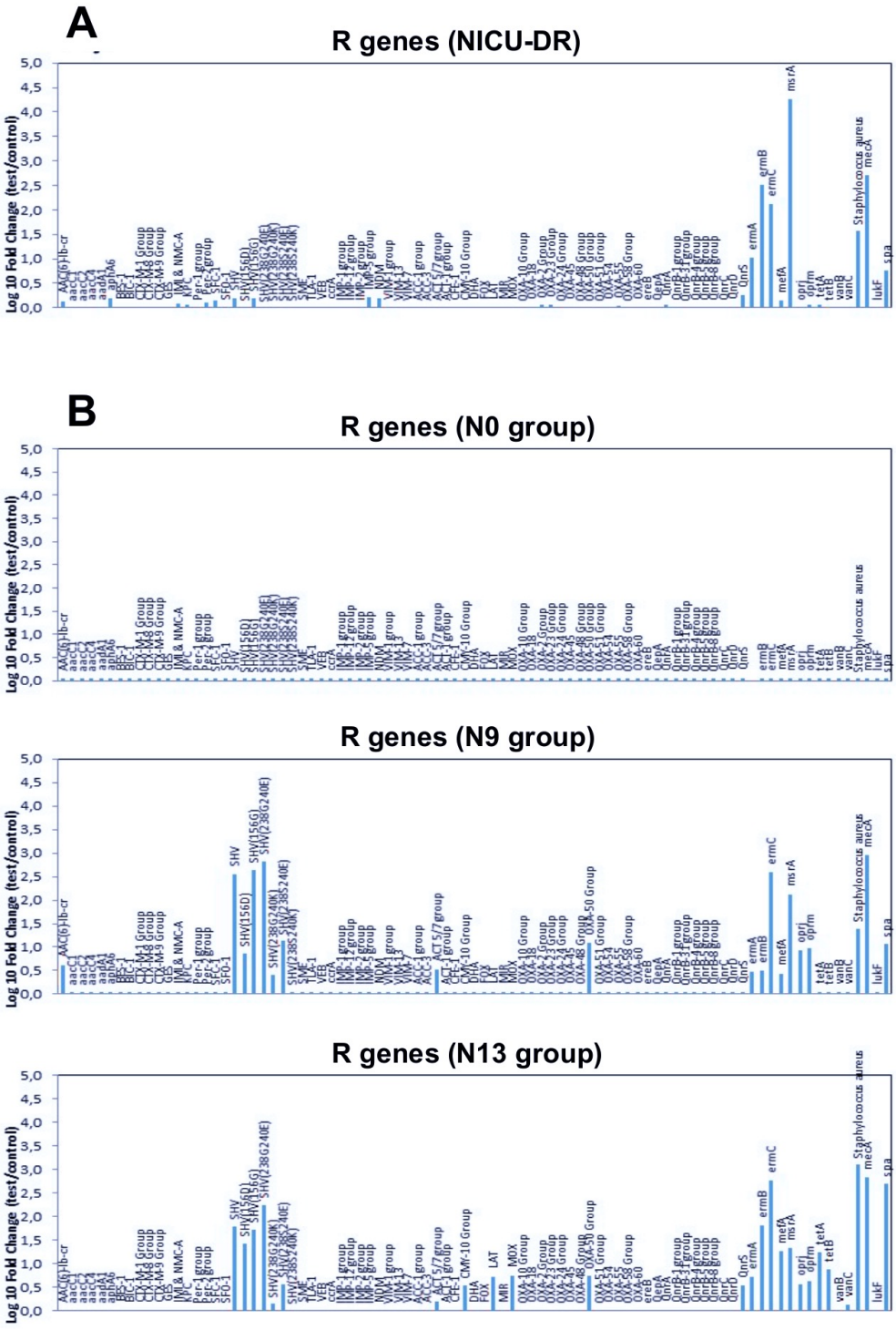

Figure 13. Resistome characterization of the microbial population contaminating NICU surfaces. Results were obtained by qPCR microarray as described in Methods, and are expressed as $\log_{10}$ fold change of each detected R gene, compared with the negative controls (NTC). The plotted data represent the mean values of duplicate samples obtained in two environmental sampling campaigns for NICU environmental duplicate sampled points **(A)** and for nasal swabs of N0, N9 and N13 newborn groups **(B)**.

The molecular methods, as reported in the first part of this study (Comar et al., 2019), have proven to be of great importance for the active monitoring of the environmental and patient microbiological composition. The in-depth characterization of the environmental microbial composition has led to the observation of a progressive increase in environmental-derived bacterial species and resistance genes in the nasal cavities of newborns, associated with the stay in the wards. This data provides further confirmation of environmental microorganisms colonization of the newborn patients (Younge et al., 2018) highlighting how hospital bacteria play a fundamental role in shaping the human microbiome starting from the first moments after birth.

The bacteria detected are potential pathogens, mainly responsible for care-related infections (Hooven and Polin, 2014; Ramasethu, 2017; Zingg et al., 2017), many of which are associated with the severity of the diseases, particularly the resistant strains (Bassetti and Righi, 2013; Facciolà et al., 2019). Furthermore, the fragility of the patients included in this study makes them particularly at risk of contracting HAIs (Kawagoe et al., 2001; Larson and Dinulos, 2005).

In particular, *Staphylococcus* spp., is confirmed to be one of the main contaminants of the environment (Oliveira and Damasceno, 2010; Caselli et al., 2016b; Best et al., 2018; Comar et al., 2019), and it was also the main contaminant of the nasal cavities of newborns in the *aureus* and *epidermidis* species, in concentrations and relative abundances increasing with the stay in NICU. Staphylococcal species are responsible for the majority of late-onset infections in VLBW babies (Ramasethu, 2017). In particular, in very immature preterm newborns, Methicillin-resistant *S. aureus* (MRSA), , is associated with significant mortality and morbidity (Ericson et al., 2015; Giuffre et al., 2015; Dong et al., 2018) and it was detected both in nasal swabs and in environmental samples. Strains of MRSA can be shed into the environment by positive patients (Boyce et al., 1997), surviving for up to a year

(Wagenvoort et al., 2000). Also *S. epidermidis* has emerged as a predominant pathogen of neonatal late-onset sepsis in VLBW infants (Dong and Speer, 2014).

As regard fungi, *C. albicans*, detected only in newborns after entering the NICU, is one of the principal cause of late-onset sepsis in VLBW preterm neonates (Stoll et al., 2002)

and is as well considered one of the pathogens with nosocomial transmission (Fridkin et al., 2006; Leibovitz et al., 2013; Aliaga et al., 2014).

The results obtained once again highlight the weight that an hospital environment contaminated by potential pathogens has in the aetiology of HAIs, which can be mainly transmitted via hands of health-care workers (Guidelines for Environmental Infection Control in Health-Care Facilities: (545922006-001), 2003).

It appears clear that the introduction of periodic molecular screening is of fundamental importance, both on the patient, but especially on the environment. Effective active monitoring of surfaces can be useful in order to assess the risk of hospitalized fragile patients contracting infections related to care, but above all it is a powerful tool for evaluating the efficiency of routine cleaning and therefore for their subsequent implementation (Galvin et al., 2012).

4.3 Use of molecular technologies to characterize the environmental microbiome during the COVID-19 pandemic.

The identification of a cluster of microorganisms and antimicrobial resistance genes contaminating the hospital environment has validated the introduction of a faster and less expensive molecular method based on PCR and reverse hybridization, capable of detecting in few hours 36 microorganisms and 20 resistance genes associated to HAIs. This method became part of the active monitoring of the microbial composition of the environmental surfaces of the hospital, and was also in use at the beginning of the COVID-19 pandemic, in which in the wards there was an intensification of the frequency of cleaning interventions with chlorine-based detergents, with an average increase of 1.5 hours per day for each department dedicated to sanitization. It was possible to preliminarily assess whether and what was the impact on the composition of the microbiome of the surfaces of the modification of the cleaning protocols.

As showed in **Figure 14**, the bacteria and fungi most frequently identified on the surfaces were: *S. pneumoniae, Candida* spp*. C. albicans, S. aureus, S. maltophilia, P. aeruginosa, Streptococcus* spp*., A. baumannii, Enterobacteriaceae, K. Pneumoniae and E. Coli*. With the exception of *S. Pneumoniae* and *S. maltophilia*, a moderate increase in the prevalence of all organisms was recorded, albeit without statistical significance except for *S. pneumoniae* ($p < 0,05$). *Staphylococcus* spp. also with this method turned out to be the most frequent bacterial genus, identified in 13/29 samples in pre COVID-19 era, and 15/29 samples during the COVID-19 pandemic.

The same was observed for the resistance genes (**Figure 15**), only Carbapenemase GES and NDM were detected less frequently during the COVID-19 pandemic, while the others showed a moderate increase.

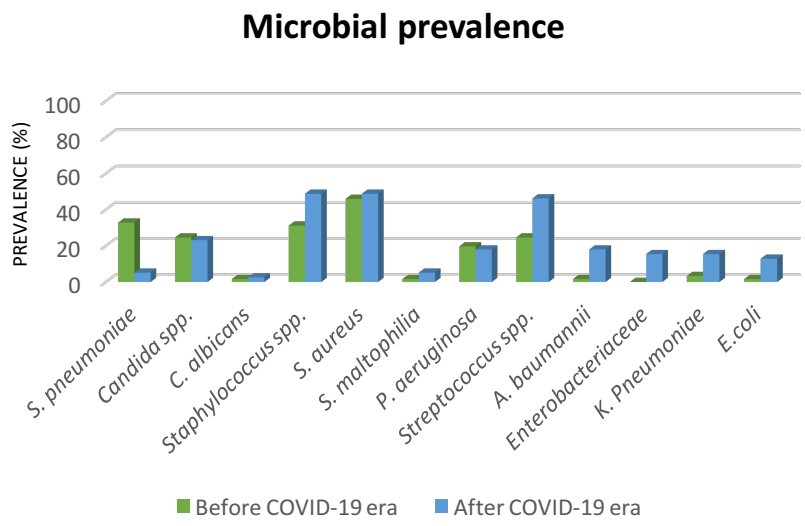

Figure 14. Prevalence of bacterial and fungal communities on hospital surfaces. Results are represented as percentage of positive samples on the total, in green for the pre COVID-19 era and in blue during the COVID-19 era.

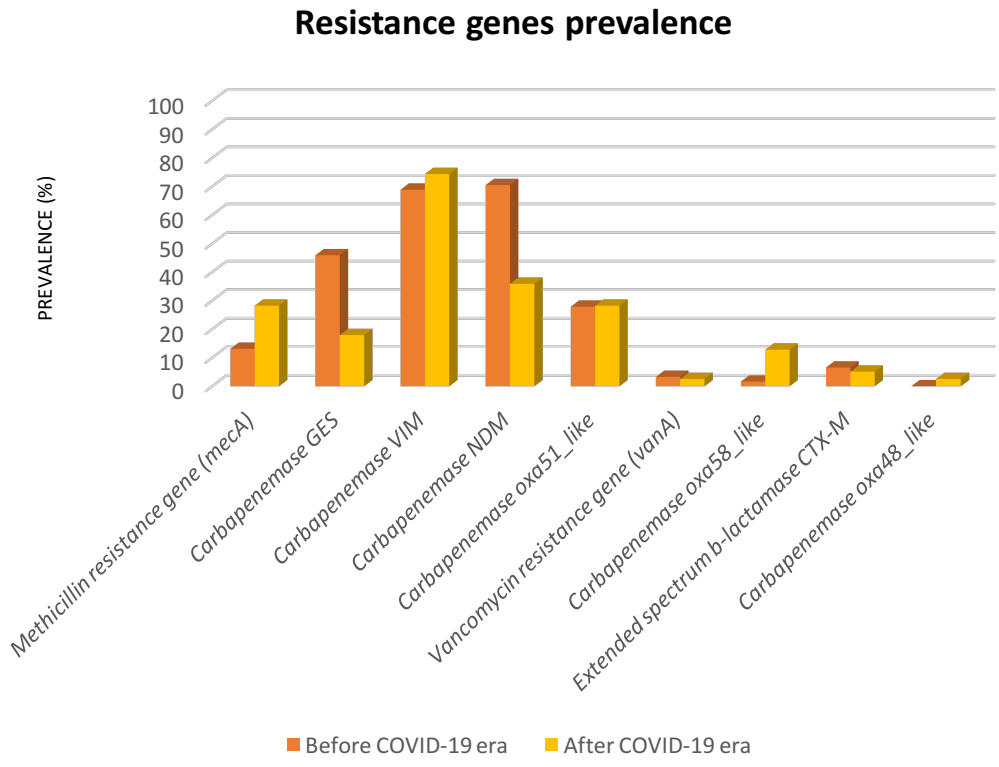

Figure 15. Prevalence of resistance genes on hospital environment. Results are represented as percentage of positive samples on the total, in orange for the pre COVID-19 era and in yellow during the COVID-19 era.

To note, in our case, the analysis of contamination of the air has not given positive values, as well as the search for COVID-19 on all of the samples.

These results are consistent with the literature, although chlorine-based cleaners are effective in eliminating SARS-CoV-2 from surfaces (Pradhan et al., 2020), they do not counteract the recontamination phenomena of surfaces by microorganisms (Rutala and Weber, 2014; Vandini et al., 2014), and moreover can favour the selection of resistant strains (Caini et al., 2013).

As regards the use of the ATP bioluminescence system, employed in parallel with the molecular analyses, it provided a positive signal in fewer environmental critical points than molecular detection method. **Figure 16**. The ATP bioluminescence system in fact gave a positive value on 24/58 of the samples analysed, while PCR and subsequent reverse hybridization detected the presence of microorganisms in 49/58 of cases.

This data corresponds to what was expected, confirming the limits of this method. The measurement of ATP in fact does not provide specific information on the type of organic material identified, it is less sensitive than molecular methods, cannot detect very low microbial counts (<10 CFU/cm^2) (Boyce et al., 2009; Whiteley et al., 2012), and can provide results that can be affected by environmental contaminants of different origin (Malik et al., 2003; Malik and Shama, 2012).

The results confirmed that ATP monitoring is not suitable to provide an indication of the quality of cleaning, but can be however useful for detecting the need for cleaning attention (Boyce et al., 2011).

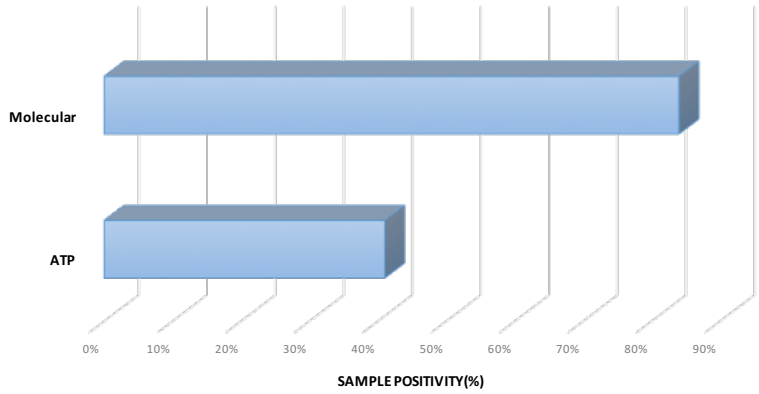

Figure 16. Comparison of ATP bioluminescence and molecular methods in the efficiency of detection environmental contamination in all analysed samples. Results are represented as percentage of positive samples on the total.

The overall results, albeit preliminary, highlighted again the inadequacy of the current sanitizations protocols and the need to introduce alternative sanitation methods, which effectively limit over time the recontamination phenomena of the surfaces by pathogenic microorganisms, with a consequent reduction in the risk of contracting HAIs. From October 2020, starting from the Emergency Room of IRCCS Burlo Garofolo, Trieste, in collaboration with the University of Ferrara, for the first time in the paediatric field, the Probiotic Cleaning Hygiene System (PCHS) was introduced as part of infection prevention and control strategies.

It is a system at low environmental impact that uses spores of the *Bacillus* genus which, due to the phenomenon of competitive exclusion, limit colonization by other microorganisms over time. In adult hospitals this system has proved to reduce pathogens compared to conventional methods by 70-96% and to reduce antibiotic resistance genes by 70-99.9% (Vandini et al., 2014; Caselli et al., 2018). It was also seen to be able to reduce HAIs by 52%

with consequent benefits not only in terms of well-being for the patient but also at an economic level (Tarricone et al., 2020).

The effectiveness of the PCHS system will be monitored with the methods developed in this study throughout the duration of its use.

5 CONCLUSIONS

The introduction of molecular methods for the analysis of the environmental microbial composition has shown how these are more effective for characterizing the environmental microbiome, not only as regards its composition in terms of microorganisms, but also with reference to the resistance genes circulating in the environment. In fact, molecular methods, in particular NGS and qPCR for the characterization of R genes, compared to traditional culture methods, can identify and quantify the microbiome with greater sensitivity and specificity. In particular High-throughput sequencing of 16S rRNA gene, not requiring to know a priori which microorganisms are to be searched, might be an effective first-step tool for monitoring the whole composition of hospital bacterial microbiome, providing a deeper knowledge of the microbial composition of hospital surfaces, possibly leading to significant improvements in the development of new protocols to fight HAIs and AMR.

It has also been shown that potentially pathogenic microorganisms can colonize the nasal cavities of extremely fragile patients such as preterm infants and that the permanence in hospital wards increases colonization by environmental pathogens. This, again, highlights how it seems a mandatory aspect to introduce a rapid and effective molecular screening of the environment as part of the infection prevention and control strategies, as a tool to be used not only for the clinical management of the patient but mainly for a remodelling of surface sanitation strategies.

The identification of a recurrent cluster of microorganisms and resistance genes has validated the introduction of a faster and less expensive molecular method for the characterization of the microbiome, for an effective, efficient and prompt response which can also be used in the event of a microbial outbreak. Even with the use of this method, the need for a reconsidering of sanitation strategies has been highlighted. Due to the spread of

the COVID-19 pandemic there has in fact been a change in the sanitation protocols in hospitals with the introduction and increase in the frequency of sanitation interventions with chlorine-based detergents. This type of detergents, while effective in the immediate abatement of the bacterial and viral load on surfaces, does not prevent the recontamination phenomena underlying the transmission of HAI. During the pandemic, attention towards antibiotic stewardship and the prevention of HAIs took a back seat, not without probable consequences for the future. The need for resorting to new surface disinfection strategies, highlighted by this study, led to the introduction of a sanitation system allowing colonization by benign microbes rather than potential pathogens with consequent stable elimination of pathogenic microorganisms and resistance genes from surfaces over time.

6. BIBLIOGRAPHY

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