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USE OF PROBIOTICS AND BACTERIOPHAGES IN HOSPITAL ENVIRONMENT FOR HAI CONTROL: POTENTIAL AS SANITIZING AGENTS

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INTRODUCTION

1. Healthcare-associated infections (HAIs)

The Centre for Disease Control and Prevention (CDC) defines an Healthcare-associated infection (HAI) as a "localized or systemic condition occurring as an adverse reaction to the presence of an infectious agent(s) or its toxin(s) that was neither present nor incubating upon the patient's admission to the acute care facility" (Horan et al., 2008).

An infection is considered HAI-associated if it develops after more than 48h after admission at healthcare setting, and it is identified by specific criteria (ECDC, 2015).

The term HAI refers to different levels of healthcare, including hospitals, ambulatory centres, outpatient clinics, long-term care facilities and replaced the previous terms, *nosocomial infections* and *hospital-acquired infections,* which have been traditionally used to indicate only acute infections occurring in inpatients during hospitalization (Archibald, 2012).

Today, HAIs represent one of the most frequent complications occurring in healthcare facilities worldwide, impairing the clinical outcome of up to 15% of all hospitalized patients (Allegranzi et al., 2011).

In particular, according to the European Centre for Disease Prevention and Control (ECDC), about 4 million patients acquire an HAI in European acute hospitals every year, and 37,000 die as a HAI direct consequence (ECDC, 2013; Brusaferro et al., 2015).

In Italy, the incidence of these infectious complications generally varies from 5-15% for hospitalized patients to 1% for home care patients, and infections caused by antibiotic-resistant microorganisms are more and more common, with a mortality rate of up to 30% of total infections (Messineo and Marsella, 2015).

In addition to a significant clinical impact, these infections have also an important influence on costs. Indeed, according to World Health Organization's first global report, HAIs are responsible of lengthening of the duration of stay, long-term disability, additional economic burden on health systems and patients and their families, and significant excess mortality (WHO, 2011). Only in Europe, about 16 million extra-days of hospital stay are caused by HAI onset, with over 1.1 billions € annual financial loss estimated annually, including only direct costs, directly ascribable to HAIs (WHO, 2011).

1.1 The "Chain" of Healthcare-associated infections

According to the 2011-2012 Point Prevalence Survey of HAIs conducted by ECDC, the most frequently reported infections in acute care hospitals are respiratory tract infections (pneumonia 19.4% and lower respiratory tract 4.1%), surgical site infections (19.6%), urinary tract infections (19.0%), bloodstream infections (10.7%) and gastro-intestinal infections (7.7%), with *Clostridium difficile* infections representing 48% of the latter and 3.6% of all HAIs (ECDC, 2013).

Outcome events (infection and/or disease) depend on different factors, as the exposure to a particular infective agent is necessary, but not sufficient to cause a disease in the host. The onset of a clinically evident disease following infection is the result of the interactions between the microorganism itself, the host conditions and the environment.

As a result, HAIs represent the result of a complicate interconnection of some elements including susceptible host, infectious agent, reservoir, mode of transmission, portal of entry/exit, together composing the so-called "Chain of Infection components", a model that is a useful framework to better understand how HAIs occur (**Figure 1**).

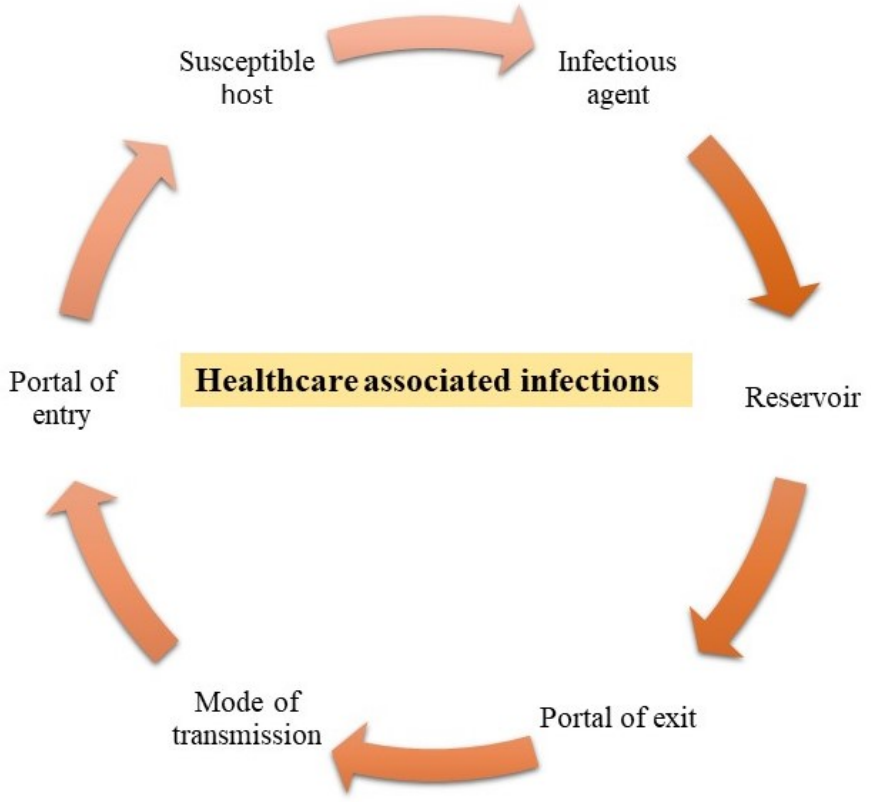


Figure 1. Schematic diagram for the Chain of healthcare-associated infections.

1.1.1 Susceptible host

A susceptible host is a person that is more prone to acquire infections, which in turn are favoured by several factors. With regard to HAIs, people at major risk for HAI onset are patients, but also healthcare professionals (HCP) and visitors can be at risk, due to continuous exposure to pathogens.

The main predisposing factor for HAI acquisition is related to the subject characteristics (age, gender, clinical status). Indeed, people with comorbidities (i.e. diabetes, malignancies), compromised immune systems, or poor physiologic reserve (i.e. advanced age), have an increased risk of infection (Siegel et al., 2007). Furthermore, the administration of antimicrobials, or the use of particular medical treatments, such as surgery or radiation therapy, can impair host defence mechanisms, leading to a subsequent increase of the risk of infection (Siegel et al., 2007).

Finally, the use of invasive devices, such as central-line catheters or urinary catheters, but even injection needles, can favour HAI onset by facilitating pathogen penetration and colonization of anatomical sites that are usually sterile (Siegel et al., 2007). According to this, some of the most frequent infections include central line-associated bloodstream infections (CLABSI), catheter-associated urinary tract infections (CAUTI) and ventilator-associated pneumonia (VAP), confirming the critical role of invasive devices as risk factors for HAI acquisition and defining such devices as important threats to patient safety (CDC, 2014).

Based on the host conditions, the exposure to an infectious agent can thus lead to different outcomes: some individuals will never develop symptomatic diseases, whereas others will become ill and eventually die in case of severe disease (Siegel et al., 2007). Importantly, an infected host becomes a source of infectious microorganisms that can be transmitted to others individuals, representing an important epidemiological ring of the chain of HAIs.

1.1.2 Infectious agents and antimicrobial resistance (AMR)

Several agents can cause HAIs, including bacteria, mycetes and viruses. Although most of the factors influencing the occurrence and severity of disease are related to the host, the microorganism features are also important, including infectious dose, virulence and pathogenicity (Siegel et al., 2007).

Infectious dose is the amount of an agent needed to cause an infection, pathogenicity refers to the ability of inducing an infection, and virulence is the degree of pathogenicity of an infectious agent (Ahmad et al., 2013).

According to ECDC, the most frequently microorganisms responsible for HAI onset and isolated from HAI patients are, in decreasing order, *Escherichia coli* (15.9%), *Staphylococcus aureus* (12.3%), *Enterococcus* spp. (9.6%), *Pseudomonas aeruginosa* (8.9%) *Klebsiella* spp. (8.7%), coagulase-negative staphylococci (7.5%), *Candida* spp. (6.1%), *Clostridium difficile* (5.4%), *Enterobacter* spp. (4.2%), *Proteus* spp. (3.8%) and *Acinetobacter* spp. (3.6%) (ECDC, 2013).

Some of such microorganisms are common commensals in human body, and may become pathogenic and cause HAIs as a result of an opportunistic behaviour.

S. aureus, for example*,* is a common member of the human microbiota, frequently found on the skin and in the upper respiratory tract, but it can also become an opportunistic pathogen in a susceptible host, and indeed it is currently the species most commonly associated to blood-stream, lung, soft tissue, and skin infections (D. J. Diekema et al., 2001).

Among *Enterobacteriaceae, E. coli*, commonly harmless in the lower intestine, can be responsible of gastroenteritis, urinary tract infections, respiratory illness and pneumonia (Rodrigo-Troyano and Sibila, 2017)

In addition, *P. aeruginosa,* a Gram-negative bacterium ubiquitous in the environment, represents a leading cause of HAIs, mainly in subjects with compromised immune response, causing important infections of the urinary tract, blood or respiratory tract (Stover et al., 2000; Rodrigo-Troyano and Sibila, 2017).

A characteristic shared by HAI-associated microorganisms is their antimicrobial resistance (AMR), due to the selective pressure exerted by the huge use of antimicrobial drugs in the hospital setting. The AMR refers to the ability of microorganisms to be resistant to the action of one or more antimicrobial agents, and today represents one of the most urgent threats to the public's health, since infections caused by multidrug resistant (MDR) microorganisms are very frequent in hospitalized patients, and very difficult, or even impossible, to treat.

In most cases, these types of infections require extended hospital stays and additional sanitary costs. AMR phenomena has become so common in the last decades, that the current period has been defined as the "post-antibiotic" era (Bragg et al., 2018), and, according to recent observations, it has been hypothesized that AMR will causing more death than cancer within 2050 (O'Neill, 2014). One of the causes of the growing AMR is the over-use of antimicrobial agents, that are administered unnecessarily or without testing the susceptibility of the infectious agent, thus leading to the spread of the MDR microorganisms, also defined 'superbugs', that further limit the therapeutic options.

It has been observed that one out of three patients receive at least one antimicrobial on any given day (ECDC, 2013), and that the consumption of antimicrobials for treatment of MDR pathogens has almost doubled between 2011 and 2014 (EARS, 2017).

Notably, a high proportion of HAI-associated microorganisms show a multidrug-resistant phenotype in healthcare settings, where the most prevalent are represented by methicillin-resistant *S. aureus* (MRSA), vancomycin-resistant *Enterococcus faecalis/faecium* (VRE), extended-spectrum cephalosporin-resistant, carbapenem-resistant *Enterobacteriaceae* (CRE), carbapenem-resistant *P. aeruginosa* (EARS, 2017).

Methicillin is a narrow-spectrum β-lactam antibiotic acting, as all β-lactams, by binding to transpeptidase enzymes and inhibiting their action, finally inhibiting the synthesis of the major component of the cell wall, the peptidoglycan. Transpeptidases have a very high affinity for β-lactams, so that they are also called penicillin-binding proteins (PBP). Methicillin was synthesized in order to circumvent the continuous growing of penicillin resistance in *S. aureus,* but early after its introduction, MRSA strains rapidly emerged and spread worldwide (Harkins et al., 2017). Resistance to methicillin occurs through the production of a modified PBP (PBP2α), encoded by the *mecA* gene, and characterized by a lower affinity for the antibiotic, thus impeding its action and allowing bacterial survival (Venter et al., 2017). MRSA strains are usually treated with vancomycin antibiotic, that also inhibits cell wall synthesis but with a mechanism independent from the binding to PBPs, as it acts by blocking the aminoacid D-Ala-D-Ala dimer needed for transpeptidase action. Resistance to vancomycin is related to a modification of D-Ala-D-Ala residues (often by substitution of a D-Ala residue with a Lactate). Modified aminoacids reduce the affinity of the dipeptide for vancomycin and prevent disruption of peptidoglycan (Venter et al., 2017). *Staphylococcus spp.* can acquire resistance to vancomycin, as reported for *S. aureus* strains isolated in 2004 in US, by acquiring the transposon Tn*1546* from vancomycin-resistant *Enterococcus faecalis*, likely through conjugation or other forms of genetic exchange between bacteria (Gardete and Tomasz, 2014).

Similarly, extended spectrum beta lactamases (ESBL) and, in particular, carbapenemases production in Gram-negative bacteria, are the main features of MDR Gram-negative bacteria, and can represent the step before the so called 'pan' drug resistance (PDR), when carbapenemase-producing Gram-negative bacteria are often resistant to all or almost all available antibiotics (Meletis, 2016).

In these cases, older antibiotics active against the external membrane of Gram-negative bacteria, such as polymixines, become a last-resort choice (Morrill et al., 2015).

One of such antibiotics is colistin (polymixin E), which represents a "last resort" antibiotic effective in treatment of MDR or PDR Gram-negative bacteria. However, this antibiotic has been used for many years in livestocks, because it is very cheap and quite toxic in humans, and this has led to the appearance of a plasmid-mediated, transferable resistance against colistin in some Gram-negative bacteria (Kempf et al., 2016). Indeed, although bacterial resistance to this antibiotic was previously considered rare, in 2016 the plasmid-mediated colistin resistance *mcr-1* was first reported in chickens (Liu et al., 2016), and afterwards a rapid spread of this resistance to bacteria infecting humans has been observed (Ye et al., 2016). The mechanism of action of colistin is similar to a detergent action, as it permeabilizes the outer membrane of Gram-negative bacteria, by interacting with the lipid A subunits present in the lipopolysaccharide (LPS), thus resulting in the bacterial cell death. In colistin-resistant bacteria, the plasmid-mediated *mcr-1* gene encodes a phosphoethanolamine transferase which is able to modify the lipid A with a phosphoethanolamine (PEP) group, preventing its interaction with colistin (Venter et al., 2017) (**Figure 2**).

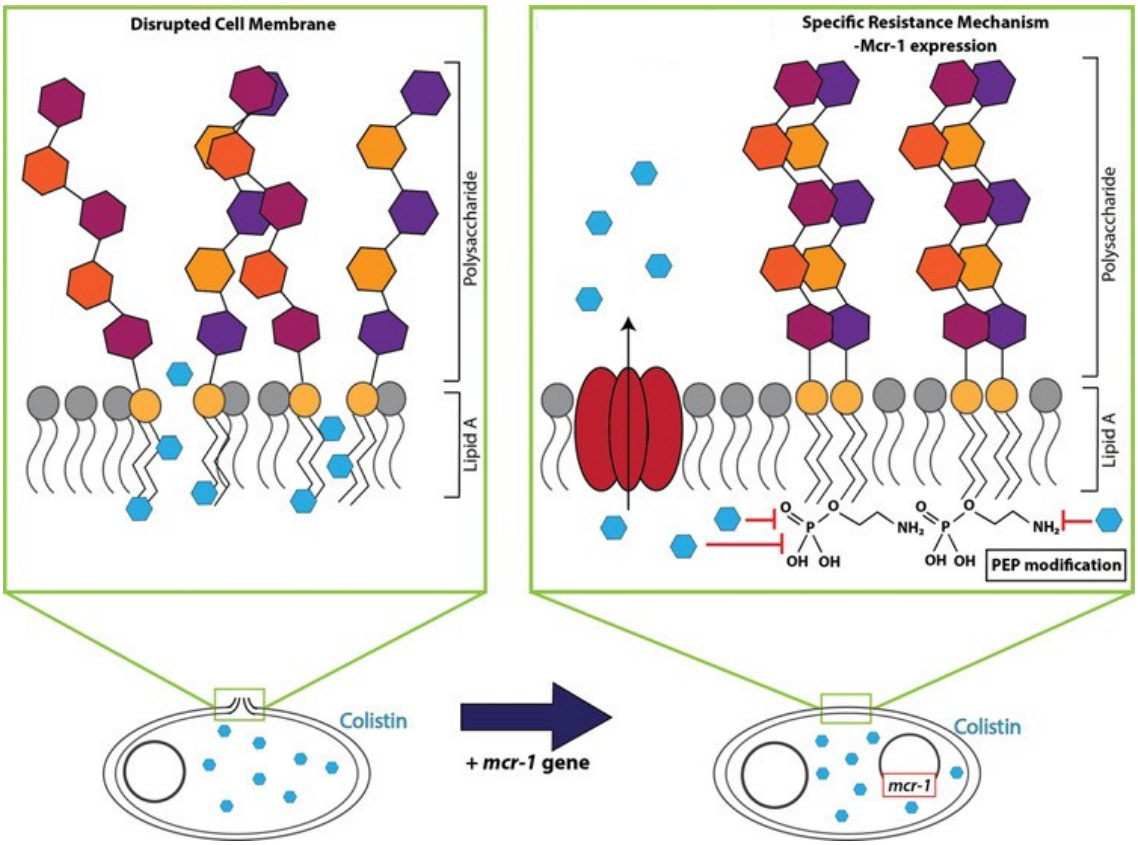


Figure 2. Mechanism of colistin resistance mediated by *mcr-1* gene (Venter et al., 2017)

This resistance, being located in bacterial plasmids which by definition promote the horizontal gene transfer, has been shown to spread rapidly and easily between bacteria (Liu et al., 2016), including *E. coli*, *P. aeruginosa*, and *Klebsiella* spp. (Erfanimanesh and Hashemi, 2016). Notably, recent studies evidenced *mcr*-1 gene in up to 2% of clinical isolates (Ye et al., 2016), and about 10% of animal isolates (Irrgang et al., 2016).

Not surprising, all the mentioned microorganisms in this chapter have been included in a global priority pathogens list (global PPL) of antibiotic resistant bacteria, published in 2017 by the World Health Organization (WHO), where the methicillin and vancomycin resistant *S. aureus* strain (MRSA, VRSA) is reported in the "high" priority group and *Enterobacteriaceae* and *P. aeruginosa* carbapenem-resistant strain in the "critical" priority group (WHO, 2017) (**Figure 3**).

Figure 3. Global Priority Pathogen List (PPL) published by the WHO (WHO, 2017).

1.1.3 Reservoir

The medical definition of reservoir is "any person, animal, arthropod, plant, soil, or substance (or combination of these) in which an infectious agent normally lives and multiplies, on which it depends primarily for survival, and in which it reproduces itself in such manner that it can be transmitted to a susceptible vector" (IOM, 2010). In healthcare setting, reservoirs are represented by patients, HCPs and visitors, and by the environment, that is colonized by the microbes spread by persons and by environmental microorganisms. This complex microbial community can be defined as a hospital 'microbiota', and recently the built environments have been considered as super-organisms, with their own microbiota (N.A.S.E.M, 2017). In the case of the hospital environment, the microbiota and its balance is particularly important, as it can be directly related to HAI transmission.

1.1.4 Mode of transmission

The mode of HAI transmission differs for different pathogens and include three main routes: by contact, through droplets, and airborne.

The transmission by contact is the most frequent way of HAI transmission, and involves a contact between the reservoir (an infected or colonized person, or a contaminated surface) and a susceptible host, with subsequent physical transfer of the microorganisms from the reservoir to the host. Contaminated surfaces can include medical devices, instruments, hard surfaces, dressings including gloves, etc. (Siegel et al., 2007).

The transmission through droplets involves respiratory droplets greater than 5µm containing pathogens that are commonly generated from talking, sneezing or coughing, or during particular medical procedures (Papineni and Rosenthal, 1997; Siegel et al., 2007).

When the droplets are smaller than 5µm, the modality of transmission is defined *airborne,* and in this case, microorganisms can be widely dispersed by air currents for hours, and may be inhaled by a susceptible host even at great distance from the original reservoir (Siegel et al., 2007).

1.1.5 Portal of entry and portal of exit

The portal of entry of a microorganism is what permits the HAIs-associated pathogen to enter the susceptible host and cause infection/disease. It is usually represented by mucous membranes, skin wounds, body cavities created by invasive procedures such as catheters and endotracheal tubes (Ostrowsky, 2007; Siegel et al., 2007). The portal of exit is the way by which microorganisms leave the host to be spread and possibly transmitted to another host.

Generally, it is represented by any patient's surface and secreted/excreted body fluids, including blood, urine and faeces (Ostrowsky, 2007). A microbe can be released by different routes, but usually only one or few are important for infection transmission, as they maintain the infectivity of the pathogen.

1.2 Prevention and control of AMR and HAIs

Infection transmission in healthcare settings often occur via contaminated hands of patients and/or hospital staff (HCP), that represent one of the major factors responsible for the transmission of infectious agents to susceptible patients in the healthcare environment, including MDRO organisms such as MRSA and VRE (Siegel et al., 2007).

The control and prevention of AMR and HAIs represent a global priority worldwide, and consistently with this, in the recent years actions have been taken to limit AMR spread and fight the growing diffusion of resistant microorganisms and associated infections (WHO, 2015).

Among the others, the global action plan adopted by the WHO against AMR includes several strategic points, aimed to (WHO, 2015):

1. improve awareness and knowledge of AMR through communication and training as well as through dedicated surveillance and research;
2. reduce the incidence of infections through infection prevention measures, including sanitation procedures;
3. optimize the use of antimicrobial drugs in human and animals health.

Point 1 highlights the importance of AMR awareness and its spread, both in humans and between animals. Toward this aim, specific communication programs have been planned, able to target different types of audience (WHO, 2015). Consistently, the education and training of HCPs represent however a major aspect, and a prerequisite for ensuring that preventive procedures are understood and practiced (Siegel et al., 2007). This would also help the development of new tools, policies and regulations able to counteract the phenomenon (WHO, 2015).

Point 2 highlights the importance of environmental and hand hygiene. Hand hygiene, in particular, has been long considered one of the most important practices to reduce nosocomial transmission. Since the early 1840s, the importance of hand washing was pointed out by Dr. Ignaz Semmelweis, who showed that the incidence of puerperal fever could be drastically reduced by the use of hand disinfection in obstetrical clinics.

Unfortunately, because of his controversial personality, Semmelweis' contribution was underestimated while he was alive, and he died in insane asylum in 1865, unaware that his concept would later be recognized as the most important mechanism for prevention of transmission of nosocomial infection (Kadar, 2019). In fact, since Semmelweis observation, many studies evidenced that hand hygiene effectively affects pathogen transmission (Gould et al., 2017), and has been shown to be associated to a significant decrease in the incidence of HAIs-associated pathogens (Larson et al., 2000).

Beside hands, however, later on also the environment itself can be a huge reservoir of resistant pathogens, especially in the hospital, and notably this aspect has been included as one of key points in the 2017 EU plan against AMR (ECDC, 2017).

Point 3 highlights the risks associated with the abuse of antimicrobials consumption. According to WHO, effective and rapid tools are needed for guiding optimal use of antibiotics in human and animal medicine, and such tools should be easily integrated into clinical, pharmacy and veterinary practices (WHO, 2015). Antibiotic stewardship interventions have been in fact included in the global action plan aimed to fight and prevent AMR, aimed to limit the general excessive use of antibiotics and to promote the selective use of the antimicrobials (ECDC, 2017).

1.3 Environmental contamination in healthcare settings: the role of hospital surfaces

Based on several observations, it is accepted that the hospital environment plays an important role in HAI transmission, as it represents a reservoir for several HAI-associated pathogens (Green et al., 1998; Martinez et al., 2003; Dancer, 2008).

HAI- associated pathogens are in fact able to persist on inanimate surfaces for long periods, increasing the risk of acquisition of infections for other patients (Kramer et al., 2006; Otter et al., 2011). Importantly, a direct correlation exists between surface contamination and HAI acquisition, and the risk to acquire a specific nosocomial pathogen increases when a patient is admitted in a room that was previously occupied by an infected or colonized patients by that specific infectious agent (Huang et al., 2006). The persistence of different pathogens on hospital surfaces has been reported in several published reports, even in the absence of infected/colonized patients. For example, MRSA has been isolated from beds of MRSA-negative patients, and VRE have been found in rooms of VRE-negative individuals (Trick et al., 2002; French et al., 2004).

This is due to the ability of microorganisms of surviving for hours, days, or even months (Kramer et al., 2006) on surfaces, from where they can be easily transmitted to patients by direct or indirect contact (Otter et al., 2013), or shed in other rooms by horizontal transmission from healthcare workers, visitors or asymptomatic carriers (Riggs et al., 2007). Sites that are more frequently touched by hands provide the greatest risk for patients, together with surfaces located right beside patients (critical surfaces) (Bhalla et al., 2004).

In vitro studies showed that inanimate surfaces at low temperature, with high humidity and high inoculum, favour the microorganisms' survival (Kramer et al., 2006). Overall, VRE can survive up to 4 months, spores of *C. difficile* can maintain infectivity for more than 5 months, *P. aeruginosa* up to 16 months, and *Enterobacteriaceae*, including *K. pneumoniae*, may survive for more than 30 months in the healthcare environment (Kramer et al., 2006).

The persistence of microorganisms is also favoured by their ability to form biofilm on surfaces. Biofilms are defined as complex aggregations of microbial cells adhered to surfaces as well-structured microcolonies surrounded by a complex matrix composed of many extracellular polymeric substances (EPS). EPS are polymeric conglomerations of extracellular polysaccharides, DNA and proteins which hold the biofilm on the surface (Donlan and Costerton, 2002; Lopez et al., 2010), allowing microorganisms to be more resistant to disinfectants or cleaning procedures, as recently reported in a study where *S. aureus* dry-surface biofilms were shown to resist to sodium hypochlorite's action (Almatroudi et al., 2018).

1.4 Surfaces sanification procedures: advantages and disadvantages

For a long time hospital cleaning has been considered mostly an aesthetical requirement, but it is clear that potential pathogens are not necessarily associated with evident dirt (Dancer, 2009). Consistently with this, even if a patient room is "clean" does not provide a reliable assessment of the infection risk, even if it may fulfil aesthetic obligations (Dancer, 2009).

Scientific evidences show that the correct and appropriate cleanliness of hospital environments is accompanied by a reduction of surface contamination and a concomitant decrease of microbe transmission to patients (Rampling et al., 2001; Martinez et al., 2003; Dancer, 2008). Importantly, the reduction of surface contamination seems to be a very important point toward control of MDR bacteria spread and nosocomial infections occurrence (Rampling et al., 2001; Tacconelli et al., 2014).

Generally, hospital surfaces are routinely cleaned according to specific timings (e.g. hourly, daily, twice weekly, etc.) or when surfaces appear visible dirty (NPSA, 2007).

Nevertheless, studies show that more than 50% of surfaces result not adequately cleaned when chemical products are used for decontamination (Carling et al., 2008), and several microbes are persistently residing on treated surfaces, as judged by measurements of general markers (Goodman et al., 2008), or specific search for individual pathogens (Kramer et al., 2006; Lawley et al., 2010; Boyce, 2016).

This is because chemical sanitizers are not able to prevent recontamination phenomena, which occur as fast as 30 minutes after sanification and are ultimately the reason of the persistence of microbial contamination (Rutala and Weber, 2014). In addition to this, other important limitations are the high environmental impact associated with their use, and the observation that the chemical-based sanitation can favour the selection of resistant strains. The resistance may be induced both against the disinfectants themselves (Caini et al., 2013; Cornejo-Juarez et al., 2015), and even against antibiotics, as recently reported for the disinfectant chlorhexidine, capable of inducing resistance against the antibiotic colistin (Wand et al., 2017).

Considering the spread of AMR in the last decades, this side effect of some disinfectants appears particurarly undesirable and unsafe.

On the pathway to improve quality of cleaning hospital surfaces, several recent automatic systems have been developed (Carling et al., 2008), including the so-called no-touch technologies, the use of hydrogen peroxide or ultraviolet (Weber and Rutala, 2013; Boyce, 2016). However, because of the yet unsolved safety risks, mainly for inpatients, these methodologies are usable only during room terminal cleaning or, in other words, during the period between patients discharge and new patients admission (Dancer, 2014).

UV irradiation has been investigated as potential decontaminant of environmental surfaces, instruments and air and its effectiveness against *C. difficile* spores, MRSA and vancomycin-resistant Enterococci (VRE) has been demonstrated in hospital rooms (Nerandzic et al., 2010). However, these antimicrobial effects are highly dependent on several parameters, such as time of exposure, the intensity of light and the position of the lamp toward the irradiated surface, and can be accompanied by toxicity after accidental exposure and potential damage of electronic equipment. Consistently with this, UV irradiation is currently regarded as an effective adjunct, and not as a stand-alone technology (Memarzadeh et al., 2010). On the other hand, the HP system exists in different formulations (e.g., HP vapours and dry aerosols) and was shown to be effective against several microbes, such as multi resistant Gram-negative bacteria, including *Acinetobacter* spp. (Chmielarczyk et al., 2012).

However, like UV systems, HP instruments are economically expensive; they require a trained staff and can not be used in occupied rooms.

In addition, antimicrobial surfaces have been proposed. Such surfaces contain agents able to inhibit bacterial growth, like iron, copper and silver (Lansdown, 2006; Noyce et al., 2006; Casey et al., 2010). It is know that silver binds thiol groups present in proteins of microbial cell wall leading to death (Weber and Rutala, 2013), inhibiting also colonization of implanted device (Lansdown, 2006); similarly, copper may generate reactive oxygen radicals that damage nucleic acid and proteins (Weber and Rutala, 2013), and have been demonstrated to have an antimicrobial effect when applied to surfaces, reducing the rate of HAIs (Salgado et al., 2013). Light-activated antimicrobial coatings can be also used by generating reactive oxygen radicals and performing a nonselective toxicity towards microbes. However, all the above-mentioned methods are not suitable for all types of surfaces or settings, are poorly sustainable, and indeed the observed reduction of surface contamination was not so good, being only around 10 to 100 folds compared to untreated surfaces (Weber and Rutala, 2013).

1.4.1 How to measure cleanliness in environmental healthcare settings?

How to define the hygiene of a healthcare setting? Stating that a hospital is clean generally means to assume that it looks clean, but this could not mean that it is safe for patients. In fact, as already mentioned, microorganisms are not visible to the naked eye, so the only visual assessment does not provide the real idea of "*hygienization*", and especially, it cannot predict the infection risk for patient (Dancer, 2009).

Assessing environmental cleanliness in hospitals represents a key point in the management of infectious risk, by evaluating the effectiveness of sanitation interventions that are aimed to reduce microbial contamination on surfaces.

Consequently, the assessment of environmental cleanliness in hospitals has been often performed by using fluorescent markers, microbiological and chemical methods (Dancer, 2009; Dancer, 2014). Fluorescent markers are based on the use of invisible transparent gel that rapidly dries on surfaces following application, and that is easily removable with cleaning. The gel is visible only under a UV irradiation, so it is possible to determine the thoroughness of cleaning by using a UV lamp to illuminate surfaces where the gel is applied (Arvanitakis et al., 2018) (**Figure 4**).

Figure 4. Example of a fluorescent marker (TIPCU, 2012)

However, this method, as the visual inspection, evaluates cleaning performance rather than environmental bioburden, but it can provide a more standardised approach to process evaluation compared to visual inspection (Arvanitakis et al., 2018).

Microbiological methods evidence the total or specific pathogen load by using culture isolation followed by CFU (Colony Forming Unit) count. Devices used for this purpose include Rodac plates (Replicate Organism Detection and Counting plates, containing general or specific selective media), dip slides and nitrocellulose membranes, or swabs, sponges, or wipes able to collect microbes. Devices are incubated to allow the growth of microbes, and then CFU are counted, to provide an index of microbial contamination, expressed as CFU/m^2. However, although these conventional culture-based methods have been of great value, they show important limitations due to the need for cultural isolation, culture time, and biochemical identification (D'Accolti et al., 2019a). In fact, although having the advantage of evidencing only alive microbes, they need 48-120h to detect searched microbes. Moreover some microorganisms are difficult to grow, need specific culture media and particular growth conditions (as the temperature and time of incubation, or anaerobiosis conditions), or grow with different efficiency, thus rendering very time-consuming and complex the analysis of a whole microbial population (D'Accolti et al., 2019a).

Similarly, assays based on the measurement of adenosine triphosphate (ATP) by bioluminescence assay, are commonly used for assessing the effectiveness of cleaning procedures. Although this system is characterized by rapidity of action, being able to give results in a few seconds, is not accurate.

Studies have in fact suggested that this system is not sufficient sensitive to detect very low microbial counts (< 10 CFU/cm^2) (Aiken et al., 2011; Dancer, 2014), can identify not only alive microbes on surfaces but also organic materials misleading the correct result, and can be influenced by residues of detergents/disinfectants (Dancer, 2014; Nante et al., 2017).

By contrast, an efficient monitoring system should be able to lead a detailed characterization of the environmental bioburden in real time, providing rapid and precise description on the microbial population examined. Consistent with this, these limitations could be overcome by molecular methods based on DNA technologies, being able to provide simultaneous analysis of high numbers of different parameters and giving important information about the microbial populations from both a qualitative and quantitative point of view (D'Accolti et al., 2019a).

2. The new paradigm about environmental "health": focus on microbiota

The principle that extreme sanitization must be used in order to reduce HAIs, was tentatively applied for many years in the hospitals environment: the so-called "super sanitization".

However, recent findings provided robust evidences that a "super-sanitization" is doomed to failure (Vangay et al., 2015).

This because an "environment" free of the most skilled competitors, allow resistant pathogens to multiply and occupying more easily niches favouring the appearance of more virulent strains.

The increase of this new awareness stems from the recent acquisitions on the human microbiome, where it is known that microbiota depletions (for example after a prolonged antibiotic therapy), can favour the colonization and implant of potentially pathogenic microorganisms, hypothetically contributing to serious health conditions (Hawrelak and Myers, 2004; Deshpande et al., 2013).

For example, the skin microbiota acts as a barrier against pathogens, due to the beneficial microbiota able to antagonize the colonization of pathogen bacteria (Liu et al., 2018). Consistently whit this, chlorhexidine-based disinfectants, by removing beneficial microbial "sentinels", could facilitate opportunistic pathogen colonization, like methicillin-resistant *S. aureus* (MRSA), with a unavoidable increase of the risk to acquire an infections, rather than protection (Al-Ghalith and Knights, 2015).

Translating these considerations to the "hospital environment", a super-sanitation, aimed to eliminate almost completely microbial communities, is likely not representing a solution for the pathogen contamination problem.

By contrast, trying to manage the balance of microbial populations by restoring healthful communities and favouring good microbial allies, rather than trying to eliminate all microorganisms present, might have a greater potential for effectiveness.

The potential introduction of beneficial species in the "environment" able to counteract the colonization from pathogens was defined "Bidirectional Hygiene" or "Bygiene" (Al-Ghalith and Knights, 2015), and is based on the idea that positive microbes can displace and replace other potential pathogens in the environment, limiting their colonization (**Figure 5**).

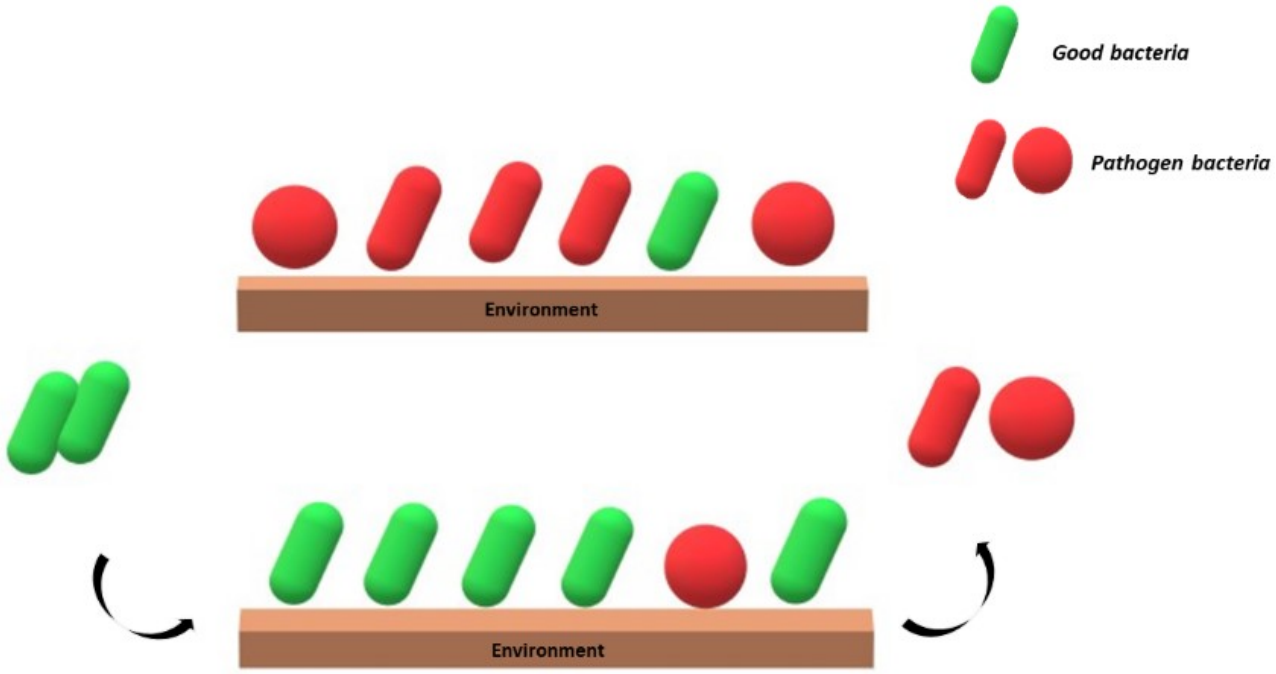


Figure 5. Schematic representation of the "Bygiene" principle. Good bacteria (green) introduced from the outside counteract the colonization of the environment by potential pathogens (red) (D'Accolti et al., 2019a).

2.1 Probiotics

Among the microorganisms potentially useful toward this aim, probiotics appear particularly suitable, as they are "live microorganisms that confer a health benefit to the host when administered in adequate amounts", accordingly with FAO/WHO guidelines" (FAO and WHO, 2002), and are able to "fill the void" disadvantaging the colonization by pathogens (Hill et al., 2014).

The original hypothesis of the positive role played by certain bacteria was first introduced in 1908 by the Russian scientist and Nobel Laureate Elie Metchnikoff, who observed the potential beneficial effects on human health given by the consumption of fermented foods, particularly with lactic acid bacteria (Vaughan, 1965).

Since Metchnikoff's original findings, several studies confirmed the positive role of probiotics on the health of the host (Fernandes et al., 1987). According to this, the use of probiotics has considerably increased in recent years, and today there is considerable interest in their use for a variety of medical conditions so much so that millions of people around the world consume them daily for perceived health benefits (D'Accolti et al., 2019a).

Generally, the most common bacterial strains used as probiotics are *Lactobacillus* and *Bifidobacterium*, since they are considered as GRAS (Generally Recognized as Safe) (Rivera-Espinoza and Gallardo-Navarro, 2010; Butel, 2014), they are the predominant groups of the gastrointestinal microbiota and have a long history of human safe of use (Doron and Snydman, 2015).

Many bacterial species belonging to genera *Lactobacillus*, *Enterococcus, Propionibacterium, Bacillus* and yeasts (i.e. *Saccharomyces boulardii),* have been used as probiotics thank to their effect promoting health (Cousin et al., 2012; Tripathi and Giri, 2014).

The "ideal" probiotic should be non-pathogenic, remain viable in the environment that has to be colonized. Thus, general criteria for probiotic selection include their safety in the host, ability to adhere to surfaces and to act by competitive antagonism against pathogenic bacteria (Collins et al., 1998; Ouwehand et al., 2002).

Many research results from human studies and animal models have shown the clinical potential use of probiotics against many pathogens. For this reason, their application in the clinical field for human disease treatment is extremely wide, and they have been effectively used for: infectious diseases, diarrhoea, obesity, intestinal infections, oral/tooth diseases, dermatological and inflammatory diseases (Sullivan and Nord, 2002).

Notably, probiotics have been also shown their efficiency in reducing the occurrence of important healthcare associated infections, including upper respiratory infections (Banupriya et al., 2015), antibiotic- associated diarrhoea (Squellati, 2018), and necrotizing enterocolitis (Patel and Underwood, 2018).

2.2 *Bacillus:* general features

Bacillus is a genus of Gram-positive, endo-spore forming, rod-shaped bacteria belonging to the phylum *Firmicutes,* including 266 named species. *Bacillus* can behave as obligate or facultative aerobic microbes, as they have the ability to live also in absence of oxygen, and they are catalase enzyme-producers.

They are ubiquitous in nature, commonly found in water, soil, air, as well as in human and animal intestine, so they can be considered part of the human microbiota (Hong et al., 2009). *Bacillus* produce compounds with antimicrobial activity, including peptides, lipopeptides, and bacteriocins (Jack et al., 1995; Lee and Kim, 2011).

They are genotypically and phenotypically heterogeneous, characterized by several different physiological properties, including the capability to decompose many heterogeneous substrates derived from animal and plants sources (i.e. cellulose, starch, agar, hydrocarbons) (Lutz et al., 2006), allowing them to colonize a wide variety of ecological habitat.

This ability to grow and persist in such different ecosystems is also due to their ability to produce endospores, quiescent cell types with a peculiar structure that allows survival at difficult environmental conditions, such as high temperatures, ionizing radiations, mechanical abrasion, chemical solvents and pH extremes (Nicholson et al., 2000).

2.2.1 Endospore structure and Sporulation mechanism

The resistance of an endospore can be explained by its structure: the genetic material is accumulated and surrounded by protective coatings, allowing them to persist in difficult environmental conditions.

From the outer to the inner part, the endospore is composed of (**Figure 6**):

- an ***exosporium***, a glycoprotein and membranous layer present only in some endospore-forming bacteria, including *Bacillus megaterium* and *Bacillus anthraces* (Lanzilli et al., 2016; Boone et al., 2018);
- a ***coat***, subdivided into an outer and an inner layer, that provides resistance and favours germination since it contains enzymes that are involved in this process (Ricca et al., 1997)
- a ***cortex,*** a thick double-layer of peptidoglycan, which is surrounded by an external and an internal membrane;
- a ***core,*** the central part of the endospore, which contains the original cell structures, such as ribosome and enzymes, and a larger amount of calcium dipicolinate (Ca^{2+}-DPA), that consists of up to 20% of the dry weight. Ca^{2+}-DPA stabilizes nucleic acids and contribute to endospore dehydration and acquisition of heat resistance. The core also contains small acid-soluble proteins (SASPs), which bind and condense DNA, protecting endospores by UV light and DNA-damaging chemicals. The core cytoplasm has a water content of less than 30 instead of 88% of that of the vegetative cell (Setlow, 1994).

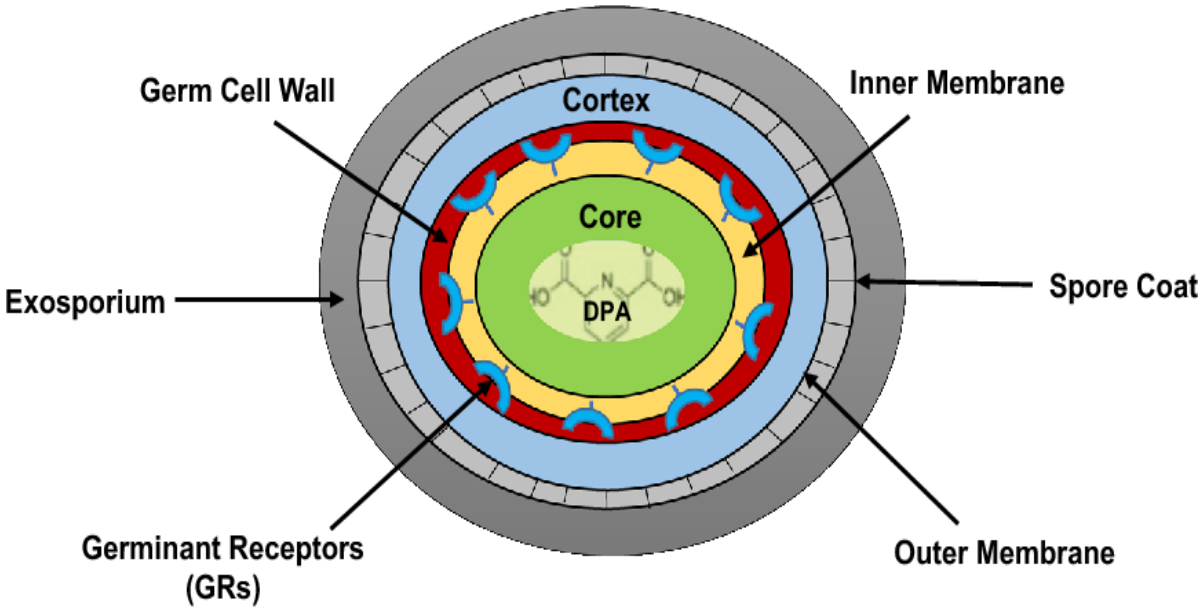


Figure 6. Schematic representation of a bacterial endospore (Tehri et al., 2018).

The mechanism of sporulation has been finely studied in the model species *Bacillus subtilis* (Piggot and Hilbert, 2004).

The process occurs in response to nutritional starvation or other difficult environmental conditions; it takes about eight hours and produces a single endospore developed within the bacterial cell (de Hoon et al., 2010). It starts with the duplication of bacterial genome that forms an axial filament that extends along the entire length of the bacterial cells (stage I). The cell starts to divide, forming an asymmetrical septum localized near to one pole of the cell, resulting in the formation of a smaller cell, the forespore, and a larger one, the mother cell (stage II); they are genetically equal, since they both get an identical chromosome, but unequal from the biological and metabolic point of view. Later, the mother cell engulfs the forespore, surrounding it with a second membrane (stage III), than the mother chromosome disintegrates and the exosporium synthesis starts to occur (stage IV).

Between the two membranes, a cortex layer of peptidoglycan is than formed (stage V), followed by dipicolinic acid synthesis and calcium incorporation into the spore coat (stage VI). The endospore becomes mature, and it is released by the mother cell (stage VII) (**Figure 7)**

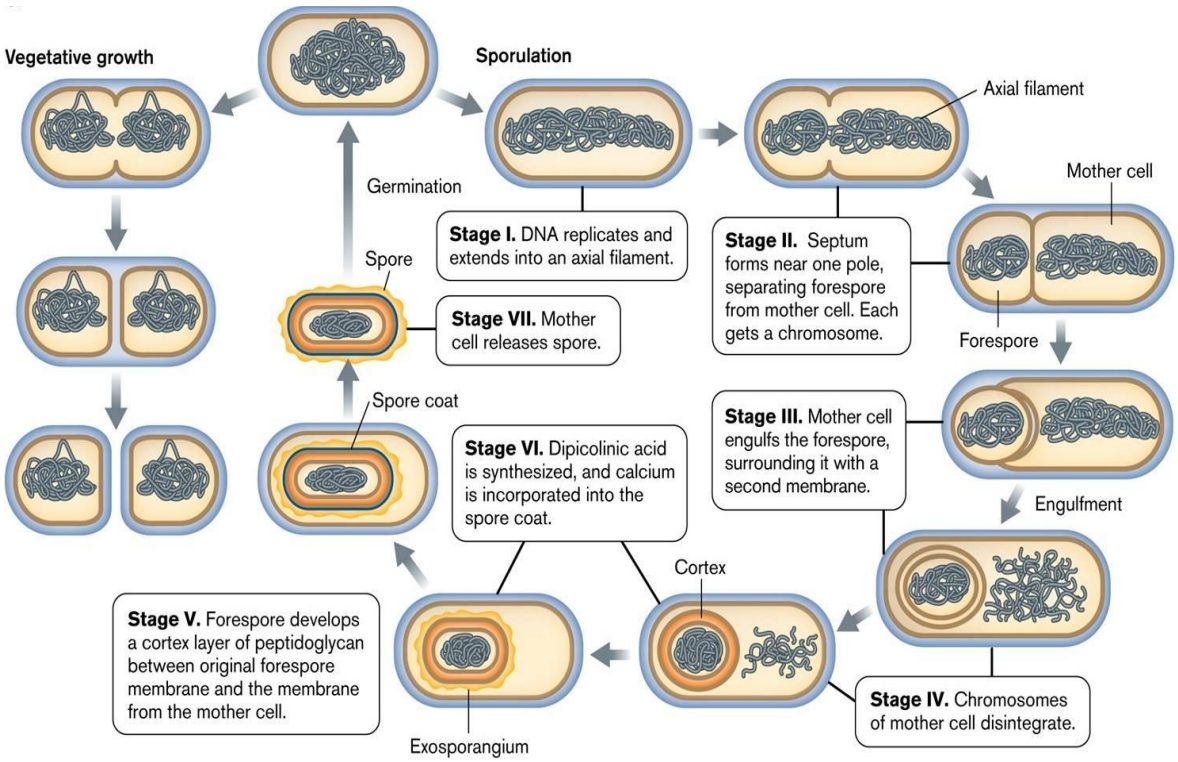


Figure 7. Schematic representation of sporulation and germination mechanisms in *Bacillus* species. Image from the website: *www.onlinebiologynotes.com/bacterial-spore-structure-types-sporulation-germination/* (last accessed in June 24th, 2019)

Sporulation of *B. subtilis* is regulated by the action of four key transcription factors, coordinated by the first regulator, the DNA-binding protein *spo0A*, responsible for entry into sporulation (Patrick J. Piggot, 2002).

The endospore can remain inactive for several years. When the environmental conditions became favourable to the vegetative growth, thanks the presence of water and nutrients, the spore can germinate originating the vegetative form. This process, named germination, involves a series of morphological and biochemical changes that lead to degradation of the spore layers, cytoplasm rehydration and loss of spore dormancy and resistance. The germination takes about 90 minutes, and requires H_20, nutritional factors and adequate temperature (**Figure 7**).

2.2.2 Pathogenicity and Safety

Bacillus genus includes a large number of species and sub-species, known to be apathogenic for humans, except for two well recognizable species that are medically significant: *B. anthracis* and *B. cereus* (Ahmad et al., 2013).

B. anthracis is the etiologic agent of anthrax, a common zoonotic disease widespread throughout the world.

Anthrax disease can occur in three different forms: cutaneous, gastrointestinal and pulmonary. The cutaneous form develops following the contact with contaminated animal products, and it affects the skin and tissues around the site of infection.

The gastrointestinal form occurs when people eat contaminated products from animals. The pulmonary form occurs when people inhale anthrax spores, and it is considered to be the most deadly form of anthrax. *B. anthracis* pathogenicity is due to the presence of several virulence factors, including the capsule, with anti-phagocytic activity.

B. cereus is a food-borne pathogen causing diarrheal disease in humans (Duport et al., 2016). In particular, it causes two different types of food poisoning: the diarrhoeal type (infection) and the emetic type (intoxication). The diarrhoeal disease is characterized by abdominal pain and diarrhoea, and it is caused by at least three different enterotoxins, which are produced by *B. cereus* during vegetative growth in the human intestine. The emetic disease is caused by the production of the emetic toxin that causes emesis generally after 0.5- 6h from ingestion of contaminated food (Granum and Lund, 1997; Ehling-Schulz et al., 2004).

By contrast, the other *Bacillus* species are considered GRAS (Generally regarded as safe) (FDA, 1999; EFSA, 2019), and their safety is extensively reported in the literature (Hong et al., 2008). *B. subtilis*, for example, is considered a Class 1 agent (no risk) in the National Institute of Health (NIH-US) Guidelines for Research Involving Recombinant DNA Molecules (DHHS, 1986). Additionally, it is not toxigenic (no toxins production), and has been granted "Qualified Presumption of Safety" status by the European Food Safety Authority (Hazards, 2010). Like *B. subtilis,* other environmental *Bacillus* species, including *B. licheniformis, B. pumilus,* and *B. megaterium* are considered safe, since they have never been associated to infection and disease in humans.

2.2.3 Uses of *Bacillus* spores

Non-pathogenic bacteria belonging to the *Bacillus* genus have a long history of safe uses, including biotechnological, medical and agriculture applications.

Bacillus species are used for the industrial production of a variety of bio-products, including proteins (Zweers et al., 2008), enzymes (Contesini et al., 2018), and biopolymers (Jin et al., 2016). *B. subtilis* is in fact used as a prolific “cell factory” for industrial enzymes and biopharmaceutical proteins (Westers et al., 2004), having excellent fermentation ability and being one of the best known bacteria useful for research about Gram-positive bacteria.

Beside, these bacteria have been used for food preparation, particularly for Asiatic food, as a particular strain of *B. subtilis (Bacillus subtilis* var. *Natto),* is used for the production of

Natto, a traditional Japanese food made from soybeans fermentation, containing up to 10^8 cells/gr (**Figure 8**) (Weng et al., 2017). Other foods, equivalent to Natto, are widely produced in other eastern countries.

Figure 8. Natto, a traditional Japanese food produced from soybeans fermented by *Bacillus subtilis* (Weng et al., 2017).

In addition, *B. subtilis* spores have been widely studied in simile-space experiments (Horneck et al., 2010), and are currently used as adjuvant in vaccines (de Souza et al., 2014). *B. subtilis QST 713* is normally used as a bio-fungicide in agriculture (i.e. Serenade, Bayer) (Leyva Salas et al., 2017), and can be introduced in soil by inoculation in horticulture and agriculture fields (Trabelsi and Mhamdi, 2013).

Similarly, *B. pumilus* spores have a fungicide effect after germination into vegetative forms: *B. pumilus* GB34 is known to be used as a fungicide, preventing infections caused by *Rhizoctoniae* e *Fusarium* in soy (EPA).

In the same way, *B. megaterium* is used for production of enzymes (Vary et al., 2007).

In medicine, *Bacillus* spores-based products have been widely used as immuno-stimulatory agents for treatment of diseases, including gastrointestinal and urinary tract disease (Ciprandi et al., 1986; Mazza, 1994). In particular, Enterogermina® (Italy) based on *B. clausii* spores, and Biosporin® (Ukraine), mixture of *B. subtilis* 2335 and *B. licheniformis 2336*, are indicated to treat and prevent intestinal disorders associated with alterations in the qualitative and quantitative composition of the normal human intestinal flora, occurring in most cases after long treatments with antibiotics (Mazza, 1994).

Bacillus species have also been proposed as food additives in poultry. Notably, numerous studies evidenced that the dietary supplementation of *B. subtilis* PB6 improves animals health, increase body weight and egg production, and significantly reduce the presence of *C. perfringens* pathogen in intestinal microbiota of animals (Teo and Tan, 2006; Jayaraman et al., 2013). Finally, *Bacillus subtilis* species are used also in aquaculture, as they have been shown to inhibit the growth of pathogen *Vibrio* spp. in marine crustacean (Vaseeharan and Ramasamy, 2003), and in parrot fish (Liu et al., 2018).

Main uses are summarized in **Figure 9**.

Figure 9. Main uses of *Bacillus* spores (Caselli et al., 2019b).

2.2.4 Probiotic-based sanitation in healthcare settings

Based on the positive effects of *Bacillus* bacteria in several human fields, and considering the big concern of contamination in hospital environments, we studied the effectiveness of *Bacillus* spores for cleaning purposes in the hospital environment.

The system (named PCHS, Probiotic Cleaning Hygiene System, Copma, Ferrara, Italy) consists in the use of an innovative sanitation strategy based on the addition of spores of *Bacillus* to eco-sustainable detergents. Notably, *Bacillus* spores, thanks to their resistance to many physical-chemical factors, are particularly suitable to be added to concentrated eco-sustainable detergents, as they maintain their activity.

In our first studies, we showed that *Bacillus* spores can germinate when the concentrated detergent (containing preservatives to prevent spore germination) is diluted in water and seeded on dry inanimate surfaces, originating the vegetative bacteria, that are responsible for the cleaning action (Caselli et al., 2016b).

Three species were included in probiotic-based cleansers, namely *B. subtilis*, *B. pumilus*, *B. megaterium,* which have been selected for their capability to remove organic dirt by enzymatic activity, and also counteracting the growth of potentially pathogenic microorganisms, including Gram-positive, Gram-negative bacteria as well as mycetes by competitive exclusion (Caselli et al., 2016b) (**Figure 10**).

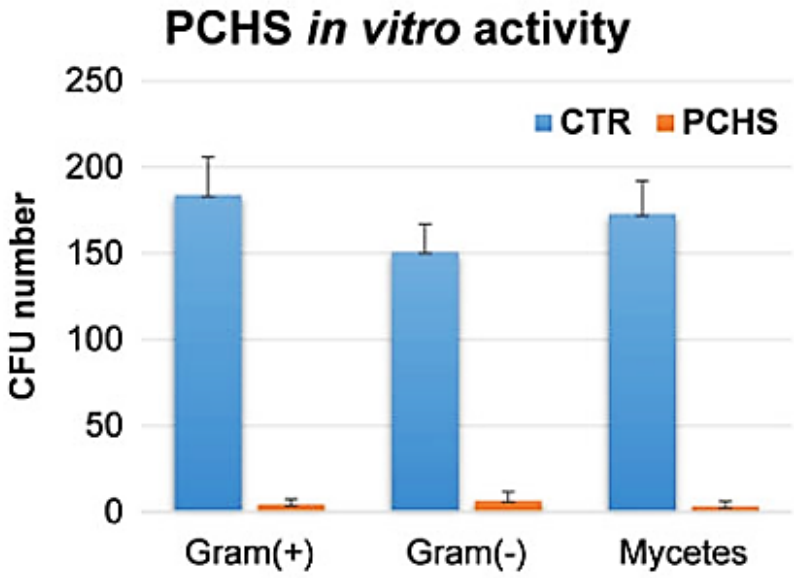


Figure 10. PCHS activity against Gram-positive, Gram-negative bacteria, and mycetes by *in vitro* tests (Caselli et al., 2019b).

The results collected in the first studies performed on field, performed since 2012 in healthcare structures in Italy and Belgium, showed a significant decrease of pathogen load on treated surfaces in all enrolled settings (Vandini et al., 2014; Caselli et al., 2016b). It is important to note that contamination monitoring (performed by CFU count) was performed at 7 hours after sanitation, a time sufficient to allow the natural process of re-growth of microorganisms. This sampling time was useful to evidence recontamination phenomena, that occurred when using chemical-based sanitation, but that were highly inhibited by the probiotic-based system.

PCHS resulted in fact able to stably remodulate microbiota on surfaces, inducing a steady abatement of pathogen contamination up to 90% more than conventional disinfectants (Vandini et al., 2014).

Most importantly, this effect was not accompanied by selection of drugs-resistant strains, but, rather, it caused an overall reduction of the antimicrobial resistance genes of the whole microbial population contaminating hospital surfaces (Caselli et al., 2016b).

Last, but very importantly, PCHS-*Bacillu*s resulted devoid of infectious risk in hospitalized patients and genetically stable. To investigate these points, a microbiological surveillance searching for PCHS-*Bacillus* has been implemented in all healthcare settings where PCHS was applied since 2011. Over 60,000 clinical specimens, derived by HAI or uninfected patients hospitalized in PCHS-treated hospitals, have been analyzed for the presence of PCHS-*Bacillus*, showing their total absence in all clinical samples, and confirming that they do not represent an infectious risk for inpatients (Caselli et al., 2016a).

In parallel, specific molecular methods have been set-up to evaluate whether PCHS-*Bacillus* could acquire new genes from pathogens, after their spread on surfaces.

The results showed that, despite the continuous contact with surface pathogenic and drug-resistant bacteria on treated surfaces, PCHS-*Bacillus* did not acquire any new resistance gene from the surrounding pathogens, suggesting a high genetic stability and the absence of mutagenicity or genetic exchange, and confirming their safety of use (Caselli et al., 2016b).

3. Bacteriophages: the enemies of our enemies

Infections by bacteria have been one of major causes of health disorders throughout human history. The discovery of penicillin in 1928 by Alexander Fleming, followed by continuous discovery and use of new antibiotics in the following decades, opened the so-called 'antibiotic era', where the management of bacteria-associated diseases was considered an obvious success. Unfortunately, the massive use of antibiotics has led to the appearance, and consequently evolution and spread of sophisticated mechanisms of drug resistance by which bacteria could resist and survive to the antibiotic attack, contributing to the continuous growth and diffusion of antimicrobial resistance (AMR). Currently, there is an urgent need to find new strategies for MDR bacteria treatment, and bacteriophages seem to be a promising tool.

Bacteriophages, as known as phages, are small viruses ranging in size from 20 to 200 nm (Jamal et al., 2019) that infect exclusively bacteria in a specific way and are harmful for humans. They represent the most abundant organisms in the biosphere, being ten times more numerous than bacteria (Hendrix, 2002) and they can be found in all environments where bacteria grow, contributing to limit their over-spreading and maintaining the right equilibrium in ecosystems.

Phages are commonly detected in water, soil, sewage (Tartera and Jofre, 1987; Kumari et al., 2010), and have been also isolated in human and animal samples, such as faeces, urine, saliva, serum (Gantzer et al., 2002; Bachrach et al., 2003).

Bacteriophages were first discovered in 1915 by the British bacteriologist William Twort, and, independently, in 1917 by the French-Canadian microbiologist Felix d'Herelle who realized the existence of some biological entities with the ability to kill bacteria.

D'Herelle proposed to name them "bacteriophages" to indicate that these viruses (phages) were able to "eat" and "devour" bacteria (Sulakvelidze et al., 2001).

After this discovery, d'Herelle had the first attempt to use bacteriophages therapeutically, and he decided to use them to restore health of a man suffering from dysentery (Sulakvelidze et al., 2001; Keen, 2012). Patient's symptoms diminished after only one a single administration of specific phages, and the man fully recovered within few days (Sulakvelidze et al., 2001). The use of bacteriophages as antimicrobial agents for human infection treatment was early proposed and preliminary data reported appeared promising.

So, during the 1920s and 1930s they were widely used for bacterial infections treatment in Eastern Europe, and in particular, in the former USSR States, with the Eliava Institute of Bacteriophage, Microbiology and Virology (Tbilisi) as one of the key centres (Sulakvelidze et al., 2001) (**Figure 11**).

Figure 11. Eliava Institute of Bacteriophage, Microbiology and Virology (IBMV), Tbilisi (Georgia).

The Eliava Institute of Bacteriophage, Microbiology and Virology (IBMV), was founded in Tbilisi (George) in 1923 and developed by the contribution of the Georgian microbiologist George Eliava and Felix d'Herelle (Chanishvili, 2012).

Curiously, despite of his important contribution to public health, in 1937, George Eliava was declared enemy of the people and he was executed. Nevertheless, the Institute continued to maintain its focus on phage therapy, and bacteriophages were used to treat bacterial infections in clinic. In first times, bacteriophages started to be largely produced in the 1940s. In fact, D'Herelle founded in Paris the "Laboratoire du bacteriophage" and started to sell various cocktails of bacteriophage.

However, the discover of penicillin followed by the beginning of the "antibiotic area", together with the scientific debate regarding the use of phages for medical treatment due to poorly controlled trials, led a sharp decrease of interest in phage therapy.

After World War II, phages were progressively abandoned in the Western World whereas in the Russia, Poland and Georgia they were continuously employed in clinic field, and they are currently still used.

Some old reports or studies from USSR countries reported successful treatments of various infections, including *P. aeruginosa* or *S. aureus*, but often they were written in Russian language, and lacked of control groups. The paucity of peer-reviewed controlled clinical trials made difficult to properly evaluate the effectiveness of such therapeutic by western standards and laws. Therefore, these studies were not able to convince the rest of the world about phage efficacy and safety.

Nonetheless, with the worldwide spreading of MDR bacteria, together with the increasing of the ineffectiveness' risk of antibiotics, the interest of therapeutic use of bacteriophages is going to remerge again in Western World (Thiel, 2004).

3.1 Morphology and classification

Bacteriophages are obligate intracellular parasites that infect only bacteria. Like eukaryotic viruses, they multiply in the host by using its biosynthetic machinery since phages have no own ribosomes and enzymes.

A typical bacteriophage particle contains a linear or circular nucleic acid (double or single DNA, dsDNA or ssDNA; double or single RNA, dsRNA or ssRNA), surrounded by a proteinaceous capsid (Weinbauer, 2004).

The current classification of bacteriophages, defined by the International Committee on Taxonomy of Viruses (ICTV), distinguishes phages based on their nucleic acid and morphology of the viral particle (Abedon, 2008) (**Figure 12**), including:

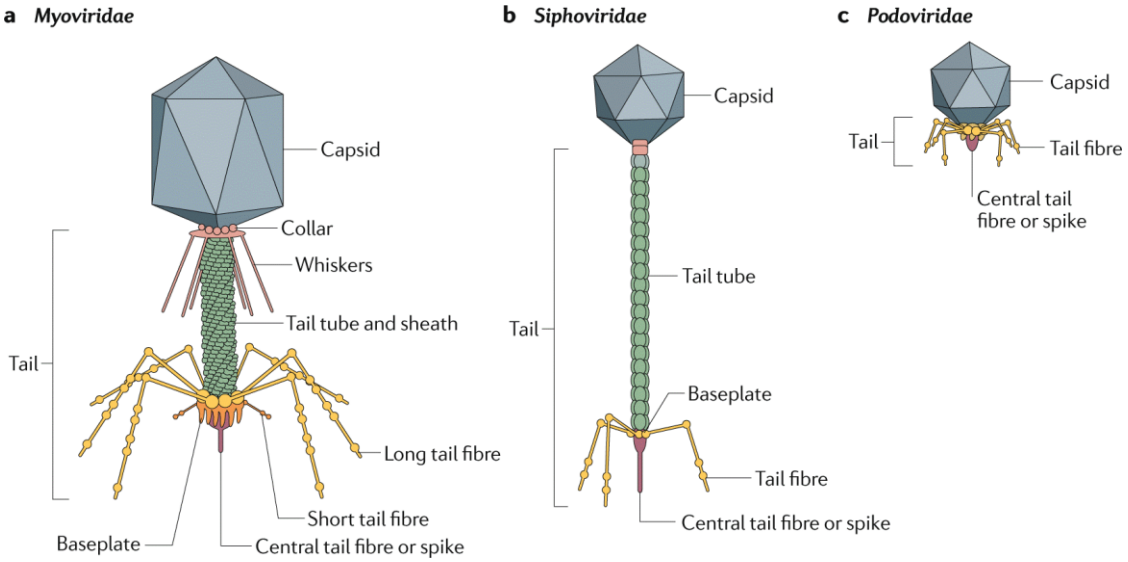
- Tailed viruses (*Caudovirales* order)
- Helical viruses (rod-shaped or filamentous)
- Isometric viruses (generally icosahedral)
- Pleomorphic viruses

The *Caudovirales* order includes tailed bacteriophages, and represents the most wide and numerous group (HW, 1998). Tailed phages have a double strand DNA genome (dsDNA) enclosed in an icosahedral head, which is attached to a tail by a specific connector proteins. The tail can have a disc or spiral shape, ending with specific fibres that facilitate phage adsorption to the bacterial host. Based on tail characteristics, the order *Caudovirales* is differentiated into three families: *Myoviridae, Siphoviridae, Podoviridae.*

- *Myoviridae* family includes phages with a long rigid tail with helical symmetry, consisting in a central tube with a contractile sheath, a collar, a base plate, tail pins and long fibres. Phage T4 is the most studied phage belonging to this family (Yap and Rossmann, 2014).
- *Siphoviridae* includes phages with long non-contractile tails usually with short terminal and subterminal fibers. Phage λ is one of the most studied phage belonging to this family (Jonczyk et al., 2011).
- *Podoviridae* family includes phages with very short non-contractile tails, where the adsorption apparatus is directly connected to the neck region. Phage T7 is one of the best studied phages of this family (Jonczyk et al., 2011)

Isometric, helical and pleomorphic phages represent the minority in comparison with tailed phages. Isometric phages include all four types of genome, including fragmented dsRNA (family *Cystoviridae*) whereas the helical and pleomorphic phages are mostly constituted by dsDNA genomes, with the only exception represented by family *Inoviridae*, which are filamentous viruses with ssDNA genomes (Abedon, 2008).

A)



B)

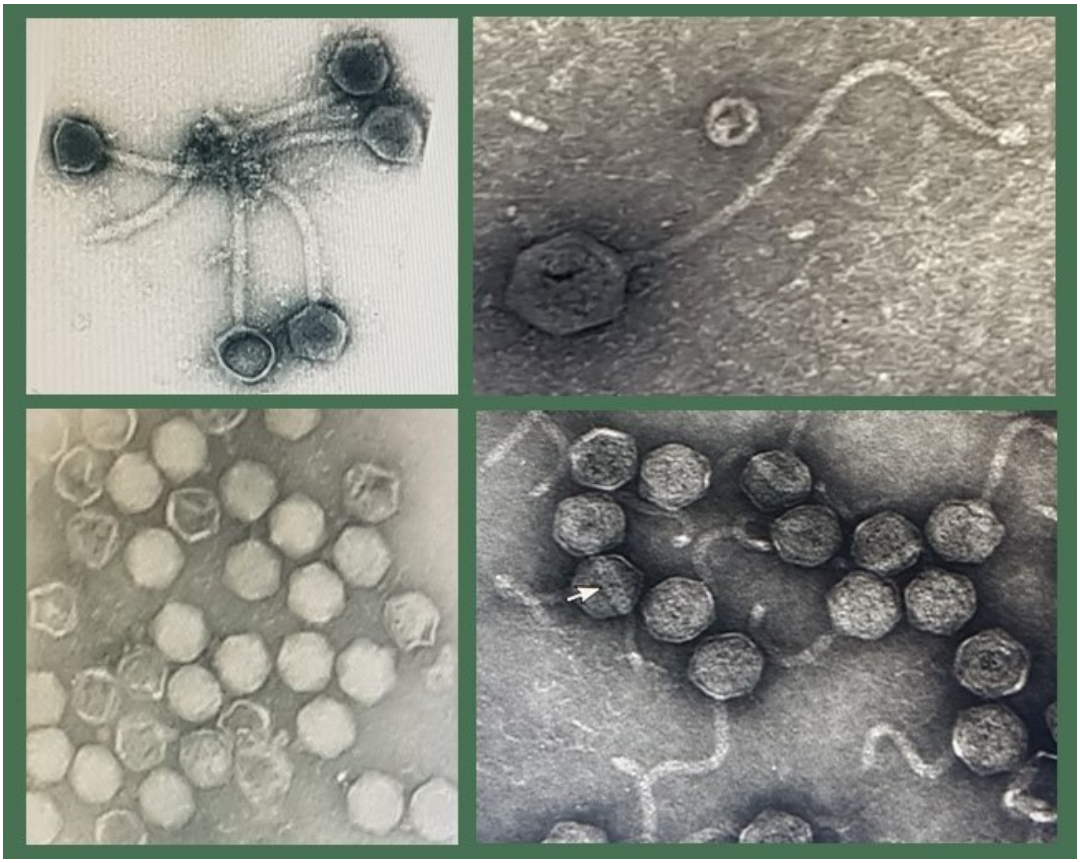


Figure 12. Bacteriophage morphology. **Panel A.** Phage families (schematic representation) (Nobrega et al., 2018). **Panel B.** Phage families (transmission electron microscopy, TEM).

3.2 Lifecycle of bacteriophages

Bacteriophages can be virulent or temperate, based on the different life cycles that they can establish in the prokaryotic host (lytic or lysogenic infection, respectively) (**Figure 13**) (Weinbauer, 2004).

The lytic cycle occurs when virulent phages infect and rapidly multiple within the host bacteria to produce viral progeny. This cycle results in the release of newly formed progeny virions by lysis of the host cell, mediated by phage-encoded enzymes able to degrade the bacterial cell wall.

By contrast, temperate phages integrate their nucleic acid into the host genome, and remain in the host in a dormant stage (prophage). This type of infection is named lysogenic, and bacterial cells that are lysogenized by temperate phages acquire resistance against infection by lytic phages. The prophage can stably reside within the host cell for long periods of time, until the appropriate physiological or environmental conditions can favour the reactivation of the lytic lifecycle (Weinbauer, 2004).

Bacteriophages have a very narrow host range, being able to infect only a specific bacterial species or even a specific microbial strain. The specificity is determined by the nature and structure of receptors that are present on bacterial cell surfaces (Rakhuba et al., 2010), which interact with the phage anti-receptors. The productive cycle includes the following phases: adsorption, penetration/uncoating, synthesis of virus components (including genome replication), assembly and release. In the adsorption phages bind the receptor molecules on the bacterial cell surfaces, represented by teichoic acids in Gram-positive bacteria, or lipopolysaccharide (LPS) and protein receptors present on the external membrane of Gram-negative bacteria (Rakhuba et al., 2010). The adsorption is the result of a random phage-cell collision, being phages not able to move autonomously (Bertozzi Silva et al., 2016).

After the adsorption, phage nucleic acid is injected into the host bacteria thanks the production of the enzyme lysozyme able to degrade bacterial cell.

After this step, phage nucleic acid is exposed and phage replication starts. The host cellular machinery (e.g. ribosomes), under viral control, is forced to synthesize viral products, by translating viral mRNAs and driving the biosynthesis of phage specific proteins. In the following step maturation and assembly of the new virions take place. Assembly can be favoured by the involvement of protein helpers, as it has been shown for T4 phage infecting *E. coli* (Ahmad et al., 2013).

Phage progeny is released via cell lysis, mediated by endolysin enzyme, able to break down the bacterial peptidoglycan.

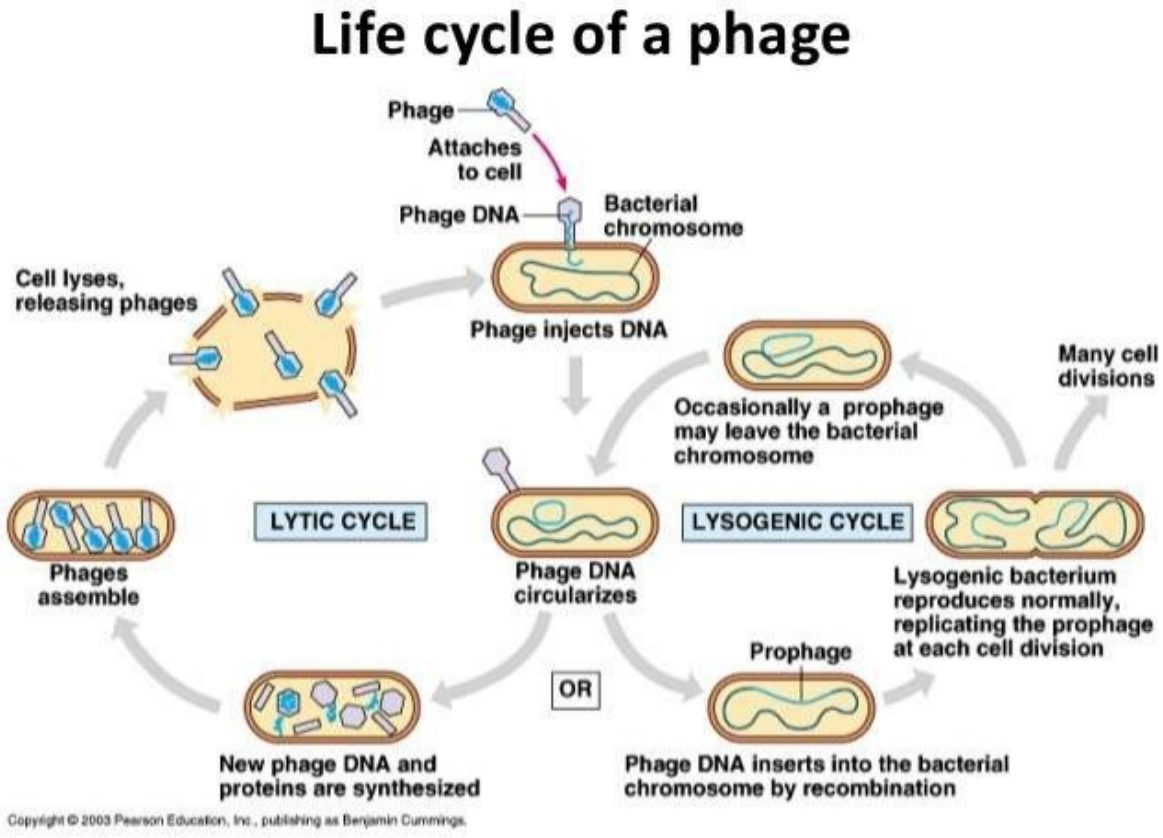


Figure 13. Bacteriophage life cycles: lytic, lysogenic. Each of the two cycles starts with phage adsorption on bacterial cell, followed by injection of viral nucleic acid into the host cell (bacterial cell). In lytic phage cycle, injected DNA starts to manipulate the host machinery in order to produce phage specific proteins and viral DNA. Phage proteins assemble to form mature phage particle. Once viral progeny has been formed, they start to produce compounds able to degrade cell wall and membrane, by causing the bacterial cell lysis and the release of new phages in the environment. During lysogenic phage cycle, following DNA injection into the host cell, phage integrates its nucleic acid in the bacterial genome originating a prophage, that can remain in this condition for long period of time, replicating its DNA together with the bacterial host. Prophage can be stimulated to exit the genome and form circular DNA (episomes) stimulating the switch to a lytic cycle. Frome the website: https://www.slideshare.net/suganyakunju/bacteriophages-71259201 (last accessed in November 24th, 2019)

3.3 Phage uses

3.3.1 Phage therapy

The bacteriophage ability to kill bacteria make them good candidates against bacterial infections. Despite being ignored for several years in Western Medicine, recent research studies re-ignited interest about their potential therapeutic employment.

Pre-clinic studies conducted in 1980's by Smith and Huggins (Sulakvelidze et al., 2001) showed that the use of anti-*E. coli* phages was able to reduce the target bacterial load in animals (Smith and Huggins, 1983; Smith et al., 1987).

These first results opened the way to other interesting studies that evidenced that phages could also be effective against other bacterial target in animals, including *P. aeruginosa* and *S. aureus* (Soothill, 1994; Matsuzaki et al., 2003). The efficacy of phage treatment against *S. aureus* in animals was also demonstrated by Wills et al. who showed that the subcutaneously injection of *S. aureus* and phage treatment in a rabbit model resulted in absence of abscesses, compared to what observed in not-treated animals (Wills et al., 2005).

In the 1980's studies, phage therapy was shown effective in patients with mixed infections caused by *S. aureus*, resulting in health improvement and bacterial eradication (Slopek et al., 1983; Slopek et al., 1987). Others well-designed clinical trials were recently launched, showing encouraging results, mainly for wound infection in burn patients, ulcers and chronic otitis (Rhoads et al., 2009; Wright et al., 2009; Rose et al., 2014).

In 2014, the first multicentre randomized controlled trial in humans (France, Belgium, and the Netherlands) studied patients with wound infections caused by *E. coli*. The project is currently running, and preliminary results seem to be very promising (France Europe Innovation, 2013-2019).

Currently, phage therapy has still not been registered for general clinical use in the Western world. However, in the US, Food Drug Administration (FDA) has recently approved the first clinical trial of an intravenously administered bacteriophage-based therapy (LaFee and Buschman, 2019). Unlike wide spectrum antibiotics, phage therapy is characterized by selectivity and specificity of action. This means that phages are able to infect only specific types of bacterial cells, maintaining the health of commensal flora. On the other hand, a precise diagnosis is needed to plan a therapeutical phage application. For these reasons, wide host range phages able to infect a large number of strains are generally more indicated for therapy.

Interestingly, phages can destroy bacterial biofilms and are effective where the antibiotic therapy fails, such as in cases of chronic infections caused by biofilms-producing bacteria (Maciejewska et al., 2018). This is because some phages produce depolymerases that degrade the biofilm matrix (Abedon, 2015), and act towards bacterial cells that are covered by this complex structure. Importantly, only lytic phages are indicated for phage therapy, as lysogenic phages have the high probability to cause horizontal gene transfer between bacteria (Kazmierczak et al., 2014).

Generally, only the fully sequenced bacteriophages are considered suitable for treatment of bacterial diseases, since the DNA sequencing guarantees the absence of lysogeny or toxic genes (Kazmierczak et al., 2014).

Phage are active against antibiotic-susceptible or resistant bacteria, however bacteria can develop resistance even against bacteriophages. The bacterial resistance to phages has been studied in detail and, according to literature, can occur in several ways. The most common mechanism is the prevention of phage adsorption. To this purpose, bacteria can modify surface receptors, and produce proteins able to block or mask receptor sites (Labrie et al., 2010).

Bacteria can also prevent phage DNA entry into the host cell, through the superinfection exclusion (Sie) systems, consisting in particular proteins blocking phage entry (Labrie et al., 2010; Maciejewska et al., 2018). Additionally, bacteria can disrupt the invading foreign DNA by using specialized restriction–modification systems at the DNA/RNA level (CRISPR/Cas system) (Labrie et al., 2010). Last, bacteria can undergo abortive infection, inducing the death of the infected host cell, and thus preventing phage multiplication (Labrie et al., 2010).

However, development of resistances can be reduced by using bacteriophage cocktails for therapy (Principi et al., 2019), since it decreases the selective pressure that can be exerted by a specific bacteriophage on its host. Consistently, phage preparation used for therapy are generally composed of multi-phage cocktails, which can be further modified and/or improved by adding more phages or replacing those already present with others (Maciejewska et al., 2018). This procedure is quite simple, since it is possible to select the phage of interest from an existing bacteriophage collection, or by isolating new bacterial viruses from the environment, being these the most abundant ubiquitous entities in the world (Chan et al., 2013; Maciejewska et al., 2018).

Interestingly, during the long history of using phages as therapeutic agents in Eastern Europe and the former Soviet Union, phages have been administrated to humans in different ways (orally, intravenous, rectally) and no reports of serious side-effects were described associated with their use (Sulakvelidze et al., 2001).

3.3.2 Bacteriophages in decontamination/sanitation procedures

Given the high specificity of action, bacteriophages have been suggested as decontaminating agents of food and surfaces used for food preparation, as many foodborne illness are caused by the consumption of foods contaminated by bacteria, including *E. coli* O157:H7,

Salmonella spp. and *Listeria monocytogenes,* that represent the most dangerous foodborne pathogens (Abdelhaseib et al., 2019).

Decontaminating foods (i.e. fruits, vegetables and meat) presents considerable challenges, since the most common strategies to clean them are washing with water or by using solution with antibacterial chemicals which are well-tolerated by humans (Abuladze et al., 2008).

However, the only treatment with water does not reduce the bacterial pathogen load, and, as reported above, chemicals have some limits that include the acquisition of resistance by microorganisms to chemicals themselves with consequent loss of their efficacy, and the alteration of organoleptic properties of treated foods (Abuladze et al., 2008).

By contrast, lytic phage cocktails may reduce pathogen load on food by acting in a very specific way targeting exclusively bacterial cells. In addition, "contaminating" food with phages does not represent a risk for humans, being these viruses completely safe for eukaryotic cells, and does not alter food qualities (Leverentz et al., 2003; O'Flynn et al., 2004; Carlton et al., 2005).

Based on several data, FDA in 2006 approved an anti-*Listeria* phage-based preparation (LMP-102, composed of a mix of six phages) for the treatment of meat and poultry against *L. monocytogenes* (Lang, 2006). Later on, in 2013, FDA also approved SALMONELEX™ (Micreos, The Netherlands), as a GRAS (Generally Recognized as safe) food processing help for *Salmonella* control on beef and vegetables.

Beside direct treatment of food, phage treatment is also indicated for decontaminating surfaces that are used for food processing (Abuladze et al., 2008; Tomat et al., 2014).

Based on these observations, our group hypothesized to use phages for decontamination of hospital surfaces, being the hospital environment colonized by bacteria potentially causing infections in the hospitalized patients.

To this regards, a study recently showed that phages against *S. aureus* could decontaminate fomites, including glass and clothes, reducing *S. aureus*, including MRSA (Jensen et al., 2015).

However, in this case, phage activity was tested using high bacterial densities (around 10^8 colony forming units (CFU) per square meter (Jensen et al., 2015)), that represent a good condition for facilitate the encounter between bacterial target and bacteriophages, but that are not consistent with the bacterial density that is normally found on hospital surfaces, where the average level of contamination is consistently lower (between 10^3 and 10^5 CFU/m^2) (D'Accolti et al., 2018).

Furthermore, studies were performed with high volumes of phage solution, in order to have a prolonged contact between phages and target bacteria, which however is not compatible with routine sanitation protocols in hospitals.

Studies aimed to analyze the feasibility and the effectiveness of a routine daily phage decontamination are lacking in literature, and represented part of the research work of the present thesis.

Phage uses are summarized in **Figure 14.**

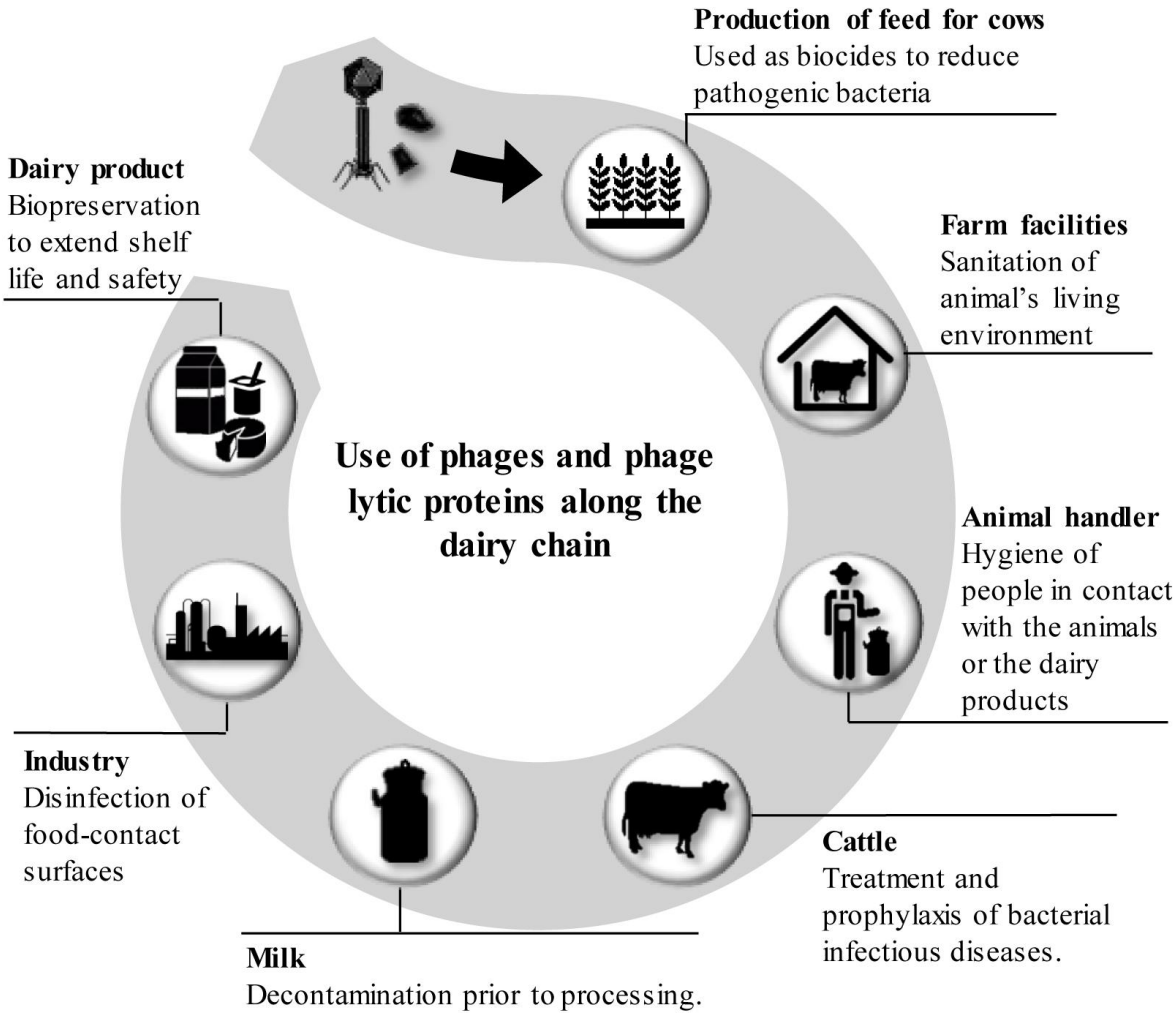


Figure 14. Representation of the current different uses of bacteriophages (Gutiérrez et al., 2019).

RESEARCH AIMS

4. General preconditions and objectives

The persistent contamination on hospital surfaces represents a global major concern due to its impact on transmission of HAIs, which are one of the most frequent complications occurring in healthcare facilities worldwide. In fact, it has been accepted that contaminated environmental surfaces represent reservoirs of several HAIs-associated microorganisms (Kramer et al., 2006; Allegranzi et al., 2011; Otter et al., 2011; Otter et al., 2013).

Several of them have been shown to persist in hospital environment for long period of times (from hours to weeks, in some cases also months), stably contaminating hard surfaces frequently touched by humans, as well as medical devices, contributing to cause potential infection outbreaks (Kramer et al., 2006).

Due to the importance of this concern, many attempts have been made in order to improve the control of pathogens contamination, including the use of conventional chemical antimicrobial compounds, which however show important limitations related to the their incapability in preventing recontaminations, and in possible induction of resistances against both chemicals and antibiotics (Caselli et al., 2016b; Wand et al., 2017).

On the other hand, antimicrobial resistance (AMR) represents a global concern, since threatens the effective prevention and treatment of an ever-increasing range of infections caused by multidrug resistant microorganisms (Caini et al., 2013; Cornejo-Juarez et al., 2015).

In attempt to find new more efficient and sustainable cleaning methods capable of fighting such pathogens, an innovative biologic-based sanitation system has been developed. Interestingly, the concept of hospital sanitation has been recently rethought, trying to manage its "health" as the health of human body, and this new perception stems from several Microbiome Project data, in which it is supported that rather than eradicating all microbes in a specific "environment", to replace pathogens by beneficial microbes might result in a more effective decrease of pathogen contamination and, consequently, HAIs incidence.

The innovative system, named Probiotic Cleaning Hygiene System (PCHS) consists of eco-friendly cleaning solutions additioned with spores of probiotic bacteria belonging to the *Bacillus* genus (*B. subtilis, B: megaterium* and *B. pumilus*).

Bacteria of the *Bacillus* genus are in fact largely not pathogenic for humans, ubiquitous, and with a long history of safe use.

Furthermore, they are spore-formers, characteristic that allows them to be easily added to concentrated detergents without losing activity, since they are able to survive in not-common environmental conditions.

Since 2014, this system has been tested in field, both by simultaneous comparison of similar hospital wards treated respectively by PCHS and chemical-based sanitation, and by pre-post comparison in the same ward (Vandini et al., 2014; Caselli et al., 2016b). Collected results evidenced the PCHS ability to induce a strong decrease of pathogen surface contamination in a stable way (Vandini et al., 2014). More importantly, reduction was not accompanied by selection of resistant strains, but rather by decrease of the whole resistance genes harbored by residual microbiota on treated surfaces (Caselli et al., 2016b). Meanwhile, PCHS resulted safe for use, being genetically stable (Vandini et al., 2014; Caselli et al., 2016b) and not representing an infectious risk for hospitalized patients (Caselli et al., 2016a).

However, being mainly based on competitive antagonism (inspired by Gause's law), PCHS action is not specific and relatively slow, since several weeks are needed in order to achieve the maximum inhibition of pathogens growth on treated surfaces.

For this reason, the probiotic system cannot be considered as a rapid way for the elimination of specific targeted pathogens, even responsible of potential outbreaks, but rather it seems to represent as a preventive and stabilizer cleaning system.

By contrast, bacteriophages, viruses that specifically target prokaryotic cells, are characterized by rapidity and specificity of action, and they have been suggested as potential decontaminating agents. Phage application has been in fact proved to be effective against foodborne pathogens, and consequently used for treatment of food and food-related surfaces. However, studies have been performed by using very high bacterial densities (Abuladze et al., 2008) in order to favor the encounter between phages and bacteria, which are not consistent with those generally detected on hospital surfaces (very lower bacterial densities). Furthermore, a prolonged contact between phages and target bacteria in high isotonic solution volumes have been used, but this is not consistent with routine hospital sanitation procedures. Based on these observations, the aim of this research was to evaluate the impact of the probiotic-based sanitation on HAI incidence, and to explore the feasibility and effectiveness of a phage decontamination added to a probiotic-based sanitation. The study was thus developing in the following lines of research.

4.1 Probiotic based-sanitation impact on AMR and HAIs incidence: a pre-post interventional and multicentre study

Based on previous data about PCHS effectiveness on microbiota remodulation (Vandini et al., 2014; Caselli et al., 2016a; Caselli et al., 2016b), the first aim was to assess the generalizability of collected results, by analyzing PCHS effectiveness in multiple healthcare settings located in different Italian regions and characterized by different levels of complexity and size. Moreover and more importantly, we wanted to assess the PCHS impact on HAI incidence, whose decrease generally represents the final goal of any sanitation procedure in hospitals. To this aim, a multicenter, prospective, pre-post interventional study, simultaneously analyzing surface contamination and HAI incidence, was conducted in six public medium to large Italian hospitals for 18 months. Both microbiological and high-sensitive molecular tests were performed in order to characterize environmental microbiota, and HAI-patients data were collected and analyzed in order to evaluate HAI incidence before and after PCHS application.

4.2 Effectiveness against hospital pathogens of a combined use of probiotics and bacteriophages: test *in vitro* and *on field*

Being the probiotic-based system non-specific and quite slow, the second aim of this thesis was to ascertain the potential use of bacteriophages for decontamination purposes in the hospital environment. This based on their characteristics of specificity and rapidity of action on specific target cells. We evaluated the potential decontamination effectiveness of a combined use of probiotics and bacteriophages, against hospital pathogens, including MDR ones, by performing both tests *in vitro* and *on field.* For *in vitro* tests, different types of surfaces were artificial contaminated by three target bacteria, chosen among those most frequently detected on hospital surfaces and associated with HAIs, namely *S. aureus, E. coli* and *P. aeruginosa*.

Then, after evaluating bacteriophages' stability in the probiotic cleaning system, *on field* tests were performed directly in the hospital environment. Briefly, the potential feasibility and effectiveness of a routine phage decontamination in addition to a probiotic sanitation, were evaluated in a monocentric study performed in a healthcare setting in Ferrara. As a proof of concept, our attention was focused on *Staphylococcus* spp. in the bathrooms of the Internal Medicine ward, as the bathrooms resulted the most contaminated areas (previous results) and the Staphylococci the most prevalent contaminating species.

MATERIALS AND METHODS

5. Probiotic based-sanitation impact on AMR and HAIs incidence: a pre-post interventional, multicentre study

5.1 Study design

The study, named SAN-ICA, was performed in six public medium-large hospitals located in different geographical Italian regions (North, Centre and South), for a period of 18 months. The following hospitals were enrolled: Tolmezzo (UD), Pavia (PV), Feltre (BL), Roma – Gemelli (RM), Foggia (FG) and Messina (ME). The following wards were included in the study: Internal Medicine, Geriatric, Cardiology, Gastroenterology and Neurology.

The trial protocol was approved by the Institutional Ethics Committees of each enrolled hospital, which fulfilled the following eligibility criteria:

- Presence of the Internal Medicine wards
- Presence of an established HAI surveillance and infection control programs
- Whole size larger than 100 in-patients beds
- Acceptance to not introduce any new interventions for infection prevention and control (IPC), except for those already existing.

Among the six hospitals, five were subjected to the intervention, which consisted uniquely in the introduction of PCHS sanitation in substitution of the conventional chemical-based one (chlorine products). Instead, one hospital was randomly selected as external control (extC) and did not receive the intervention, but was analyzed for HAI incidence and environmental bioburden, in order to measure the effect due to the mere «participating to a study» (“Hawthorn” effect).

The six hospitals were randomly subdivided, by a computer-generated choice, into two groups of Interventions (I_1, I_2), which entered the study in two different periods, trying to minimize eventual seasonal fluctuations.

I_1-group included three hospitals entering the study on January 1st 2016 (Feltre, Roma, Foggia); I_2-group included two hospitals entering 5-months later, on May 1st 2016 (Vigevano and Tolmezzo); the extC hospital was monitored starting from May 1st 2016, as for I_2 group (Messina).

The study included two phases: a six-month pre-PCHS intervention phase, when hospitals maintained the conventional chemicals-based sanitizing procedure, and a six-month PCHS intervention phase, in which PCHS was routinely applied.

Between the two phases, the study included a "stabilization period" (2 or 3 months, depending on the hospital group) in which PCHS was introduced.

Cleaning staff did not change during the study and were adequately trained for the appropriate PCHS application in all the hospitals receiving the intervention. The cleaning modalities did not change, in order to not create a different perception by wards healthcare personnel or patients, which had to be blinded about the change of the cleaning procedures.

Cleaning was performed with the Probiotic Cleaning Hygiene System (PCHS; Copma srl, Italy), by using detergents containing 10^7 spores per ml of three *Bacillus* species: *B. subtilis*, *B. pumilus* and *B. megaterium* (Chrisal, Lommel, Belgium), as previously described (Vandini et al., 2014).

During the entire period of the study, environmental samplings for hospital microbiota characterization were performed monthly, and all patients admitted at the enrolled wards were included in the study and surveyed for the development of HAIs. HAIs monitoring was conducted *in continuum,* in order to have the true value of HAI incidence. **Figure 15** shows the SAN-ICA study design.

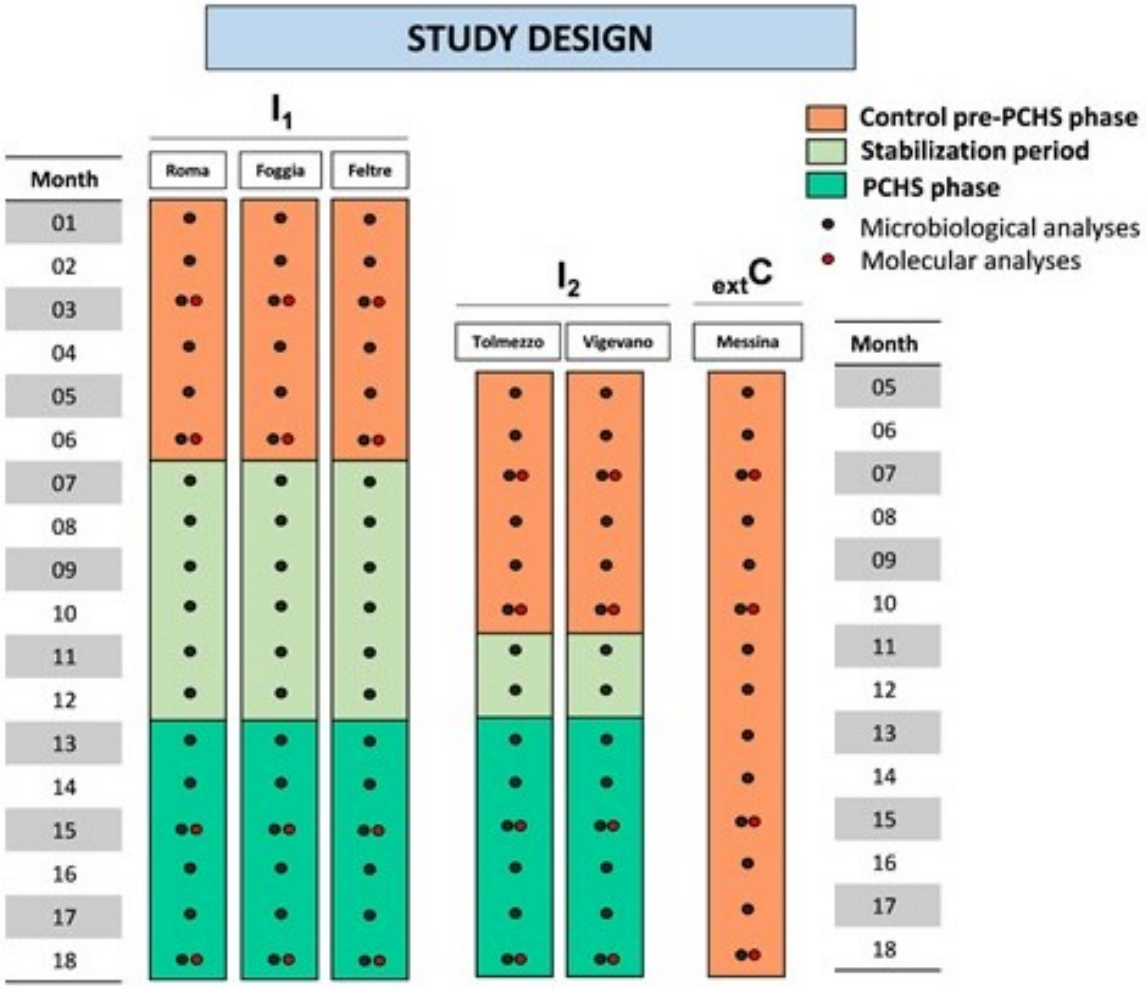


Figure 15. Study design graphic representation. Six Italian hospitals from different geographical regions were enrolled in the study (North: Feltre, Tolmezzo, Vigevano; Centre: Rome; South: Foggia, Messina). Five hospitals were randomly allocated in two Intervention groups (I_1, I_2), and one further hospital was selected as external control ($_{ext}C$, Messina). The three phases of the study are indicated by colours: pink, control 6-months pre-intervention period (pre-PCHS); light green, stabilization period characterized by PCHS introduction; blue 6-months post-intervention period (PCHS), when PCHS was routinely applied. Sampling campaigns for microbiological analyses are indicated by circles: conventional microbiological analyses were performed monthly (black circles), and molecular analyses quarterly (red circles) in all enrolled hospitals (Caselli et al., 2018).

5.2 Microbial surface contamination: environmental sampling

Collection of environmental samples was performed simultaneously and following two different methods, according to downstream microbiological or molecular analyses.

To this aim, three points/room such as floor, bed footboard and sink, representing some of the most representative surfaces in healthcare settings, were considered for sampling.

For microbiological tests, sampling was performed monthly, in duplicate by Replicate Organism Detection and Counting (RODAC™) plates (BD Diagnostic Systems, USA), used for microbiological monitoring of surfaces equivalent to 24 cm^2 (**Figure 16A**).

In particular, the following six HAI-related microorganisms were monitored: *Staphylococcus* spp., *Enterobacteriaceae* spp., *Acinetobacter* spp., *Mycetes*, *Pseudomonas* spp., and *Clostridium difficile;* and the following microbial media were used:

- Tryptic Soy Agar with Lecithin, Tween and Histidine (Merck Millipore, Darmstadt, Germany) general growth medium for total bacterial count;
- Baird Parker Agar (Merck Millipore, Darmstadt, Germany), moderately selective medium staphylococci;
- MacConkey Agar (Merck Millipore, Darmstadt, Germany), selective for *Enterobacteriaceae*;
- Herellea agar (Likson, Italy), selective for *Acinetobacter* spp. detection;
- Sabouraud Dextrose Contact Agar (SDA) with chloramphenicol (Merck Millipore, Darmstadt, Germany), selective for mycetes and *Candida albicans;*
- Cetrimide agar (Incofar, Italy), selective for *Pseudomonas* spp;
- Clostridium difficile agar (Likson, Italy), selective for *C. difficile.*

The method consisted of exerting an uniform and costant pressure for 10 seconds on plates that are put into contact with the surface. After sampling, incubation was performed aerobically at 37°C for 24-48 hours for Tryptic Soy Agar, Baird Parker Agar, MacConkey Agar, Herellea agar and at 25°C for 72h-5 days for Sabouraud Dextrose Contact Agar. Incubation of Clostridium difficile agar plates was performed anaerobically at 37°C for 48-72h by using anaerobic jars (Thermo Fisher Scientific Inc.) with Anaerogen™ Systems.

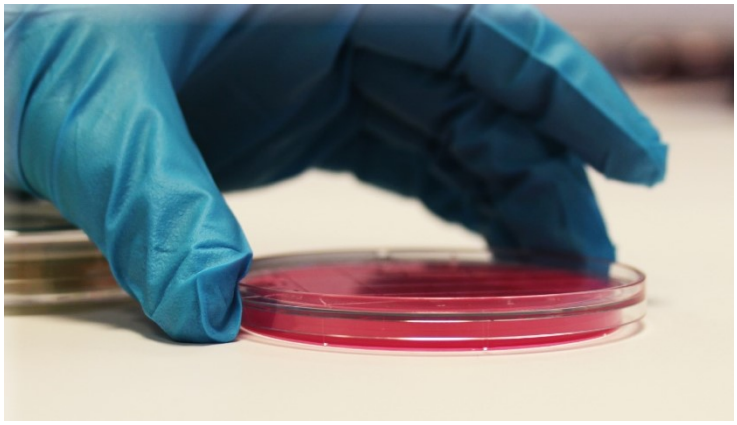
Colony Forming units (CFU) on all agar plates were manually counted after the incubation period and individual plate photos have been took and collected.

For molecular analyses, sampling was performed quarterly (twice in the Pre-PCHS and twice in PCHS phases), by using sterile rayon swabs (Copan, Brescia, Italy).

Briefly, swabs were first premoistened in sterile TSB broth (Biolife, Monza, Italy), and then used to collect a sample surface, delimited by a sterile 10x10 cm disponsable plastic template (Copan, Brescia, Italy) corresponding to 100 cm^2 (**Figure 16B**). Swabs were finally put in 5 ml of sterile TSB, immediately refrigerated and put in thermal incubator within 24 hours from sampling.

After incubation at 37°C for 24 hours, useful to allow a controlled amplification, a microbial quote was added to 50% sterile glycerol and immediately frozen at -80°C. Remained microbial suspension was pelletized by centrifuge at 14000xg for 5 minutes and pellet immediately refrigereted and kept at -20°C until use.

A



B

Figure 16. Methods of environmental sampling. **Panel A**, Replicate Organism Detection and Counting (RODAC™) plates.
Image from the website http://www.2030lab.com/labservices/cleanroom-sterile-services/environmental-monitoring/ (last accessed in July 22nd , 2019).
Panel B, sterile swabs, tubes with sterile culture media and disposable plastic templates. Image from https://products.copangroup.com/ (last accessed in July 22nd, 2019).

5.2.1 Culture Isolation and microbial identification

Microorganisms grown on Rodacs were collected and re-cultured on new agar plates in order to obtain culture isolation and species identification.

Seeding should been take place in extreme sterility condition by using disposable sterile loops in order to spread microbial suspension on agar surface following several directions, to create a progressive reduction of the same and generate isolated colonies.

Identification of isolates was first assessed by standardized biochemical galleries, following the manufacturer's instructions (API, BioMerieux, Inc; Liofilchem, Teramo).

For confirmation of identification, specific isolates were also typized by Maldi Tof (Matrix Assisted Laser Desorbtion Ionization Time-of-Flight) mass spectrometry technology (AccuPRO-ID; Charles River Lab Europe SaS, Ecully, France) (**Figure 17).**

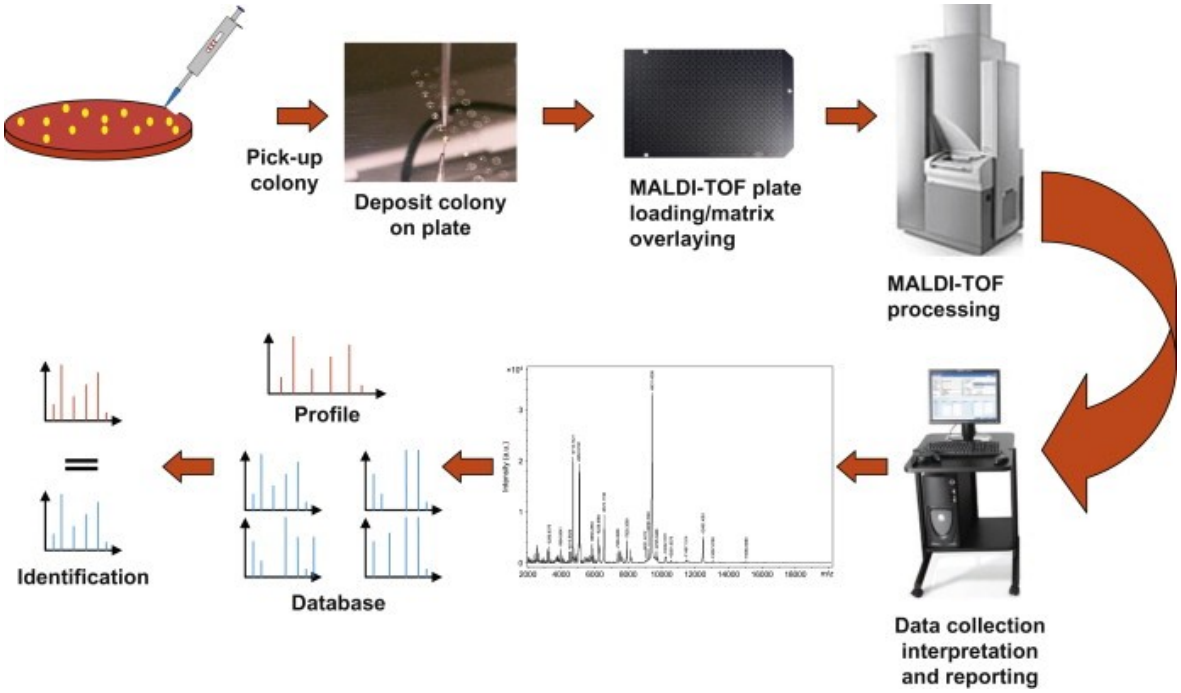


Figure 17. Schematic representation of the Maldi-Tof analysis performed on isolates (Dridi and Drancourt, 2011).

5.2.2 Antimicrobial susceptibility: Kirby Bauer test and microdilution broth

After microbial identification, antibiotic resistances of *Staphylococcus aureus* and *Enterobacteriaceae* isolates were evaluated by antibiograms.

In particular, the two following methods have been performed:

- Kirby-Bauer disk diffusion antimicrobial susceptibility test
- Broth microdilution assay

The diffusion method is the analysis of microorganism sensitivity to one or more types of antibiotics on Mueller-Hinton Agar plates. The experimental method consists of spreading a single bacterial inoculum on the plate by sterile swabs and then placing the appropriate standard concentration antimicrobial-impregnated disks on the surface of the agar.

In particular, for *S. aureus* the following antibiotics were tested: ampicillin, vancomycin, oxacillin, cefotaxime, imipenem, and penicillin G.

For *Enterobacteriaceae* the Entero 1 Multodisc (Liofilchem, Teramo, Italy) was used with the following eight antibiotics: Amikacin, Aztreonam, Piperacillim-Tazobactam, Chloramphenicol, Trimethorim-Sulfamethoxazole, Netilmicin, Cefotazime, and Nitrofurantoin.

Inhibition zone diameters were interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoint tables for the interpretation of minimum inhibitory concentration (MIC) and inhibition zone diameters and the Clinical and Laboratory Standards Institute manual (28^{th} edition) (CLSI, 2018).

Broth microdilution assay is a method to evaluate the microorganism susceptibility to serial dilutions of specific antibiotic. The experimental protocol consisted of preparing tubes with the same volume of liquid culture medium to which were added scalar dilutions of the antibiotic to be tested. A standard amount of a microorganism was then inoculated, and after an incubation period of 24 h, the presence of microbial growth evaluated, being evidenced by the turbidity of the suspension.

For *Enterobacteriaceae* susceptibility to colistin, the sensiTest^{TM} Colistin (Liofilchem, Teramo, Italy) was used. It consisted of a compact panel containing the antibiotic in seven two-fold dilutions (0.25-16 µg/ml), that allowed the simultaneously test of four samples. Bacterial growth was evidenced by turbidity or sediment at the bottom of the well, and antimicrobial activity assessed by comparing each well with the negative control.

The highest drug dilution with no bacterial growth corresponded to the MIC value, expressed as mg/dl and corresponding to the lowest antibiotic concentration able to inhibit bacterial growth.

5.2.3 Growth inhibition by stab overlay assay and in *Bacillus*-conditioned medium

To test bacterial growth inhibition by antimicrobial compounds produced by PCHS-*Bacillus,* two methods were performed: *stab overlay assay* (solid) and *growth inhibition in Bacillus-conditioned medium* (liquid).

In the stab overlay assay, PCHS-*Bacillus* (producer bacteria) were seeded on TSA and grown for 24h at 37°C. Then, grown colonies were removed by scraping, and plates were inverted to seed Gram-positive (i.e., *S. aureus*) or Gram-negative (i.e. *E. coli*) ATCC strains (American Type Culture Collection strains), as well as wild-type environmental strains (both ATCC and wild-type strains were considered indicator bacteria).

Specifically, after agar inversion, overnight culture indicator bacteria (100µl) were added to 3 ml of soft top agar (0.75% agar, as opposed to the usual 1.5% for agar plates), which has previously been melted at 100°C and cooled to 45°C (this condition allowed agar to remain liquid).

The melted agar/bacterial suspension was mixed, poured evenly across the top of the agar plate and allowed to solidify. After incubation for 24h at 37°C, the indicator bacteria spread on the agar were grown to produce a homogeneously lawn, except for the zones corresponding to previous grow of producer strains, in which typical inhibition areas would be observed caused by the eventual production of antimicrobial compounds.

In the second method, PCHS-*Bacillus* culture, namely *B. subtilis*, *B. pumilus* and *B. megaterium* were grown in liquid Luria broth for 14 h, then bacterial cells were removed by 0.45µm filtration, and conditioned media were used for the growth of the indicated target bacteria. After 24h incubation bacterial growth was evaluated by spectrophotometric reading at 660 nm and compared to that obtained in unconditioned Luria broth medium.

The following indicator bacteria have been tested for this method: *S. aureus* ATCC-25923*, S. pyogenes* ATCC-19615*, E. coli* ATCC-25922*, P. aeruginosa* ATCC-BAA-47, *K. pneumoniae* ATCC-27736*, P. mirabilis* ATCC-29906*, E. cloacae* ATCC-13047 and *S. marcescens* ATCC-14756.

5.2.4 Microbial DNA extraction

For molecular analysis, total microbial genomic DNA was extracted from pelletized environmental samples by using the QIAmp UCP Pathogen Mini Kit (Qiagen, Hilden, Germany).

The original manufacturer's protocol for DNA extraction was modified to obtain an optimal lysis of Gram-positive bacteria. In particular, mechanical disruption by sonication and enzymatic lysis by lysozyme digestion have been added.

The concentration of extracted DNA was evaluated by spectrophotometric reading by "Nanodrop" instrument (Thermo Scientific, Milan, Italy), determining the optical density (OD) at the wavelength of 260nm and 280nm. OD 260nm/280nm ratio was evaluated to check the purity of extracted DNA (it must be 1.8-1.9). After reading, DNA samples were stored at -20°C until use.

5.2.5 Polymerase Chain Reaction (PCR)

Four PCR reactions were performed on extracted microbial DNA, to check the amplificability of DNA (by a *PanB* PCR), the presence of *mcr-1* gene in *Enterobacteriaceae* genome (CLR PCR), the sequence of 16S rRNA gene in *Bacillus* strains (BK1 PCR), and the sequence of 16 rRNA gene in *B. subtilis* group strains (*Bsub* PCR).

The amplified product was visualized by agarose gel electrophoresis. The 100 bp DNA ladder marker (Norgen Biotek Corp, Canada) was inserted to verify that the observed bands DNA corresponded to the expected molecular weight. Electrophoretic runs have been conducted at an appropriate voltage, which ranged from 20 to 100V. DNA in the gel was visualized by exposure to UV light and photographed with a digital capture system (Gel Doc, Bio-Rad, California).

5.2.5.1 Panbacterial PCR (*PanB* PCR)

A universal panbacterial PCR was performed as a control of amplificability of extracted bacterial DNA. The PCR is designed in a conserved regions of 16S rRNA gene, which is conserved in all prokaryotic microorganisms. Amplification reactions were performed using 10 ng of bacterial DNA as template. *E. coli* DNA was used as positive control, whereas eukaryotic DNA (extracted from Jurkat T cell line) was used as negative control.

Tables 1 and **2** show specific *PanB* primers and thermal conditions of *PanB* PCR.

Table 1. Specific primers for *PanB* PCR

Gene	Primers	Sequence	Size
PanB	PanB-F	5′-TGG AGC ATG TGG TTT AAT TCG -3’	**160 bp**
	PanB-R	5′-TGC GGG ACT TAA CCC AAC A -3	

Table 2. *PanB* PCR thermal conditions

GENE	Thermal conditions	Cycles
PanB	95°C 3 min.	1 cycle
	95°C 30 sec, 59°C 30 sec, 72°C 1 min	30 cycles
	72°C 10 min., 4°C pause	1 cycle

5.2.5.2 *CLR* PCR and Nested PCR (nCLR)

Enterobacteriaceae DNA was analyzed for the presence of ***mcr-1*** plasmid-mediated colistin resistance gene by nested PCR. To this purpose, a first-round *mcr-1* gene amplification was performed using *CLR*-F and *CLR*-R primers, and the thermal conditions reported in **Tables 3** and **4** (Liu et al., 2016).

Table 3. First-round CLR PCR: specific primers for *mcr-1* gene

Gene	Primers	Sequence	Size
mcr-1	CLR-F	5′- CGG TCA GTC CGT TTG TTC -3’	**308 bp**
	CLR-R	5′- CTT GGT CGG TCT GTA GGG -3	

Table 4. First-round CLR PCR: thermal conditions

GENE	Thermal conditions	Cycles
mcr-1	94°C 5 min.	1 cycle
	94°C 30 sec, 54°C 30 sec, 72°C 3 min	35 cycles
	72°C 10 min., 4°C pause	1 cycle

Nested CLR-PCR amplification was carried out using the following primers and thermal conditions (**Tables 5** and **6**).

Table 5. Nested CLR PCR: primers specific for *mcr-1* gene

Gene	Primers	Sequence	Size
mcr-1	nCLR-F	AAA CCT ATC CCA TCG CGG AC-	**208 bp**
	nCLR-R	CCG CGC CCA TGA TTA ATA GC	

Table 6. Nested CLR PCR: thermal conditions

GENE	Thermal conditions	Cycles
mcr-1	94°C 5 min. 94°C 30 sec, 57°C 30 sec, 72°C 30 sec. 72°C 10 min., 4°C pause	1 cycle 35 cycles 1 cycle

5.2.5.3 *Bacillus* genus PCR (*BK1* PCR)

A group-specific primer pair was used to amplify the 16S rRNA gene of spore-forming *Bacillus* bacteria, as previously reported (Wu et al., 2006). Amplification reactions were performed using 10 ng of presumptive *Bacillus* DNA as template. *Bacillus clausii* ATCC DNA was used as positive control, whereas *Escherichia coli* ATCC DNA was used as negative control. **Tables 7** and **8** show specific *BK1* primers and PCR thermal conditions.

Table 7. Specific primers for *BK1* PCR

Gene	Primers	Sequence	Size
BK1	*BK1*/F	5′-TCA CCA AGG CRA CGA TGC G-3’	**1114 bp**
	BK1/R1	5′-CGT ATT CAC CGC GGC ATG-3	

Table 8. *BK1* PCR thermal conditions

GENE	Thermal conditions	Cycles
BK1	94°C 3 min. 94°C 30 sec, 63°C 30 sec, 72°C 2 min. 72°C 10 min, 4°C pause	1 cycle 25 cycles 1 cycle

5.2.5.4 *Bacillus subtilis*-group PCR (B-sub PCR)

A specific PCR was used to analyze the sequence of *Bacillus subtilis* isolates, as previously reported (Wattiau et al., 2001). The PCR was based on the 16S rRNA gene amplification, able to identify any of the five species of the "*Bacillus subtilis* group" (*B. subtilis, B. pumilus, B. atrophaeus, B. licheniformis* and *B. amyloliquefaciens*).

Amplification reactions were performed using 10 ng of presumptive *Bacillus* DNA as template. Prior to use, specificity and amplification efficiency of PCR was verified on PCHS-derived *Bacillus* strains and on bacteria belonging to other groups (*S. aureus, S. pyogenes, E. coli*, *P. aeruginosa, Clostridium difficile*), to ascertain lack of unspecific amplification.

Bacillus subtilis ATCC DNA was used as positive control, whereas *Escherichia coli* ATCC DNA was used as negative control.

Tables 9 and **10** show specific primers for *B-sub* PCR and thermal conditions of amplification.

Table 9. Specific primers for *B-sub* PCR

Gene	Primers	Sequence	Size
B-sub	Bsub-5F	5′-AAG TCG AGC GGA CAG ATG G-3'	**595 bp**
	Bsub-3R	5′-CCA GTT TCC AAT GAC CCT CCC C-3	

Table 10. *B-sub* PCR: thermal conditions

GENE	Thermal conditions	Cycles
B-sub	95°C 3 min.	1 cycle
	95°C 30 sec, 65°C 2min, 72°C 2 min.	30 cycles
	72°C 10 min, 4°C pause	1 cycle

5.2.6 *Bacillus* DNA digestion and sequencing

Amplified products generated by *BK1* PCR were digested by specific restriction enzymes in order to perform an initial identification of *Bacillus* strains isolated from hospital surfaces.

Prior to digestion, *BK1*-PCR products were purified by GeneAll ® Expin^{TM} columns, according to the manufacturer's protocol (Gene All, Tema Ricerca, Italy), and were eluted in a final volume of 50µl of elution buffer (10mM TrisHCl, pH 8.5).

Purified amplicons were then digested with the enzymes *Alu1* (AG'CT) and *Taq1* (T'CGA) (both New England Biolabs, MA, USA): 10µl of purified samples were digested with 5U of individual restriction enzyme in a 20µl reaction volume for 4 hours at 37°C for *Alu1* and 65°C for *Taq1*. Digestion pattern was evaluated by electrophoretic migration on agarose gel (2%). Observed fragments ranging in size from 76 to 804 base pairs were compared with the profiles of amplicons obtained from *Bacillus* reference strains described in literature (Wu et al., 2006). In parallel, DNA samples which resulted positive for the presumptive identification of PCHS-*Bacillus*, as detected by *BK1* PCR, were also analyzed by DNA sequencing for a more precise *Bacillus* species identification. Briefly, the amplicons obtained by *B-sub* PCR were purified as already described and sequenced by Sanger technology (BMR Genomics, University of Padua, Padua, Italy). Nucleotide sequences of isolates were then compared with those obtained for the three PCHS-*Bacillus* contained in the original PCHS product.

5.2.7 Quantitative Real Time PCR (qPCR)

Three different quantitative real-time PCR (qPCR) have been performed for these research project:

a. Panbacterial qPCR (*PanB* qPCR), used for the quantification of the whole bacterial population collected from environmental surfaces, by amplifying the gene encoding the 16S rRNA gene, conserved in all bacteria;
b. qPCR specific for *Bacillus* genus (*spo0A* qPCR), for the quantification of the population of *Bacillus,* by amplification of the specific *spo0A* gene, responsible for entry into sporulation and uniquely present in *Bacillus* genus;
c. Antibiotic Resistance Genes qPCR microarray (Qiagen), for the detection and quantification of 84 antibiotic resistance genes in the total microbial population (resistome).

The instrument used for all amplification reactions was the QuantStudio5 (Thermo Fisher, Life Technologies, Milan, Italy).

5.2.7.1 Panbacterial qPCR (PanB qPCR)

Panbacterial qPCR (*panB* qPCR) was performed by using a sequence-specific TaqMan probe labelled with the fluorescent reporter FAM at the 5'end, and the quencher TAMRA at 3' end.

The amplification reaction was carried out in a 96-wells plate, by using 30µl final reaction volume, including 20µl of reaction mixture (mix) and 10µl of DNA template at appropriate concentration (0,1 ng/µl).

Samples were tested in duplicate and they included standards (ST), unknown (UNK) and negative control (NTC). ST samples were obtained by 10-fold serial dilutions (from 10^7 to 10^2 DNA copies) of *Escherichia coli* DNA with known titre in order to create the standard curve. Absolute quantitation of bacterial DNA was thus obtained.

Tables 11 and **12** show *PanB* primers and probe sequences, and qPCR thermal conditions (Gentili et al., 2012).

Table 11. *PanB* qPCR primers and probe sequences

Oligonucleotides	Sequence
Primer F (P891F)	5'-TGG AGC ATG TGG TTT AAT TCG A- 3'
Primer R (P1033R)	5'- TGC GGG ACT TAA CCC AAC A-3'
TaqMan probe	5'-(FAM) CAC GAG CTG ACG ACA RCC ATG CA- (TAMRA)-3'

Table 12. Thermal condition of *PanB* qPCR

Phase	Thermal conditions	Cycles
Enzyme activation	95°C 10 min.	1 cycles
Denaturation	95°C 15 sec.	40 cycles
annealing/elongation	60°C 1 min.	

5.2.7.2 *Bacillus*-specific qPCR (*Spo0A* qPCR)

The *Bacillus*-specific qPCR (*Spo0A* qPCR), based on the amplification of the ***spo0A*** key gene regulating bacterial sporulation, was used to quantify *Bacillus* spp. in environmental samples.

The reaction was performed in a 96-wells plate, in 25µl final volume for each sample/well, including 15µl of reaction mix and 10µl of DNA (0,1ng/µl).

The reaction was carried out by using the SYBR® Green fluorescent DNA-binding dye (Thermo-Fisher, Applied Biosystems, Milan, Italy), already present in the Master Mix.

ST samples were obtained by 10-fold serial dilutions (from 10^7 to 10^2 DNA copies) of *Bacillus subtilis* ATCC 6633 DNA with known titre in order to create the standard curve. Absolute quantitation of *Bacillus* spp. DNA was thus obtained.

Tables 13 and **14** show *spo0A* primers sequences, and qPCR thermal conditions (Bueche et al., 2013).

Table 13. *Spo0A* qPCR primers

Primers	Sequence
Spo0A655f	5'-GGH GTD CCN GCN CAT ATH AA -3'
Spo0A923r	5'- GCD ATG AAY TCD GAG TTN GTN GG -3'

Table 14. Thermal conditions of *Spo0A* qPCR

Phase	Thermal conditions	Cycles
Enzyme activation	95°C 10 min.	1 cycle
Denaturation	95°C 30 sec.	45 cycles
Annealing	52°C 30 sec.	
Elongation	72°c 30 sec	

5.2.7.3 Antibiotic Resistance Genes qPCR microarray

Total DNA extracted from environmental samples was also analyzed by the Antibiotic Resistance Genes qPCR microarray (Cat. no. 330261 BAID-1901ZRA, Qiagen), to detect and quantify the resistome of the whole population contaminating surfaces.

The microarray contained TaqMan assays for 84 antibiotic resistance genes belonging to different classes of antibiotics, including aminoglycosides, β-lactams, erythromycin, fluoroquinolones, macrolide-lincosamide-streptogramin B, tetracyclines, and vancomycin, together with reactions for the detection and quantification of the following genes/species:

- *Staphylococcus aureus*;
- *Staphylococcus aureus* Panton–Valentine leukocidin
- *Staphylococcus aureus* IgG Binding Protein A

Moreover, two different panbacterial assays were included as positive controls (Pan Bacteria 1 and Pan Bacteria 3), together with a Positive PCR Control (PPC) assay, to test the efficiency of the reaction and the eventual presence of PCR inhibitors. Reactions were performed in the QuantStudio5 instrument (Thermo Fisher, Life Technologies, Milan, Italy).

Table 15 shows the list of the antibiotic resistance genes and bacterial virulence genes analyzed in qPCR microarray (modified by the author, and adapted from the website www.qiagen.com/~/media/genetable/ba/antibioticresistancegenes).

Table 15. List of antibiotic PCR reactions included in the Antibiotic Resistance genes qPCR microarray: resistance genes, bacterial virulence factors and controls.

Position	*Species/ Genes*	*Antibiotic classification/ gene description*
A01	AAC(6)-Ib-cr	Fluoroquinolone resistance
A02	aacC1	Aminoglycoside-resistance
A03	aacC2	Aminoglycoside-resistance
A04	aacC4	Aminoglycoside-resistance
A05	aadA1	Aminoglycoside-resistance
A06	aphA6	Aminoglycoside-resistance
A07	BES-1	Class A beta-lactamase
A08	BIC-1	Class A beta-lactamase
A09	CTX-M-1 Group	Class A beta-lactamase
A10	CTX-M-8 Group	Class A beta-lactamase
A11	CTX-M-9 Group	Class A beta-lactamase
A12	GES	Class A beta-lactamase
B01	IMI & NMC-A	Class A beta-lactamase
B02	KPC	Class A beta-lactamase
B03	Per-1 group	Class A beta-lactamase
B04	Per-2 group	Class A beta-lactamase
B05	SFC-1	Class A beta-lactamase
B06	SFO-1	Class A beta-lactamase
B07	SHV	Class A beta-lactamase
B08	SHV(156D)	Class A beta-lactamase
B09	SHV(156G)	Class A beta-lactamase
B10	SHV(238G240E)	Class A beta-lactamase
B11	SHV(238G240K)	Class A beta-lactamase
B12	SHV(238S240E)	Class A beta-lactamase
C01	SHV(238S240K)	Class A beta-lactamase
C02	SME	Class A beta-lactamase
C03	TLA-1	Class A beta-lactamase
C04	VEB	Class A beta-lactamase
C05	ccrA	Class B beta-lactamase
C06	IMP-1 group	Class B beta-lactamase
C07	IMP-12 group	Class B beta-lactamase
C08	IMP-2 group	Class B beta-lactamase

C09	IMP-5 group	Class B beta-lactamase
C10	NDM	Class B beta-lactamase
C11	VIM-1 group	Class B beta-lactamase
C12	VIM-13	Class B beta-lactamase
D01	VIM-7	Class B beta-lactamase
D02	ACC-1 group	Class C beta-lactamase
D03	ACC-3	Class C beta-lactamase
D04	ACT 5/7 group	Class C beta-lactamase
D05	ACT-1 group	Class C beta-lactamase
D06	CFE-1	Class C beta-lactamase
D07	CMY-10 Group	Class C beta-lactamase
D08	DHA	Class C beta-lactamase
D09	FOX	Class C beta-lactamase
D10	LAT	Class C beta-lactamase
D11	MIR	Class C beta-lactamase
D12	MOX	Class C beta-lactamase
E01	OXA-10 Group	Class D beta-lactamase
E02	OXA-18	Class D beta-lactamase
E03	OXA-2 Group	Class D beta-lactamase
E04	OXA-23 Group	Class D beta-lactamase
E05	OXA-24 Group	Class D beta-lactamase
E06	OXA-45	Class D beta-lactamase
E07	OXA-48 Group	Class D beta-lactamase
E08	OXA-50 Group	Class D beta-lactamase
E09	OXA-51 Group	Class D beta-lactamase
E10	OXA-54	Class D beta-lactamase
E11	OXA-55	Class D beta-lactamase
E12	OXA-58 Group	Class D beta-lactamase
F01	OXA-60	Class D beta-lactamase
F02	ereB	Erythromycin resistance
F03	QepA	Fluoroquinolone resistance
F04	QnrA	Fluoroquinolone resistance
F05	QnrB-1 group	Fluoroquinolone resistance
F06	QnrB-31 group	Fluoroquinolone resistance
F07	QnrB-4 group	Fluoroquinolone resistance
F08	QnrB-5 group	Fluoroquinolone resistance

F09	QnrB-8 group	Fluoroquinolone resistance
F10	QnrC	Fluoroquinolone resistance
F11	QnrD	Fluoroquinolone resistance
F12	QnrS	Fluoroquinolone resistance
G01	ermA	Macrolide Lincosamide Streptogramin_b
G02	ermB	Macrolide Lincosamide Streptogramin_b
G03	ermC	Macrolide Lincosamide Streptogramin_b
G04	mefA	Macrolide Lincosamide Streptogramin_b
G05	msrA	Macrolide Lincosamide Streptogramin_b
G06	oprj	Multidrug resistance efflux pump
G07	oprm	Multidrug resistance efflux pump
G08	tetA	Tetracycline efflux pump
G09	tetB	Tetracycline efflux pump
G10	vanB	Vancomycin resistance
G11	vanC	Vancomycin resistance
G12	Staphylococcus aureus	
H01	mecA	Beta-lactam resistance
H02	lukF	Panton-Valentine leukocidin chain F Precursor
H03	spa	Immunoglobulin G binding protein A Precursor
H04	Pan Bacteria 1	
H05	Pan Bacteria 1	
H06	Pan Bacteria 1	
H07	Pan Bacteria 3	
H08	Pan Bacteria 3	
H09	Pan Bacteria 3	
H10	PPC	
H11	PPC	
H12	PPC	

In **Table 16,** thermal conditions of qPCR microarray for resistance genes are shown.

Table 16. Thermal conditions of the Antibiotic Resistance genes qPCR microarray

Phase	Thermal conditions	Cycles
Enzyme activation	95°C 10 min.	1 cycles
Denaturation	95°C 15 sec.	40 cycles
annealing/elongation	60°C 2 min.	

5.2.7.4 Antibiotic resistance genes qPCR microarray data analysis

After amplification, the analysis of obtained data was performed by the web-based data analysis software available on Qiagen website (http://www.qiagen.com/Products/Catalog/Assay-Technologies/Real-Time-PCR-and-RT-PCR-Reagents/Microbial-DNA-qPCR-Array) through the comparative ΔΔCT method (Livak and Schmittgen, 2001), that compares for each gene target, the Ct value of the test samples (environmental samples collected after PCHS treatment) with the Ct value of the reference sample (environmental samples collected before PCHS treatment).

In particular, Ct values obtained for each target gene were normalized to the values of the housekeeping genes (Pan Bacteria 1 and 3), both for test and control samples, according to the following formulae

$$\Delta Ct_{(test)} = Ct_{(\text{target gene; } test)} - Ct_{(\text{housekeeping genes, } test)}$$

$$\Delta Ct_{(ctr)} = Ct_{(\text{target gene; } ctr)} - Ct_{(\text{housekeeping genes, } ctr)}$$

where Ct (target gene) indicates the value of the threshold cycle for the gene of interest, and Ct (housekeeping gene) indicates the value of the threshold cycle for the housekeeping gene used as normalizer.

Subsequently, the normalization between $\Delta Ct_{(test)}$ and $\Delta Ct_{(ctr)}$ was calculated as follows:

$$\Delta\Delta Ct = \Delta Ct_{(test)} - \Delta Ct_{(ctr)}$$

Finally, ratio is calculated as follows:

$$RE = 2^{-\Delta\Delta Ct}$$

where RE indicates the relative expression, ΔCt (test) indicates the difference between Ct values of the target gene and the housekeeping gene(s) calculated for the test sample, ΔCt (control) indicates the difference between the Ct values of the target gene and the Ct values of the housekeeping gene(s) obtained from environmental samples collected before PCHS treatment (control).

The obtained results quantified the *decrease* or the *increase* of a specific target gene of test samples compared to control ones, and normalized compared to housekeeping genes. This difference is indicated in terms of "Fold change", both in normal or in logarithmic form (Log_{10} Fold change).

5.3 HAIs data collection, management and analysis

Clinicians of the University of Udine, with whom we collaborated for the clinical part of the research project, performed HAIs data collection and analysis.

All new patients admitted at the enrolled wards in the pre-PCHS and PCHS periods were included in the study and surveyed for the development of HAIs, without distinction of age or gender and keeping their identity completely anonymous, so that informed consent was not needed. Patients already present at the beginning of pre-PCHS and PCHS periods, as well as in the window period between pre-PCHS and PCHS phases, were excluded from the study. HAIs data were collected in *continuum* in order to obtain the true incidence value, from patients' clinical records by specialized and specifically trained healthcare professionals, who were blinded to intervention time (consisting in PCHS treatment), but only aware of an incidence study to be conducted during the 18 months-study.

Recording was performed through a touch screen tablet specially designed and realized for this study, containing two pre-defined formats (S1 and S2), and data were instantly submitted centrally via a secure, password protected website.

The *S1* format was filled out for each patient, and contained anonymised general data of each patient, such as gender, age, provenience, admission date, admission cause, risk factors at admission time, antibiotics therapy in the two weeks preceding admission, presence of colonization by alert microorganism and eventual presence of HAI and etiological agent.

The *S2* format was filled out in case of HAI onset, and it included information about HAI onset and localization, admission phase at HAI onset (initial, intermediate, advanced), HAI etiological agent, drug therapy, infection resolution or outcome. Data were analyzed in anonymous way, by expert analyzers that were blinded to the intervention time and hospital's allocation.

Each HAI occurring during the observation periods in the patients admitted to the enrolled hospital wards in the two observed periods was identified according to the criteria defined by the European Centre for Disease Prevention and Control (ECDC, 2015). All HAI types were included in the study, namely: urinary tract infections (UTI), bloodstream infections (BSI) including those central-vascular catheter (CVC)-related, systemic-clinical sepsis,

gastrointestinal infections (GI), skin and soft tissue infections, pneumonia, lower respiratory tract infections (LRI), surgical site infections (SSI), reproductive tract infections, EENT (eye, ear, nose and throat or mouth) infections, bone and joint infections, intra-abdominal infections, and non-specified infections.

HAI etiological agents were identified by microbiology laboratories of each hospital, based on routine diagnostic tests. Overall, the study surveyed 11,842 patients, 11,461 from intervention I_1-I_2 hospitals and 381 from the external control hospital.

5.4 Statistical analyses

The study power was estimated based on admissions and HAI incidence rates in Italian hospitals (Messineo and Marsella, 2015; Mancini et al., 2016). The sample size was calculated considering an 80% power to detect an infection incidence reduction of at least 25% starting from a hypothesized rate of 4%, assuming a two sided test with an alpha level of 0.05, and corresponded to 10,476 patients. Statistical analyses were performed using chi-square test, Kolmogorov-Smirnov test for evaluating normality, parametric (Student's t test) and non-parametric (Mann-Whitney) tests, chi-square test of association, and multivariable analysis (logistic regression), assuming as statistically significant a p value at least <0.05.

Multivariable model was developed including all the parameters which showed a statistically significant correlation with HAI occurrence by univariate analysis. Bonferroni correction for multiple comparisons was applied for analysis of microarray data (a p_c value <0.05 was considered significant).

Analyses were performed using the software IBM1SPSS201Statistics (IBM, Bologna, Italy).

6. Evaluation of the effectiveness against hospital pathogens of a combined use of probiotics and bacteriophages: test in vitro and in situ

6.1 Test *in vitro*

6.1.1 Bacterial species, culture isolation and microbial identification

The bacterial strains used for *in vitro* tests included both ATCC strains and wild-type strains collected from hospital surfaces.

ATCC strains included *S. aureus* (ATCC 25923), *E. coli* (ATCC 25922) and *P. aeruginosa* (ATCC BAA-47), whereas hospital isolates included three drug-resistant strains of the same species, previously collected and identified by Maldi-Tof (AccuPRO-ID; Charles River Lab Europe SaS, Ecully, France), namely *S. aureus* (SA2-R73), *E. coli* (EC-R60), *P. aeruginosa* (PA-V6).

6.1.2 Antimicrobial resistances characterization of bacterial species

The selection of wild-type strains for the subsequent tests was performed based on their antimicrobial resistance features. To this aim, each isolate was characterized for antibiotic resistance by conventional disc-diffusion Kirby Bauer antibiograms, using Mueller-Hinton agar plates.

The following antibiotics were tested: penicillin G, ampicillin, vancomycin, oxacillin, cefotaxime, cefoxitin, gentamicin, imipenem, aztreonam, meropenem and colistin (Oxoid, Altrincham, UK; Liofilchem, Teramo, Italy). Inhibition zone diameters were interpreted according to the EUCAST breakpoint tables for the interpretation of MIC, and inhibition zone diameters and the Clinical and Laboratory Standards Institute manual (CLSI, 2018). In addition, MIC of resistant strains were also measured, accordingly to EFSA (European Food Safety Authority) guidelines, by using antibiotic stripes containing serial dilutions of each antibiotic (Liofilchem, Teramo, Italy).

6.1.3 Bacteriophages

Two bacteriophage preparations obtained from Eliava Institute (Tbilisi, Georgia) were used for this study and included “Staphylococcal bacteriophage”, a filtrate of the purified lytic active against *S. aureus* strains (concentrated 10^7 plaque forming unit per ml, PFU/ml) and “Pyophage”, a mixture of selected lytic phages directed against *Staphylococcus* spp., *Streptococcus* spp., *Proteus* spp., *E. coli* and *Pseudomonas* spp. (concentrated 10^5-10^6 PFU/ml).

Phage mixtures were stored at 4°C until use, as indicated by manufacturer instructions. In **Figure 18** "Staphylococcal bacteriophage" and "Pyophage" preparations are shown.

Figure 18. "Staphylococcal bacteriophage" and "Pyo bacteriophage" preparations (Eliava Institute). Modified image created by the Author, from the websites http://phage.ge/products/staphylococcal-bacteriophage/ and http://phage.ge/products/pyo-bacteriophage/ (last accessed in October 3rd, 2019).

Each individual phage component of the mixture was first titrated by PFU counting on the correspondent ATCC bacterial target including *S. aureus* ATCC 25923 for anti- *S. aureus* phages, *E. coli* ATCC 25922 for anti-*E. coli* phages, *S. pyogenes* ATCC 19615 for anti-*S. pyogenes* phages, *P. mirabilis* ATCC 29906 for anti-*P. mirabilis* phages and *P. aeruginosa* ATCC 10145 for anti-*P. aeruginosa* phages.

Briefly, phage stock preparations were serially diluted in TSB, then 100µl of phage dilutions were mixed with 100µl of bacterial target suspension in logarithmic growth phase (OD_{600nm}= 0,4 by using spectrophotometer DU-640B, Beckman Coulter, Brea, CA, USA).

The bacteria and phages mixture was incubated for 10-15 minutes at room temperature, then it was added to 3 ml of soft top agar (0,75% agar) poured evenly across the top of the TSA plate and allowed to solidify for 15 minutes. Samples were performed in triplicate.

After incubation at 37°C for 24 hours, PFU were counted at appropriate dilution and the phage titer was determined by multiplying the number of lytic transparent zones (plaques) with the dilution index.

Individual phage preparations specifically targeting each single bacterial species were obtained from the mixtures by the lysis plaque elution method (**Figure 19**), which consisted to collect plaques by the plate by using sterile loop, subsequently disrupted by pulse vortexing in 1 ml of TSB, and added to 5 ml of the bacterial target suspension at 0.4 OD_{600nm}.

The suspension was then incubated at 37°C under mild agitation until the solution became clear, indicating bacteriophage amplification and reduction of bacterial growth. The solution was finally centrifuged in order to remove bacterial debris and the supernatant was collected and filtered through a 0.22 µm pore size membrane filter, added with 15% sterile glycerol and stored at -80°C until use. Each individual stock was titrated before experiments, as already described, , obtaining a 10^{10} PFU/ml final titer of each individual phage preparation.

Figure 19. Lysis plaque elution method steps description.

6.1.4 Host range analysis: double-layer plate assay

The host range of single phage preparation stock was determined by double-layer plate assay (soft agar spot tests), performed against all the bacterial species previously described.

Briefly, overnight bacterial cultures in TSB were subcultured by 1:10 dilution and grown at 37°C under agitation in order to reach $OD_{600nm=}$ 0.4. Aliquots (100µl) of subculture were added to 3ml of soft agar, then overlaid on TSA plates, and allowed to solidify at room temperature for 15 minutes. Phage stocks were serially diluted (with 10-fold increments) in phosphate buffered saline (PBS) and 10µl aliquots of phage dilutions were added to bacterial lawns. Assays were performed in triplicate. The lytic activity was checked after 24 hours of incubation at 37°C. The presence of clear zones in the phage spot area indicated *positive results*, distinguishable in:

CL: Complete Lysis;

SCL: Semi-confluent Lysis;

OL: Overgrowth, bacterial lawn completely broken, but presence of singular bacterial colonies on spot;

IPO/IPC: Countable number of multiple small phage plaques. The presence of transparent phage negative colonies is recorded as IPC. The presence of opaque negative colonies is recorded as IPO.

By contrast, the absence of zones of bacterial growth inhibition indicates the resistance (R) of bacterial strain to tested bacteriophages and is considered as a *negative result*.

6.1.5 Bacteriophage stability in PCHS detergent

Phage preparations were diluted in PCHS detergent, previously diluted 1:100 (v/v) in distilled sterile water to obtain the work dilution as indicated by the manufacturer. Phage stability was measured after 1, 2, 3 and 7 days at room temperature, by PFU titration) on the specific bacterial targets (10^3 and 10^2 PFU were considered for tests), after removing the bacterial component of detergent (bacilli) by centrifugation and filtration.

Bacterial target included: *S. aureus* ATCC 25923, *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 10145.

In parallel, phage preparations were also diluted in PBS, as a positive control, and direct comparison between PFU number of the two preparations (phages in PBS and in PCHS) was performed.

6.1.6 Decontamination tests on hard surfaces

The ability of phages to lyse *S. aureus, E. coli, and P. aeruginosa* (both ATCC and wild type strains) on different kinds of hard nonporous surfaces (plastic, glass, and ceramic respectively represented by irradiated sterile plastic plates, or glass plates, and ceramic tiles sterilized by autoclave) was assessed by *in vitro* decontamination assays.

Briefly, target bacteria were grown in TSB until reaching the logarithmic growth phase (checked by spectrophotometric reading, OD_{600nm} =0.4), and then diluted in order to obtain a specific final bacterial concentration of 4×10^6 CFU/ml.

To mimic bacterial densities comparable to those found on hospital surfaces, 10µl of target bacteria corresponding to 10^2 CFU/24 cm^2 (4×10^4 CFU/m^2), representing a realistic contamination value based on previous studies, were seeded on surfaces by using sterile spatula. Seeded bacteria were allowed to dry for 15 minutes at room temperature, then, 50 µl of the concentrated individual phage solution diluted in PBS at a multiplicity of infection (MOI) of 10, 100, and 1000:1 (10^3, 10^4 and 10^5 PFU respectively for 10^2 bacterial cell) were spread on the surface and allowed to dry in a maximum drying time of 10 minutes, limiting the contact time between phages and bacteria as low as far as possible in order to mimic routine sanitation procedures.

Mock treatment with phage buffer alone was used as a control whereas denatured alcohol was used as a positive control.

Each sample was performed in triplicate. After 1, 3, 6, and 24 hours, surface sampling was directly performed by contact Rodac plates containing the appropriate selective medium, to collect residual viable bacteria. Each plate, containing samples taken at the different time points, was then incubated for 24 hours at 37°C and bacterial load was evaluated by enumerating CFU.

The same assays were performed by diluting phage preparations in PCHS detergent, using 100 CFU/24 cm^2 of target bacteria and phage at 100 and 1000:1 MOI (phage/bacteria ratio).

6.2 Test *in situ*

6.2.1 Probiotic/phage treatment

Decontamination ability of phages was tested *in situ*, using the ceramic sink of a bathroom. Briefly, the sink was artificially contaminated with 10^2 CFU/ml of *S. aureus* ATCC strain, uniformly spread and allowed to dry for 24 hours.

After 24 hours, the artificially contaminated surface was treated by spraying water (CTR), PCHS detergent alone, anti-staphylococcal phages in PBS alone (phages), or probiotic detergent added with 10^5 PFU/mL of anti-staphylococcal phages (PCHS + phages), at 1000:1 MOI, based on previous results obtained *in vitro* experiments.

The detergent solution sprayed on the tested surfaces was kept low enough to dry completely in <10 minutes, in order to mimick routine surface cleaning.

6.2.2 CFU enumeration

After treatment, surface contamination was assayed by application of Baird–Parker Rodac plates after 1 hour, and 1, 3, and 15 days, that were incubated at 37°C for 48°C.

Both *S. aureus* and PCHS-Bacilli able to grow on Baird–Parker agar, but they are clearly distinguishable as *S. aureus* originates black round colonies surrounded by a clear zone, whereas Bacilli give rise to irregular gray-brown colonies. After incubation, *Bacillus* and *Staphylococcus* colonies were counted. Three independent experiments were performed, with duplicate samples.

6.3 Statistical analysis

Statistical significance was measured by the unpaired onetailed Student's t-test (STAT View software; SAS Institute Inc., Cary, NC, USA). Values of $p<0.05$ were considered statistically significant.

7. Evaluation of Staphylococcal decontamination on hospital surfaces by a bacteriophage–probiotic sanitation strategy: a monocentric study

7.1 Study design and sanitation procedure

The study was performed in the private hospital Quisisana (Ferrara, Italy), routinely sanitized since 2014 by the probiotic-based PCHS system, after obtaining approval by the local Ethics Committee and authorization of the Hospital Medical Director. As a proof of concept, Staphylococcal contamination was assayed in the bathrooms of Internal Medicine ward. Eight rooms of the Internal Medicine ward were enrolled, and randomly included in the "Intervention" group or in the "Control" group (four room each).

All enrolled rooms received PCHS sanitation both in the room and in the bathroom, performed as already described (Vandini et al., 2014; Caselli et al., 2016b). Prior to introduction of combined probioic-phage sanitation, all rooms were monitored for Staphylococcal contamination for 1 week, by performing three total samplings on alternate days, to quantify Staphylococcal load (representing the most prevalent bacteria contamination in bathrooms). Then, four bathrooms started receiving PCHS sanitation plus anti-Staphylococcal bacteriophages (Intervention group), whereas four bathrooms received PCHS sanitation alone (Control group). All sanitation procedures were performed in early morning and phage application was performed by nebulization with atomizer, placed in the middle of the bathroom so that all surfaces were treated in the same way. The whole procedure took about 10 minutes during which the bathroom was not available for inpatients. Based on preliminary tests, executed in order to guarantee a homogeneous dispersion on bathroom surfaces and a time of water persistence on surfaces of 10 minutes, nebulization was performed for 4 minutes, using 500 ml of solution with phages (2×10^8 PFU).

Phages were were applied daily for seven days, then discontinued for 4 days, and then re-introduced for a further 7 days. Staphylococcal contamination was assessed for 2 weeks at the following times: days 1, 3, 5, 7, 9, 10, 11, 14, 16 and 18 (**Figure 20**), by monitoring 5 points in duplicate at each sampling time: bathroom floor, sink, shower plate, room floor and bed footboard.

The study design thus included an internal control for each room (comparison between phases in the same room) and parallel controls between groups (comparison between Intervention and Control group).

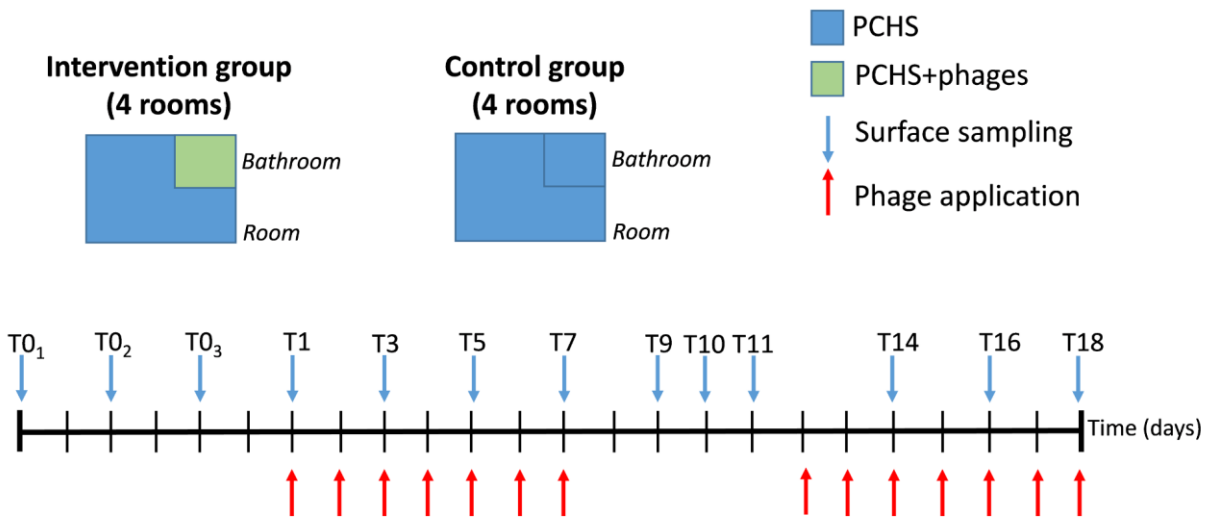


Figure 20. Study design and timeline. Eight rooms were enrolled in the studied and randomly divided into two groups: Intervention group, having the bathroom sanitized by PCHS plus anti-staphylococcal phages (green), and Control group, having the bathroom sanitized by PCHS alone (blue). Phage applications are indicated with red arrows, samplings with blue arrows (D'Accolti et al.,2019b)

7.2 Bacteriophages used for treatment

A concentrated phage product used was specifically obtained from Eliava Institute (Tbilisi, Georgia), consisting of a concentrated solution (10^{10} PFU/ml) of the "Staphylococcal Bacteriophage" preparation, active against *Staphylococcus aureus* strains (Sb-1 phage, usually available at the concentration of 10^7 PFU/ml).

Preparation was maintained at 4°C until use and titred as previously described. For phage application on hospital surfaces, it was diluted in 0.5 µm filtered PCHS detergent at work dilution (1:100 in water), obtaining a final concentration corresponding to $4x10^8$ PFU/l; 500ml (2×10^8 PFU) were used per bathroom (considering a bathroom floor surface of 4.5 m^2), in order to obtain a MOI of 1000:1.

7.3 Environmental sampling, culture isolation and species identification

Surface Staphylococcal contamination was evaluated by CFU counts on Rodac plates, with Baird Parker agar medium, selective for *Staphylococcus* spp.

After sampling, performed at 2.00 pm to allow the natural recontamination processes, plates were immediately refrigerated and incubated at 37°C for 48h within 2 hours of sampling.

At the end of the incubation time, CFUs were counted and results expressed as CFU/m^2.

A total of 400 samples were collected and analyzed. Since *Bacillus* can also grow on Baird Parker medium, the same plates were also used for their enumeration.

Staphylococcus spp. were re-cultured on plates and species identification was performed by Maldi-Tof (AccuPRO-ID; Charles River Lab Europe SaS, Ecully, France).

7.4 Host range analysis: double-layer plate assays and spot test

Prior to phage application on field, Staphylococcal contamination was characterized, and each *Staphylococcus* isolate was characterized for susceptibility to 'Staphylococcal bacteriophage' by spot test and double-layer plate assays (soft agar spot test).

Double-layer plate assays were performed as previously described (**6.1.4** "Host range analysis: double-layer plate assay" paragraph), using $10,10^2,10^3,10^4$ PFU per spot (10 µl per spot)

For spot tests, bacteria in the logarithmic growth phase (OD_{600nm}= 0.4) were seeded on agar plates, and 10 µl of phage preparation was added in a single drop at the centre of the seeded bacteria. Lysis plaques were observed after 24 h of incubation at 37°C and results interpretation was performed according description in **6.1.4** "Host range analysis: double-layer plate assay" paragraph.

7.5 Molecular analysis

The same points monitored for microbiological analyses were also sampled by sterile swab, using a 10x10 cm surface, as already described in **5.2** "Microbial surface contamination: environmental sampling" paragraph.

After sampling, swabs were put in sterile tubes containing 0.4 ml of sterile PBS, immediately refrigerated and stored at -80°C within 2 hours.

Subsequently, samples were thawed and vortexed in order to detach microorganisms from swabs, and total DNA was extracted directly from PBS suspension by the commercial kit "Exgene™, Viral DNA and RNA" (Gene All, Tema Ricerca, Italy), following the manufacturer's instructions.

The concentration of the extracted viral DNA was finally evaluated by spectrophotometric reading by "Nanodrop" instrument (Thermo Scientific), and samples were stored at -20°C until use.

7.5.1 qPCRs: *Sb-1*, *PanB* and *spo0A* qPCRs

Three different qPCRs were performed in this study: *PanB* and S*po0A* (respectively used for quantitation of total bacterial contamination and of *Bacillus* quote, and previously described in paragraphs **5.2.7.1** and **5.2.7.2**), and a further qPCR specifically set up for the detection

and quantitation of anti-Staphylococcal bacteriophage load on treated surfaces. The amplification reaction was designed in **ORF79** of the *Staphylococcus aureus* Sb-1 phage genome, coding for the major capsid protein (NCBI Ref. Seq. NC_0230091).

Prior to use, specificity of *Sb-1* qPCR was checked by analysing the DNA extracted from phages targeting other bacteria, such as *Escherichia coli*, *Pseudomonas aeruginosa* and *Salmonella enteriditis.*

Primers, probe and thermal conditions of *Sb-1* qPCR are shown in **Tables 17** and **18** respectively.

Table 17. Primers and probe of *Sb-1* qPCR

Oligonucleotides	Sequence
Primer Sb-1F	5'-GTG ATA TCT CAC GCC GTC C- 3'
Primer Sb-1R	5'- TTT GGG TCA GAT ACT GGT GC-3'
TaqMan probe	5'-(FAM) GTC ATG GTA ACG TAG GTC A-MGB-3'

Table 18. Thermal conditions of *Sb-1* qPCR (fast technology)

Phase	Thermal conditions	Cycles
Denaturation	95°C 1 sec.	1 cycle
Annealing/Elongation	60°C 20 sec.	40 cycles

The amplification reaction was carried out in a 96-wells plate, by using 20µl of volume for each sample/well, including 11µl of reaction mixture (mix) and 10ng of DNA template.

Samples were tested in duplicate and they included standards (ST), unknown (UNK) and negative control (NTC). ST samples were obtained by 10-fold serial dilutions (from 10^7 to 10^2 DNA copies) of Sb-1 phage DNA with known titre.

Reactions were performed with fast technology to obtain the raw CT values for each assay by using the QuantStudio5 instrument (Thermo Fisher, Life Technologies). Absolute quantitation of anti-Staphylococcal phage was thus obtained.

PanB and *Spo0A* qPCRs were performed as previously described in **5.2.7.1** "Pan Bacterial qPCR (*PanB* qPCR) and **5.2.7.2** "*Bacillus*-specific qPCR (*Spo0A* qPCR)" paragraphs.

7.5.1.1 Customized qPCR microarray for microbial characterization

Characterization of the surface microbial contamination was also assessed by performing a customized qPCR microarray (Qiagen, Hilden, Germany) detecting simultaneously the presence of the following 13 microbes (**Figure 21**): *S. aureus*, *S. epidermidis*, *E. faecalis*, *E. faecium, E. coli, K. pneumoniae*/*Enterobacter*, *A. baumannii, P. mirabilis, P. aeruginosa, C. difficile, A. fumigatus* and *C. albicans*. *Pan Bacteria (panB)* and *Pan Mycetes (panM)* reactions were also included as controls.

The amplification reactions were carried out in a 96-wells plate; up to 6 environmental samples could be analyzed simultaneously. A total amount of 120ng of DNA per each sample, corresponding to about 7ng/well were seeded into 16 wells of the array plate, in a final volume of 25µl. Amplification reactions were performed by using the QuantStudio5 instrument (Thermo Fisher, Life Technologies).

	1	2	3	4	5	6	7	8	9	10	11	12
A	Staphylococcus Aureus BPID00314A	Pseudomonas aeruginosa BPID00288A										
B	Staphylococcus Epidermidis BPID00316A	Clostridium perfrigens BPID00112A										
C	Enterococcus fecalis BPID00142A	Clostridium difficile BPID00110A										
D	Enterococcus faecium BPID00143A	Aspergillus fumigatus BPID00038A										
E	Escherichia coli BPID00146A	Candida albicans BPID00092A										
F	Klebsiella/Enterobacter BPID00139A	Pan Aspergillus/ Candida BPCL00359A										
G	Acinetobacter baumannii BPID00002A	Pan Bacteria 1 BPCL00360A										
H	Proteus mirabilis BPID00287A	PPC BPCL00465A										

Figure 21. Schematic representation of the customized array used for the detection of the indicated 13 microbes (and 2 controls). Qiagen codes for each qPCR are also reported.

RESULTS

8. Probiotic based-sanitation impact on AMR and HAI incidence: a pre-post interventional multicentre study

8.1 Impact of probiotic based-sanitation on hospital surface microbiota

The impact of the *Bacillus*-based detergent on hospital surfaces microbiota was analyzed by conventional microbiological tests and molecular assays. The microbiological analysis of surface bioburden were performed by CFU count on specific Rodac contact plates, including selective media allowing detection and quantification of *Staphylococcus* spp (**Figure 22A**), *Enterobacteriaceae* spp. (**Figure 22B**), *Pseudomonas* spp., *A. baumannii, C. difficile* and *Candida* spp. Overall, >32,000 environmental samples were analyzed. Pathogen contamination was expressed as the sum of the CFU/m^2 detected for each searched pathogen by CFU count on Rodac plates.

The results showed a persistent contamination in all the wards of enrolled hospitals in pre-PCHS period, with an overall pathogen load corresponding to 22,737 CFU/m^2 (median value, range 17,053-60,632).

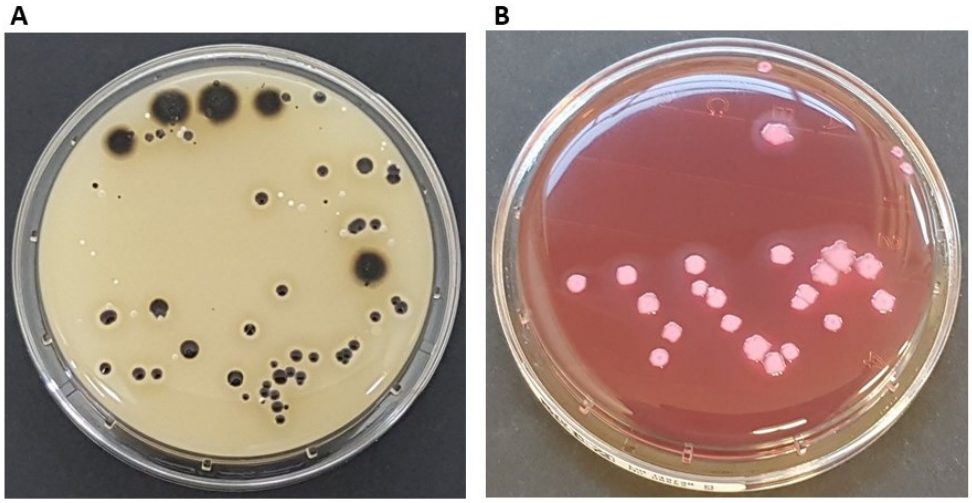


Figure 22. Photographs of representative bacteria colonies grown on Rodac plates. **A**) Baird Parker Agar (selective for *Staphylococcus* spp.): the plate evidences the growth of coagulase-positive and coagulase-negative staphylococci (respectively forming brilliant medium-size colonies surrounded by clear halos, and small/medium grey-black colonies without halos), and *Bacillus* spp. (brown colonies with irregular margins). **B**) MacConkey agar (selective for *Enterobacteriaceae)*: the plate evidences the growth of pink *E. coli* colonies, surrounded by a zone of acid precipitated bile.
All colonies of interest were identified according to what described in **5.2.1** "Culture Isolation and microbial identification" paragraph.

Pathogen load during pre-PCHS was mostly attributable to Staphylococcal contamination that represented up to 90% of the total surface microbiota; however, other genera were present, unless less abundant (**Table 19**) (Caselli et al., 2018).

Table 19. Pathogens' load during pre-PCHS period (Caselli et al., 2018)

Microbial genera	Median load (CFU/m^2)	Range (CFU/m^2)
Staphylococcus spp.	21,895	13,684-57,263
Enterobacteriaceae	1,784	444-3,015
Pseudomonas spp.	361	43-2,125
Acinetobacter	2,538	214-3,836
Clostridium difficile	286	137-842
Candida spp.	1,480	1,075-5,508

The introduction of PCHS in the five hospitals subjected to intervention (I_1 and I_2 groups) induced a statistically significant decrease of pathogen contamination from 22,737 CFU/m^2 to 4,632 CFU/m^2 (median value, range 842-12,632 CFU/m^2), corresponding to a general mean decrease of 83% of pathogens. No variations were observed in the external control hospital (extC) between the two periods of the study (**Figure 23)** (Caselli et al., 2018).

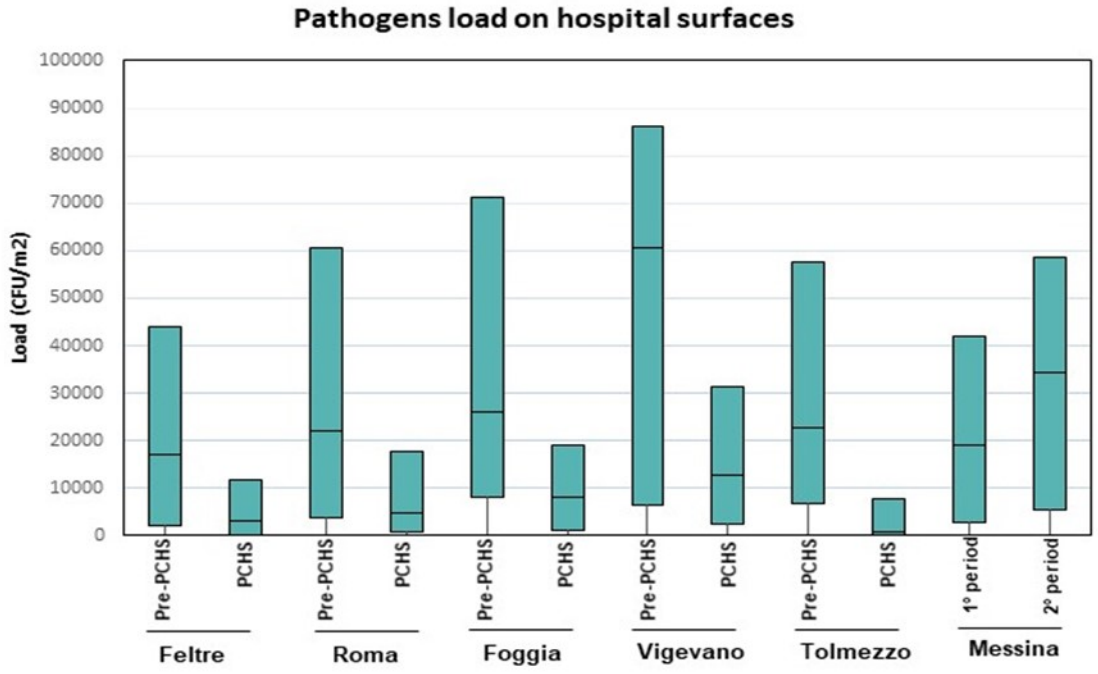


Figure 23. Schematic representation of hospital pathogen load on hospital surfaces, expressed as CFU/m^2 referred to the sum of the median value of the CFUs obtained for each of the six pathogens, as described in Methods (*Staphylococcus* spp., *Enterobacteriaceae* spp., *Acinetobacter*, *Candida* spp., *Pseudomonas* spp., *Clostridium* difficile). For pre-intervention (pre-PCHS) and intervention (PCHS) phases, and for each hospital enrolled, median values (lower part of the box) and Q3 (upper part of the box, representing the 75% percentile values), are shown. Values reported for the external control hospital, corresponded to those detected in the 1st and 2nd 6-month periods of the study. Modified from Caselli et al., 2018.

In parallel, the analyses performed by molecular assays on the same sampled surfaces by *PanB*-qPCR and *Spo0A*-qPCR (as previously described in **5.2.7.1** and **5.2.7.2** paragraphs), evidenced a concomitant significant increase in the number of *Bacillus* bacteria on the surfaces of Intervention-group hospitals. In fact, the *Bacillus* quote increased from 0% in the pre-PCHS period (median value, range 0-30%) to 69.8% in the PCHS phase (median value, range 39.9-86.8%) of the total surface microbiota ($p<0.0001$). These results suggest that the PCHS-*Bacillus* displayed the ability of competing with the persisting pathogenic microbiota on treated hospital surfaces, finally replacing most of the microbial species originally present on the surfaces. No increase in *Bacillus* counts was observed in the extC control hospital (**Figure 24**) (Caselli et al., 2018).

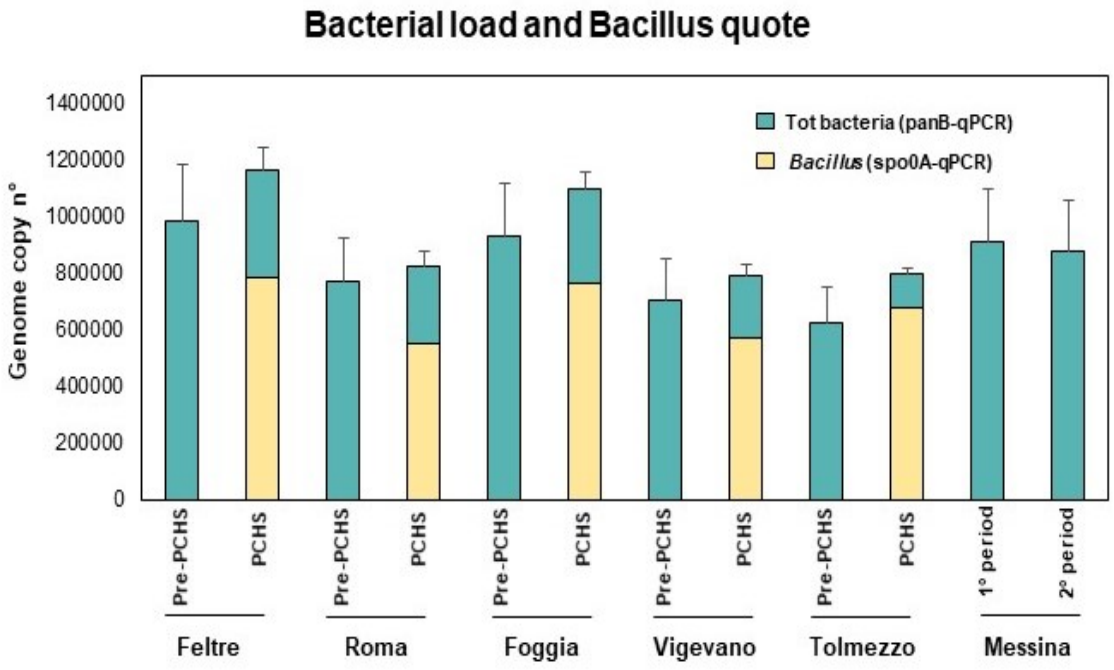


Figure 24. Total bacterial load and PCHS-*Bacillus* count, respectively measured by a pan-bacterial qPCR (*PanB*) and a specific qPCR for *Bacillus* genus (*spo0A*). Results are expressed as genome copy number per 100ng of tested DNA. The median values ± SD pf pre-PCHS and PCHS phases are shown. Values reported for the external control hospital (extC, Messina) correspond to those detected in the 1st and 2nd 6-month periods of the study. Modified from Caselli et al., 2018.

8.2 Impact of probiotic based- sanitation on AMR of the hospital surface microbiota

To characterize the antibiotic resistance profile of the entire microbiota contaminating hospital surfaces, the total DNA extracted from the environmental population was analyzed by using a qPCR microarray able to detect and quantify simultaneously 84 different antibiotic-resistance genes, including all the classes of antibiotics, as described in **5.2.7.3** "Antibiotic Resistance Genes qPCR microarray" paragraph.

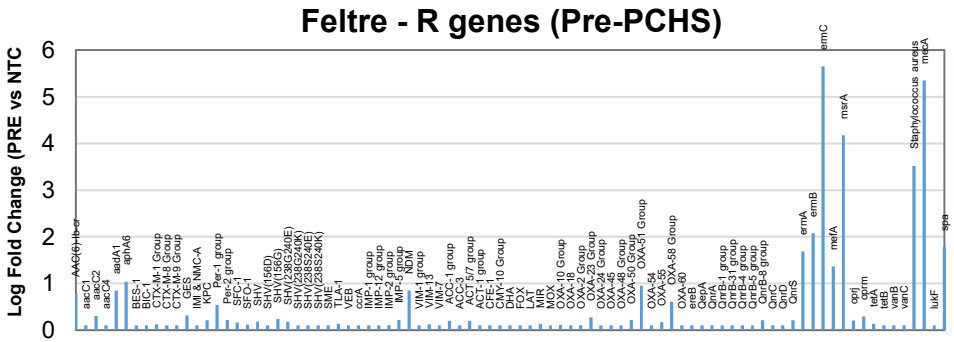
The analysis showed that in the pre-PCHS period of the study, several resistance (R) genes were detectable in the contaminating population, at different levels in the in the enrolled hospitals, potentially reflecting the selective pressure exerted in each healthcare setting by the different use of antimicrobials (**Figure 25, left panels**)(Caselli et al., 2018; Caselli et al., 2019).

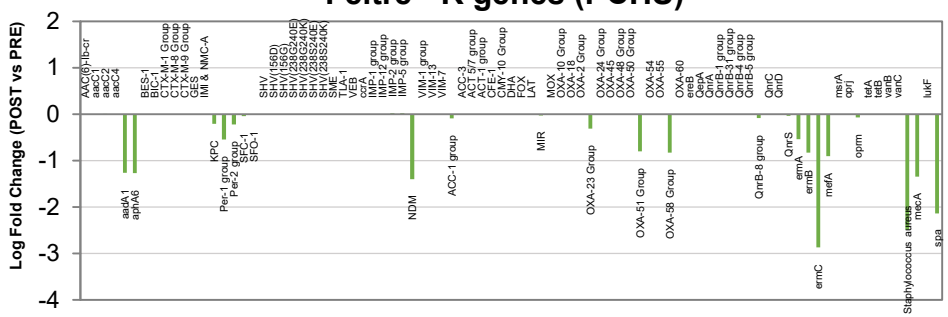
Overall, the most prevalently detected R genes included genes coding for the resistance against *aminoglycosides, fluoroquinolones, macrolides, methicillin, vancomycin* and for *class-A, class-C,* and *class-D β-lactamases*.

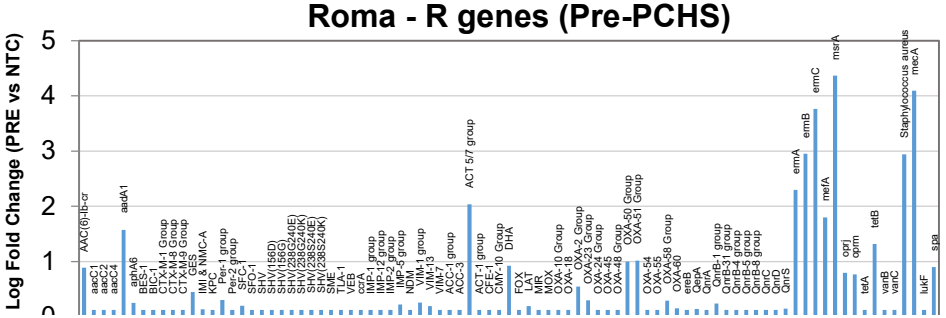
Following PCHS application, a significant decrease in the R genes was observed in each hospital of the Intervention-group, compared to those detected in the corresponding pre-PCHS period ($p_c < 0.01$), independently of the relative abundance of specific R genes in the pre-intervention phase (**Figure 25, right panels**) (Caselli et al., 2018; Caselli et al., 2019). Taken together, the results showed a significant global decrease of resistance genes in all the I_1-I_2 hospitals during PCHS phase, compared to what detected in the pre-PCHS period ($p< 0.0001$; $p_c = 0.008$). By contrast, no differences were observed in the extC hospital between the 1st and in the 2nd 6-month periods of the study (**Figure 26) (**Caselli et al., 2018).

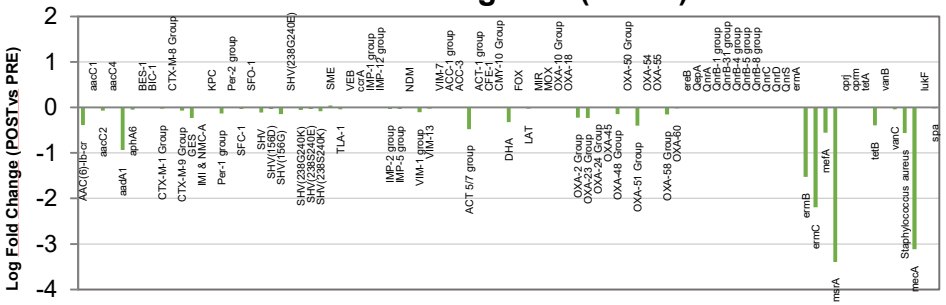
According to the high levels of contamination by *Staphylococcus* spp, the most prevalent R gene detected in pre-PCHS period was *mecA,* coding for methicillin resistance in such bacterial species (MSRA, methicillin-resistant *S. aureus*) and representing the 42.7% of all of the detected R genes in the surface microbiota. Additionally, *ermC* (26.9%, coding for erythromycin resistance) and *msrA* (26.6%, coding for macrolide resistance) genes were also detected at high frequency, whereas all the other identified genes represented only 3.8% of the resistome (**Figure 27**) (Caselli et al., 2019).

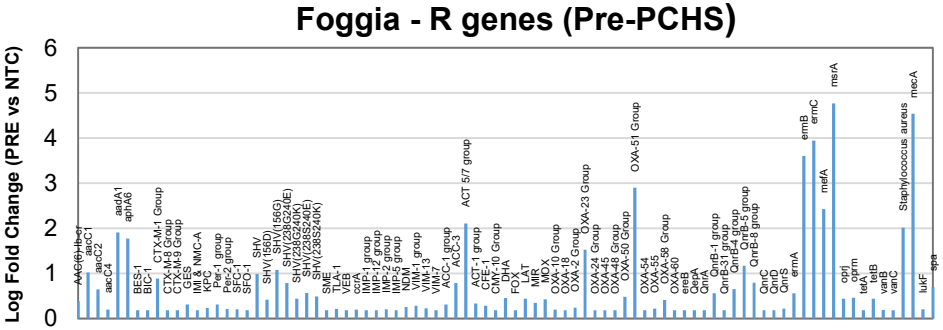
Limiting the analysis exclusively to the most prevalent R genes detected in the enrolled settings (i.e., those whose amounts were at least one log higher compared with negative controls), results showed that the introduction of PCHS appeared to be associated with a significant 70%–99.99% decrease, depending on the type of R gene (**Figure 28**) (Caselli et al., 2019).

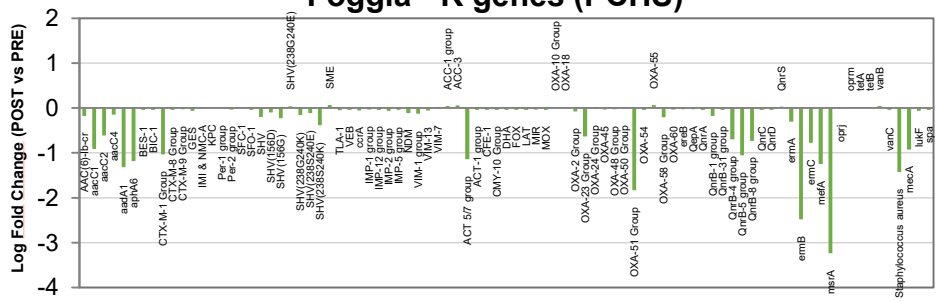

Feltre - R genes (Pre-PCHS)
Log Fold Change (PRE vs NTC)


Feltre - R genes (PCHS)
Log Fold Change (POST vs PRE)


Roma - R genes (Pre-PCHS)
Log Fold Change (PRE vs NTC)


Roma - R genes (PCHS)
Log Fold Change (POSTvs PRE)


Foggia - R genes (Pre-PCHS)
Log Fold Change (PRE vs NTC)


Foggia - R genes (PCHS)
Log Fold Change (POST vs PRE)

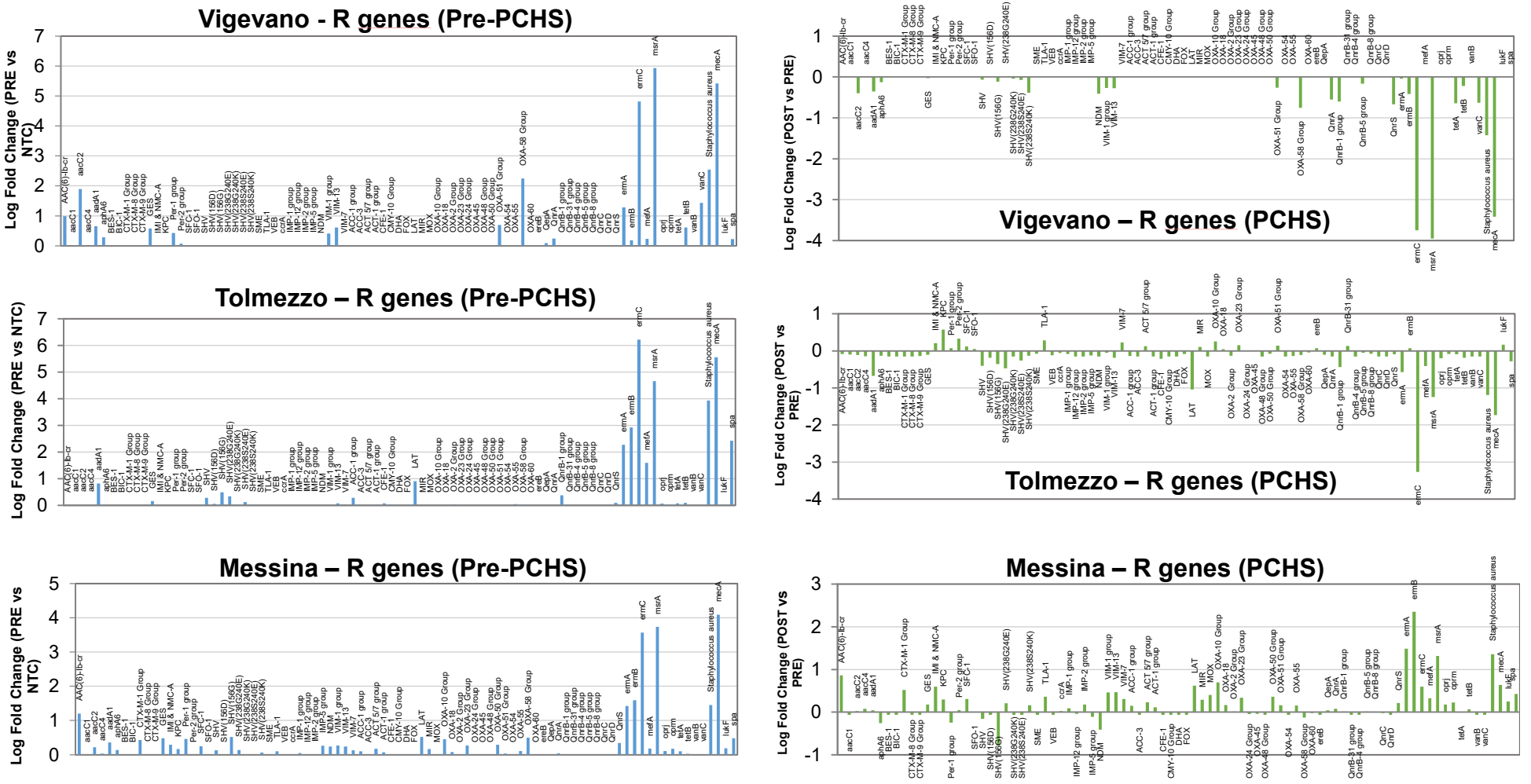


Figure 25. Resistome analysis of the surface microbiota in enrolled hospital settings. The results of the pre-intervention (pre-PCHS, left panels) and intervention (post-PCHS, right panels) phases are shown for each setting receiving intervention (Feltre, Roma, Foggia, Vigevano, Tolmezzo) and for extC hospital (Messina). The results of the pre-PCHS phase are expressed as the $\log_{10}$ fold change of each detected R gene compared with the negative controls (NTC), and the results of the PCHS phase are expressed as the $\log_{10}$ fold change of each detected R gene compared with the pre-PCHS phase. The plotted data are the mean values obtained in monthly environmental sampling campaigns (12 sampling campaigns) for all of the sampled points (18 sampled points per hospital per sampling campaign) (Caselli et al., 2019)

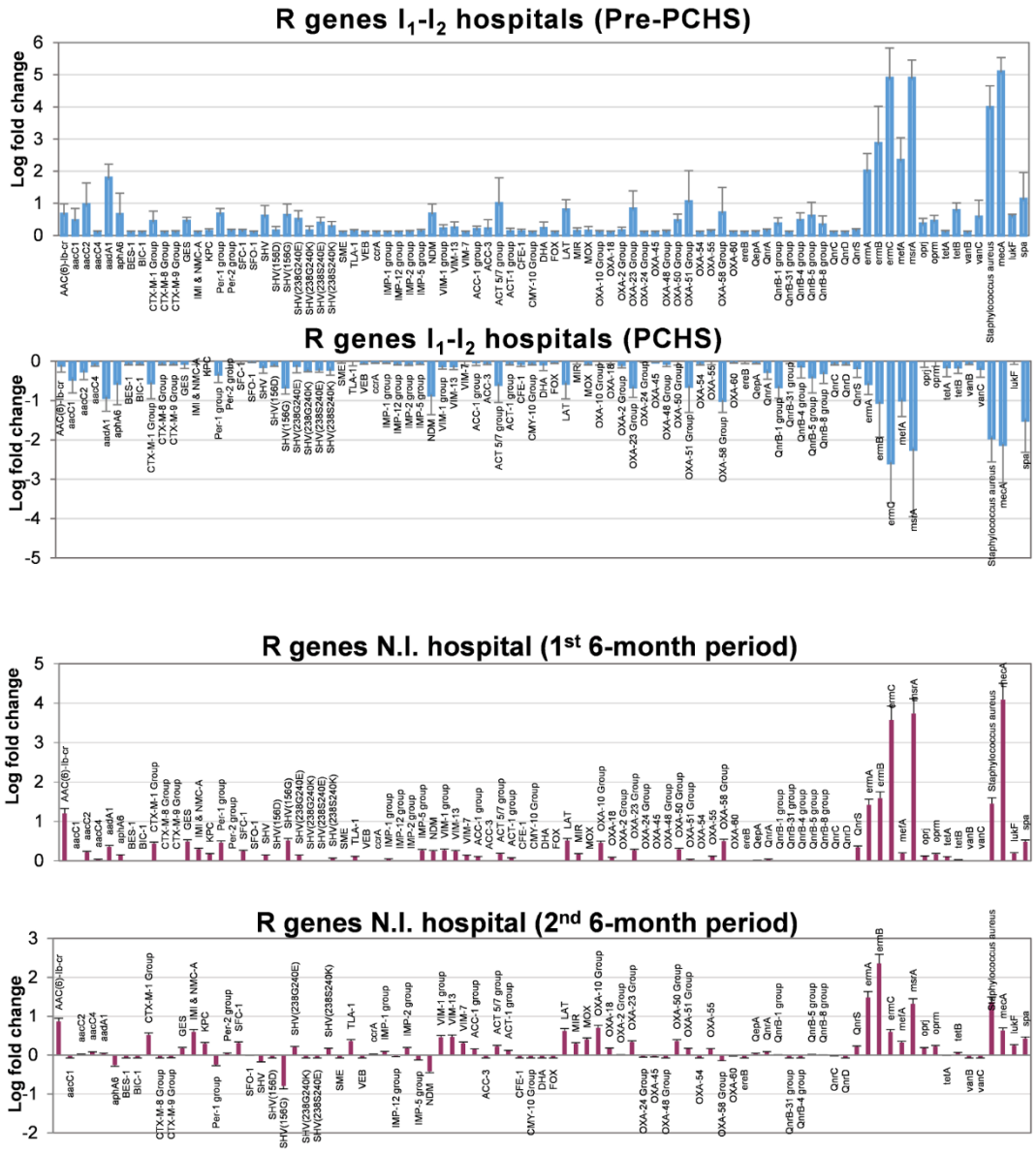


Figure 26. The results of pre-PCHS and PCHS phase are displayed for the whole Intervention-group (upper panel) and for the extC hospital (lower panel). Results are expressed as mean ± SD fold changes, compared to negative control values (for the pre-PCHS phase) and to pre-PCHS values (for PCHS phase) (Caselli et al., 2018).

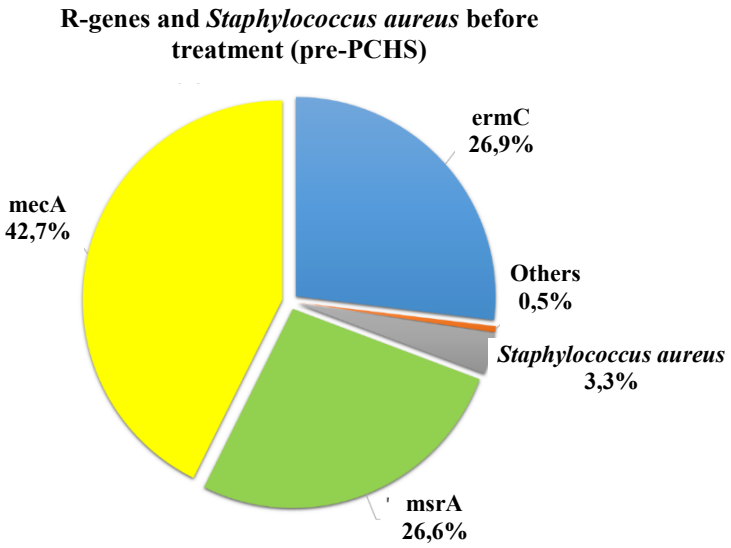


Figure 27. Pie chart representing the most prevalent R genes during pre-intervention phase (pre-PCHS). Results are expressed as percentage of the total R genes.

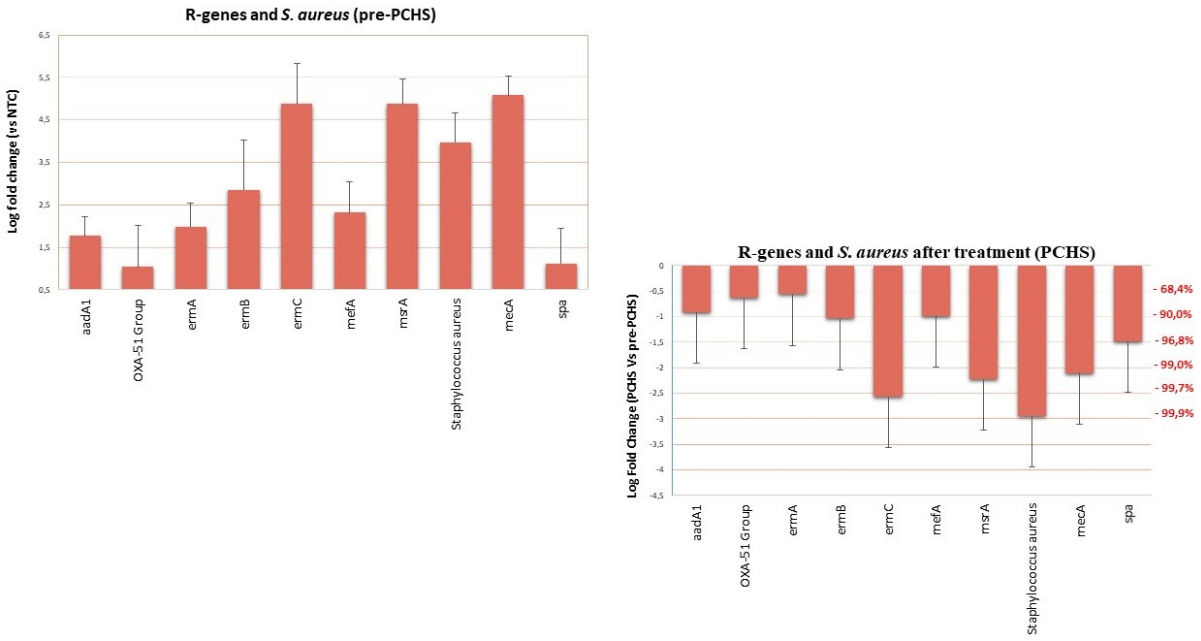


Figure 28. Representation of the % variations of the most prevalent R genes of the hospital surface microbiota following PCHS introduction. Results are expressed as Log_{10} fold and percentage change of each detected R gene compared to pre-PCHS amount (Caselli et al., 2019).

In addition, as staphylococcal contamination was prevalent in all of the enrolled hospitals, and due to the important role of *S. aureus* in HAIs, the drug susceptibility of all the *S. aureus* strains isolated from the hospital surfaces during the study was also investigated.

To this aim, all *S. aureus* isolates were analyzed using standard Kirby–Bauer antibiograms, as described in **5.2.2** "Antimicrobial susceptibility: Kirby Bauer test and microdilution broth" paragraph.

In total, 111 *S. aureus* isolates were analyzed. The results, revealed that *S. aureus* isolates from the pre-PCHS phase exhibited a high AMR (**Table 20**) (Caselli et al., 2019), with 58 strains (71.6%) exhibiting a MDR phenotype. By contrast, in the post-intervention phase *S. aureus* isolates were 63.9%–93.5% less resistant to antibiotics compared with those detected in the pre-intervention phase, depending on the antibiotic type. In addition, a global 72.4% decrease of MDR *S. aureus* isolates (defined as those resistant to three or more antibiotics), from 58/81 (71.6%) in the pre-PCHS phase to 16/30 (53.3%) in the PCHS phase, was observed (**Table 20**).

Table 20. Antibiotic resistance in *S. aureus* isolates of pre-PCHS and PCHS phases (Caselli et al., 2019)

Study period	Isolates (n)	Resistant isolates						
		Penicillin G	Ampicillin	Vancomycin	Oxacillin	Cefotaxime	Imipinem	**MDR**
Pre-PCHS	81	53 (65.4%)	58 (71.6%)	31 (38.2%)	50 (61.7%)	61 (75.3%)	42 (51.8%)	58 (71.6%)
PCHS	30	18 (60%)	20 (66.6%)	2 (6.6%)	18 (60.0%)	22 (73.3%)	13 (43.3%)	16 (53.3%)
% Decrease		–66.0	–65.5	–93.5	–64.0	–63.9	–69.0	–72.4

Notes: MDR was defined as those strains resistant to three or more antibiotics. Penicillin G (10 IU), ampicillin (10 µg), vancomycin (30 µg), oxacillin (1 µg), cefotaxime (30 µg), imipenem (10 µg).

Finally, the prevalence of *mcr-1* plasmid-mediated colR, which was not included in the microarray assay, was also investigated, being the growing plasmid-driven resistance against colistin a current concern in carbapenem-resistant *Enterobacteriaceae.*

Briefly, all environmental samples from both pre-intervention and intervention phases of the study were also grown in MacConkey broth, selective for the *Enterobacteriaceae* family, to amplify selectively such population. Total DNA extracted from grown *Enterobacteriaceae* was first checked by nested-PCR for the presence of *mcr-1* gene, as described in **5.2.5.2** **"**CLR PCR and Nested CLR PCR (nCLR)" paragraph. 452 total *Enterobacteriaceae* isolates (223 in the pre-intervention phase and 229 in the intervention phase) were analyzed. **Figure 29** shows an example of nCLR and first-round CLR PCR results, as detected for 12 environmental samples collected during the study.

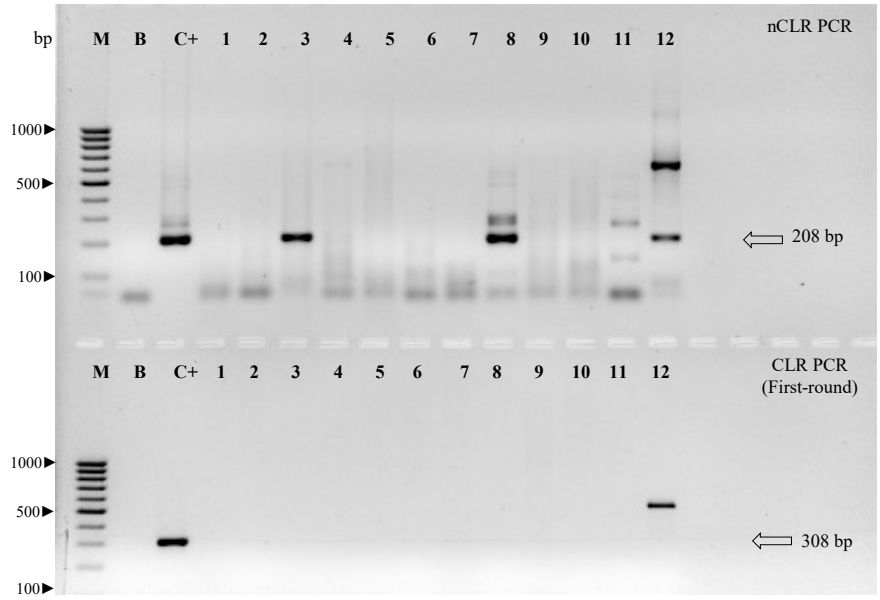


Figure 29. Results of first round CLR (lower section) and nCLR (upper section) PCRs for 12 environmental samples. Agarose gel electrophoresis was performed in 2% agarose gels. The arrows indicate the expected molecular weight for positive samples (208 bp for nCLR PCR and 308 bp for first-round PCR). As the first-round PCR is less sensitive than nCLR PCR, only C+ resulted positive. M: molecular weight marker; B: blank; C+: positive control; 1-12: *Enterobacteriaceae* environmental samples.

These molecular findings were functionally confirmed for each individual *Enterobacteriaceae* colony after culture isolation, using a broth microdilution assay (sensiTest^{TM} Colistin; Liofilchem), as described in **5.2.2** "Antimicrobial susceptibility: Kirby Bauer test and microdilution broth" paragraph.

Identification results (by Maldi-Tof) indicated that different species harboring the *mcr-1* gene, included *K. pneumoniae, K. oxytoca, E. coli, A* i*woffii, E. cloacae, E. agglomerans, C. freundii*, *P. aeruginosa,* and *P. putida* (Caselli et al., 2018b). These results suggest that this gene is silently spreading to many Gram-negative bacteria responsible for infections in clinical settings.

Importantly, results revealed that, in contrast to the pre-intervention phase, where 21/223 (9.2%) *Enterobacteriaceae* harbored the *mcr-1* plasmid, only 6/229 (2.6%) of these isolates tested positive for the *mcr-1* plasmid R gene in the intervention phase, with a whole decrease of 71.7% (**Figure 30**)(Caselli et al., 2019). In addition, microdilution assays showed that all PCR-positive strains displayed MIC values varying from 4 to 16 mg/L and exhibited the MDR phenotype, being resistant to three or more antibiotics.

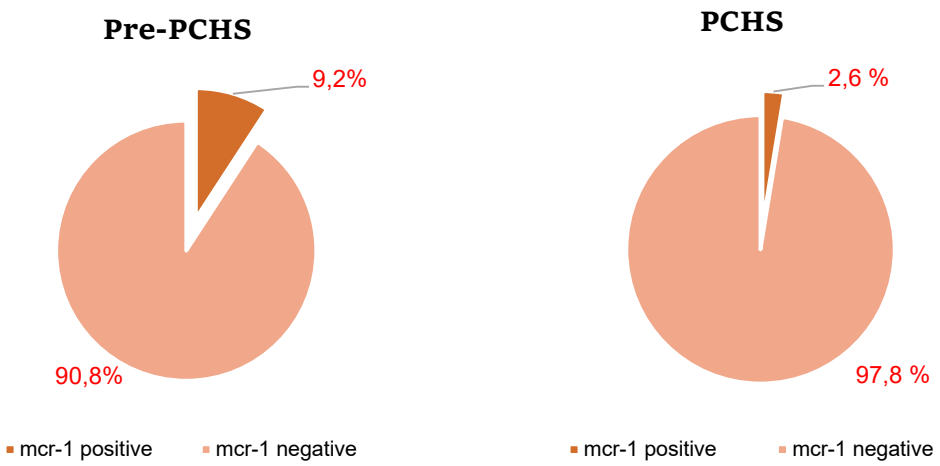


Figure 30. Prevalence of *mcr-1* plasmid presence in *Enterobacteriaceae* isolates from hospital surfaces, as judged by nested PCR results. Results are expressed as percentages of *mcr-1* positive *Enterobacteriaceae* on the total of isolates, in the pre-PCHS (21/223, 9.2%) and PCHS (6/229, 2.6%) phases.

8.3 Resistome analysis of PCHS-*Bacillus* strains collected from treated surfaces

To verify the genetic stability of PCHS-*Bacillus* despite the continuous contact with potential pathogens on treated surfaces, we analyzed the resistome of the PCHS-*Bacillus* isolated from treated hospitals. To this purpose, the *Bacillus* colonies grown on Rodac plates were identified and subsequently analyzed by qPCR microarray for R genes. Briefly, the genetic sequence of isolated *Bacillus* species was first characterized in order to distinguish between PCHS-derived *Bacillus* and similar environmental *Bacillus* species (Caselli et al., 2016b). Throughout the entire study, 4 *Bacillus* isolates were collected from each sampling campaign in the PCHS-phase for each enrolled hospital of the Intervention-group. Each isolate was identified by the following methods:

- *BK1* PCR amplification followed by amplicon digestion with AluI/TaqI restriction enzymes (as described in **5.2.5.3** "*Bacillus* genus PCR (BK1 PCR) and in **5.2.6** "*Bacillus* DNA digestion and sequencing" respectively);
- Direct DNA sequencing of the *Bsub* PCR amplification product (as described in **5.2.5.4** "*Bacillus subtilis*-group PCR (Bsub PCR) and in **5.2.6** "*Bacillus* DNA digestion and sequencing" respectively).

These two methods allowed clear distinction of the *Bacillus* strains contained in the PCHS detergent from other environmental *Bacillus* strains (data not shown).

After identification, resistome microarray analysis of PCHS-*Bacillus* isolates was performed (as described in **5.2.7.3** "Antibiotic Resistance Genes qPCR microarray**"** paragraph), and results were compared with those obtained in the PCHS-*Bacillus* contained in the original detergent.

Results showed no acquisition of new R genes in any of the *Bacillus* isolate tested during the whole study period, suggesting that these bacteria did not undergo gene transfer events, despite the continuous contact with other microorganisms contaminating surfaces, thus confirming previous studies supporting the high genetic stability of the PCHS-*Bacillus* strains (**Figure 31**) (Caselli et al., 2018).

Notably, the R genes detected in PCHS-*Bacillus* (coding for resistance against macrolides, fluoroquinolones and beta–lactams), are chromosomal and not plasmid-driven, thus the risk of transferring them to other microorganisms is very rare.

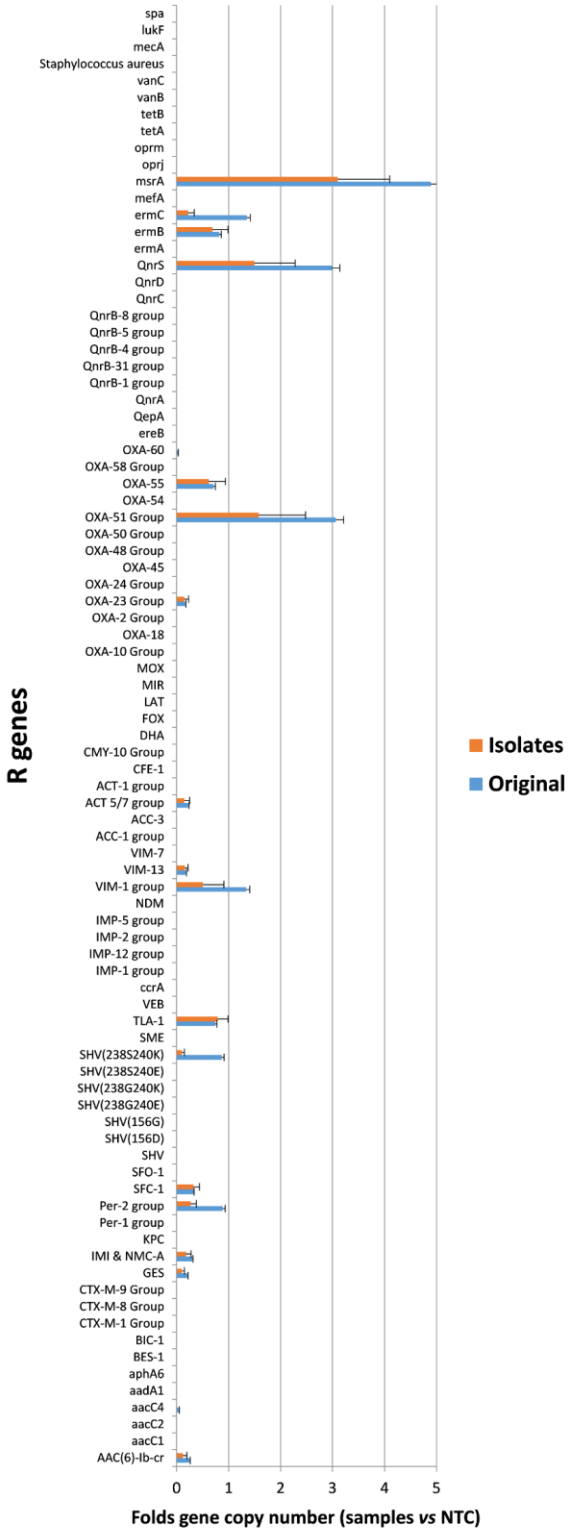


Figure 31. Resistome analysis of PCHS-*Bacillus* strains isolated from PCHS-treated surfaces.. Antibiotic resistance genes were analyzed by microarray both in the PCHS detergent prior to application, containing a blend of three *Bacillus* species (Original) and in the Bacillus isolates (Isolates) collected from hospital surfaces in the PCHS phase of I_1 and I_2 hospital groups. For original PCHS Bacilli, results are expressed as mean values ± SD of six replicates. For Isolates, results are expressed as the mean value ± SD of 120 *Bacillus* isolated from hospital surfaces. Both Original and Isolates values were compared to negative control values (NTC). Each *Bacillus* isolate was identified by PCR and sequencing prior to microarray analysis (Caselli et al., 2018).

8.4 Analysis of PCHS-*Bacillus* mechanism/s of action

Based on previous observation of our group, the main mechanism of action of PCHS-*Bacillus* is associated with competitive exclusion (Caselli et al., 2016b). However, specific assays performed to test the antimicrobial activity of PCHS-*Bacillus* evidenced that their effectiveness may at least in part attributable to the production of antibacterial compounds, as shown by results obtained both by stab overlay assays and growth tests of several indicator strains in PCHS-*Bacillus* conditioned medium, as described in **5.2.3** "Growth inhibition by stab overlay assay and in *Bacillus*-conditioned medium" paragraph.

In fact, when target microorganisms (such as *S. aureus* and *E. coli*, both ATCC and wild-type strains) were seeded on plates where PCHS-*Bacillus* were grown and then removed, a clear growth inhibition zone was evident after 24 hours of incubation (**Figure 32**).

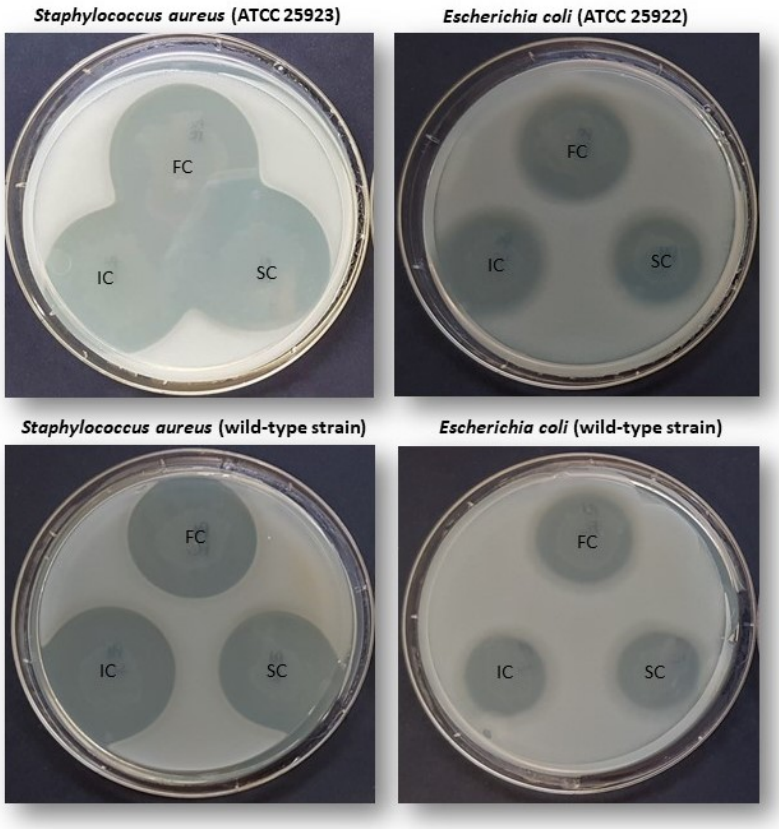


Figure 32. Bacterial growth inhibition by antimicrobial compounds produced by PCHS-derived *Bacillus* species in stab overlay assays; Bacilli were grown for 24 h on PCA plates (FC, *Floor Cleaner*; IC, *Interior Cleaner*; SC, *Sanitary cleaner*), then removed by scraping, and plates used to seed *S. aureus* and *E. coli* (both ATCC and wild-type strains): typical inhibition areas were observed after 24 h.

Similarly, the growth of several target indicator microorganisms potentially pathogenic was inhibited when cultured in PCHS-*Bacillus* conditioned medium, as shown in **Table 21** (Caselli et al., 2019b).

Table 21. Growth inhibition observed in the indicator strains cultured in PCHS-*Bacillus* conditioned medium (Caselli et al., 2019b).

Group	Species	Percentage of growth inhibition (mean ± SD)[1]		
		B. subtilis	***B. pumilus***	***B. megaterium***
Gram +	***S. aureus 25923***	94±5%	93±6%	29±9%
	S. pyogenes 19615	97±2%	95±3%	35±12%
Gram -	***E.coli 25922***	57±8%	53±9%	28±6%
	P. aeruginosa 10145	62±10%	42±11%	19±4%
	K. pneumoniae 27736	52±12%	44±10%	20±6%
	Proteus mirabilis 29906	55±9%	39±10%	22±5%
	E. cloacae 13047	92±5%	75±9%	31±7%
	S. mercescens 14756	78±15%	49±7%	21±3%

[1] The conditioned medium from 24 h PCHS-*Bacillus* cultures, depleted by residual bacteria by centrifugation and 0.45µm filtration, were inoculated with the indicator strains and incubated at 37°C for 24 h with mild agitation. The growth of indicator strains was evaluated by spectrophotometric reading at 660 nm. Results are expressed as percentages of growth inhibition compared to controls (indicator strains grown in non-conditioned medium), and represent mean value ± SD of duplicate samples from three independent experiments.

Each of the three *Bacillus* species contained in the PCHS detergent were actively inhibiting the growth of indicator strains, although to a different extent. Furthermore, similar to what observed in spot assays, the antimicrobial activity exerted by *Bacillus* was more evident against Gram-positive bacteria compared to Gram-negative ones. Additionally, PCHS *B. subtilis* and *B. pumilus* were shown to have the most effective inhibition activity compared to *B. megaterium,* suggesting that these three bacteria produce different compounds having dissimilar effect of indicator strains.

At the moment, no further information is available, but it would be interesting in the future to study the chemical and biological features of each antimicrobial compound released by PCHS-*Bacillus* strains.

8.5 Impact of probiotic based-sanitation on HAI incidence

One of the main outcomes of the study was to ascertain the effect of PCHS sanitation on the HAI incidence, being this the main goal of every sanitation procedure.

To this aim, HAI onset and characterization was evaluated in collaboration with the clinicians of the University of Udine (Prof. Silvio Brusaferro research group).

Globally 11,842 patients were surveyed, all hospitalized in the Internal Medicine wards of the enrolled hospitals. The results obtained in the pre-PCHS period showed that the mean value of HAI incidence was 4.8% (range 2.1-11.0%), confirming literature data on the HAI prevalence in Internal Medicine wards in Italy.

After implementation of PCHS, the results showed that the HAI cumulative incidence (number of patients with HAI/total surveyed patients) decreased significantly compared to the pre-PCHS phase in the I_1-I_2 hospitals, namely from 4.8% (284/5,930 patients) to 2.3% (128/5,531 patients) (range 1.3±3.7%) ($p<0.0001$) (Caselli et al., 2018). The decrease was observed in each setting regardless of the geographical location and entering time in the study (**Figure 33**).

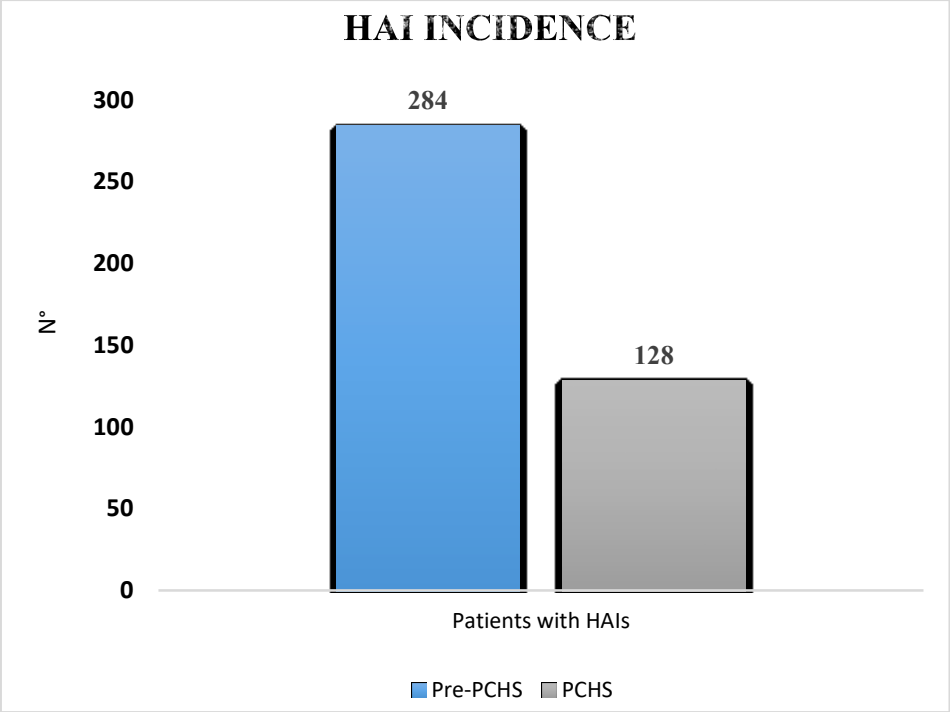


Figure 33. Number of patients with at least a HAI during pre-PCHS and PCHS periods in the Intervention-group of hospitals.

The decrease was evident in each individual hospital, including the structure with a previous very low HAI incidence (Tolmezzo, from 2.1% to 1.7%).

A slight not significant reduction was observed also in the external control hospital (extC), where the total number of HAIs was 15infections in 12 patients in the first 6-month period, and 16 infections in 16 patients in the second one, with a respective cumulative incidence rate of 8.2% and 6.8% (OR = 0.82; 95% CI, 0.37±1.78; p = 0.6) (Caselli et al., 2018).

The univariate analysis results confirmed as risk factors for HAI occurrence those already reported in the literature, indicating for example a positive correlation with the presence of urinary or central venous catheters and increasing age, whereas a protective effect emerged for being a male and self-sufficient subject.

Among all the observed HAIs, urinary tract infections (UTI) represented the most prevalent infection type, followed by bloodstream infections (BSI), systemic clinical sepsis, gastrointestinal infections (GI), skin and soft tissue infections, and respiratory infections.

Following PCHS intervention, the cumulative incidence of the most frequent HAIs decreased as follows: UTI, from 3% (179/5,930) to 1.2% (70/5,531); bloodstream infections-BSI, from 0.9% (54/ 5,930) to 0.6% (31/5,531); clinical sepsis, from 0.4% (22/5,930) to 0.1% (5/5,531); gastro-intestinal infections from 0.3% (17/5,930) to 0.1% (6/5,531); and skin/soft tissue infections from 0.3% (16/5,930) to 0.1% (6/5,531) (Caselli et al., 2018).

Instead, the relative amount of each HAI type did not change significantly in the PCHS phase compared to the pre-PCHS one, suggesting a general effect rather than a specific one.

Similarly, the number of HAI-associated microorganisms (identified in HAI patients) decreased significantly from 332 in the pre-PCHS phase to 137 in the PCHS phase, whereas the relative percentages of isolated microorganisms remained unaltered: *E. coli, E. faecalis, S. aureus, P. mirabilis and P. aeruginosa* were in fact the most frequently isolates in both phases (**Table 22**) (Caselli et al., 2018).

Table 22. Microorganisms isolated from HAIs during pre-PCHS and PCHS phases in I_1-I_2 hospitals (Caselli et al., 2018).

	Pre-PCHS	**PCHS**	
*Infections**	301	135	
Exam not available or negative	27	19	
Exam available	274	116	
Isolated microorganisms	**Samples (n, %)**	**Samples (n, %)**	**PCHS *vs* pre-PCHS**
S. aureus	21 (6.3%)	16 (11.6%)	-23.8%
***Staphylococcus* spp.**	30 (9.0%)	10 (7.2%)	-66.6%
***Enterococcus* spp.**	57 (17.2%)	24 (17.5%)	-57.8%
***Streptococcus* spp.**	7 (2.1%)	4 (2.9%)	-42.8%
C. difficile	9 (2.7%)	3 (2.2%)	-66.6%
E. coli	93 (28%)	27 (19.7%)	-70.9%
***Klebsiella* spp.**	19 (5.7%)	12 (8.7%)	-36.8%
P. mirabilis	15 (4.5%)	6 (4.3%)	-60.0%
P. aeruginosa	15 (4.5%)	10 (7.2%)	-33.3%
***Enterobacter* spp.**	8 (2.4%)	1 (0.7%)	-87.5%
***Citrobacter* spp.**	3 (0.9%)	0	-100%
A. baumannii	8 (2.4%)	5 (3.6%)	-37.5%
***Morganella* spp.**	3 (0.9%)	0	-100%
Other Enterobacteriaceae	1 (0.3%)	0	-100%
Candida spp.	26 (7.8%)	11 (8.0%)	-57.7%
Virus	5 (1.5%)	3 (2.1%)	-40.0%
Others	12 (3.6%)	5 (3.6%)	-58.3%
Total	**332 (100%)**	**137 (100%)**	**-**

During pre-PCHS, 301 HAIs included 13 co-infections; during PCHS phase, 135 HAIs included 6 co-infections.

Importantly, no infections sustained by PCHS-derived *Bacillus* spp. were detected in any of the hospitalized patients in the enrolled structures, further supporting the absence of infectious risks associated with PCHS-*Bacillus* use indicated by previous studies.

Finally, the relative role of PCHS in the reduction of HAI onset was explored by a multivariable model including all the parameters emerged as variables positively associated with HAI occurrence by univariate analysis.

The results, while confirming as statistically significant risk factors the presence of urinary or central venous catheters (respectively OR = 2.68; 95% CI, 2.10±3.41 and OR = 1.99; 95% CI 1.40±2.82), evidenced the PCHS use as a statistically significant independent protective effect (OR = 0.44; 95% CI, 0.35±0.54) ($p<0.0001$), practically halving the risk of contracting an HAI during hospitalization (Caselli et al., 2018).

8.6 Limitations of the study

Although showing a strong protective effect of PCHS, this study had some potential limitations.

a) The size of the sample and the magnitude of the resulting reduction seem to indicate a clear role of PCHS, however further studies could be performed based on larger samples and different healthcare settings in order to better understand the generalizability of the obtained results;
b) The number of patients surveyed in the external control hospital is small, and the no significant reduction observed might be due to this limitation;
c) A further potential bias might be the awareness of the sanitary staff about the study itself. However, healthcare professionals were only informed of an incidence study conducted in their hospital (for the whole 18-month period of the study). Furthermore, the information were exclusively limited to hospital managers, as data collectors and data extractors were external. The addition of an external control hospital to monitor the potential impact of being aware to be included in a study was also addressed to this point. At the same time it should be considered that the period of the study was very long, thus limiting the potential attention bias of the healthcare workers teams;
d) The seasonal variability, as well the lack of control settings from all the three regions may be another potential limitation, as the 6-month follow-up period may not be sufficient to avoid the effects of seasonality, and the external control was from only one of the three regions surveyed. Further studies might therefore include a longer follow-up period and more controls. However, the percentage of HAI reduction and the effect on microbiota modulation seem to indicate that these potential bias could have a limited effect;

e) Another potential bias may be represented by the lack of measurement for hand hygiene (use of hydro-alcoholic hand detergent); in fact, although each setting agreed not to introduce measures to improve infection control in the enrolled hospitals, this might be an uncontrolled confounding factor.

9. Evaluation of the effectiveness against hospital pathogens of a combined use of probiotics and bacteriophages: test in vitro and in situ

9.1 Phage susceptibility of tested bacteria

Since the effects of PCHS sanitation, although positive, were clearly non-specific and quite slow, we tried to improve the system by taking into consideration the potential decontaminating action of bacteriophages. In fact, they are viruses of bacterial cells, acting in a very rapid and specific way, able to kill with high efficiency their specific bacterial target. With the aim to evaluate their potential effectiveness as sanitizing agents against hospital pathogens, we focused our studies on *Staphylococcus aureus, Escherichia coli* and *Pseudomonas aeruginosa*, being those the most common pathogens detected as persistent contaminants on hospital surfaces, and frequently associated to HAI onset.

Namely, we used both ATCC strains and wild-type MDR hospital isolates belonging to the same bacterial species, for phage activity evaluation. In particular, tested species included *S. aureus* (ATCC 25923 and SA2-R73 clinical isolate), *E. coli* (ATCC 25922 and EC-R60 clinical isolate), and *P. aeruginosa* (ATCC BAA-47 and PA-V6 clinical isolate).

Each clinical isolate was collected from hospital surfaces, identified by Maldi-Tof (AccuPRO-ID; Charles River Lab Europe SaS, Ecully, France) as reported in **6.1.1** "Bacterial species, culture isolation and microbial identification" paragraph, and characterized for antimicrobial susceptibility as described in **6.1.2** "Antimicrobial resistances characterization of bacterial species" paragraph.

Table 23 summarizes drug susceptibility of bacterial targets used in the study, showing that all clinical isolates were MDR (MIC values are also reported in parentheses) (D'Accolti et al., 2018).

Table 23. Bacterial strains used in this study (D'Accolti et al., 2018)

Bacteria	Drug resistance											
	AMP	**ATM**	**CTX**	**FOX**	**COL**	**CN**	**IPM**	**MEM**	**OFX**	**OX**	**P**	**VA**
***S. aureus* ATCC 25923**	S	R	S	S	R	S	S	S	S	S	S	S
***E. coli* ATCC 25922**	I	S	S	S	S	S	S	S	S	R	R	R
***P. aeruginosa* ATCCBAA-47**	R	S	S	R	S	S	S	S	S	R	R	R
***S. aureus* SA2-R73**	R (128)	R (>256)	R (>256)	R (>256)	R (>256)	I (0.25)	R (>256)	R (48)	R (8)	R (>256)	R (>256)	S (3)
***E. coli* EC-R60**	R (>256)	S (0.47)	S (0.64)	R (96)	R (128)	S (2)	S (0.38)	S (0.64)	S (0.19)	R (>256)	R (>256)	R (>256)
***P. aeruginosa* PA-V6**	R (>256)	S (24)	R (48)	R (>256)	S (8)	R (12)	S (4)	S (0.75)	S (2)	R (>256)	R (>256)	R (>256)

Note: MICs (µg/mL) of hospital isolates are also reported in parentheses.
Abbreviations: AMP, ampicillin 10 µg; ATM, aztreonam 30 µg; CTX, cefotaxime 30 µg; FOX, cefoxitin 30 µg; COL, colistin 10 µg; CN, gentamicin 10 µg; IPM, imipenem 10 µg; MEM, meropenem 10 µg; OFX, ofloxacin 5 µg; OX, oxacillin 1 µg; P, penicillin 10 IU; VA, vancomycin 30 µg; MIC, minimal inhibitory concentration; ATCC, American Type Culture Collection; S, susceptible; I, intermediate; R, resistant.

Both ATCC strains and MDR isolates were analyzed for phage susceptibility by spot testing on soft agar as described in **6.1.4** "Host range analysis: double-layer plate assay" paragraph, and using individual phage stocks, obtained as described in **6.1.3** "Bacteriophage" paragraph. For each individual phage product, we tested 3-5 serial dilutions based on the type of phage preparation used (from 10^4 PFU to 10 PFU for anti-*S. aureus* phages and from 10^3 to 10 for anti-*E. coli* and anti-*P. aeruginosa* phages).

Results showed that both ATCC strains and MDR isolates were susceptible to phage lysis, although MDR clinical isolates were slightly more resistant to phage killing, especially at low phage concentration. (**Figure 34**) (D'Accolti et al., 2018). Based on this preliminary results, we performed decontamination assays, by testing phages at MOI 10,100, and 1000:1 (phage/bacteria ratio) against ATCC strains, and at 1000:1 MOI against MDR isolates.

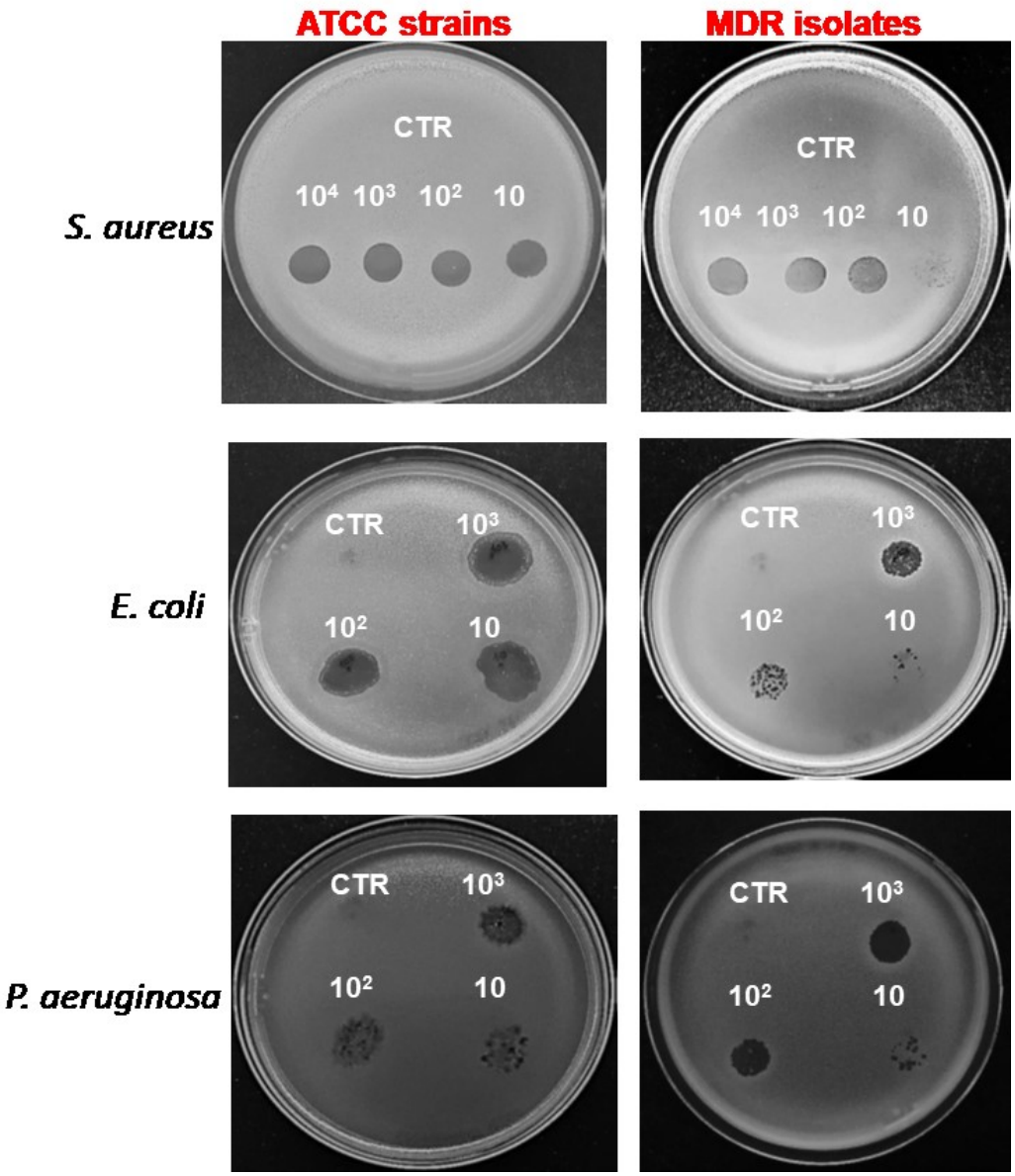


Figure 34. Phage activity against *S. aureus, E. coli, and P. aeruginosa* (ATCC or MDR strains). Bacteriophage activity was verified by spot tests. Briefly, after suspension in soft agar, bacterial cultures were overlaid on TSA plates; serially diluted phage stocks were added to bacterial lawns, checking their lytic activity after 24 hours of incubation at 37°C. Results are representative of triplicate samples (D'Accolti et al., 2018)

9.2 Phage decontaminating potential on hard surfaces

Decontamination tests were first performed on the ATCC bacterial strains. To mimic a contamination level similar to that detected in clinical settings, we took the bacterial load usually detected on hospital surfaces, as a reference (Vandini et al., 2014; Caselli et al., 2016b). To this aim, bacterial cells were spread on different types of hard non porous surfaces at a density of 10^2 CFU/24 cm^2, corresponding to $4x10^4$ CFU/m^2.

Surfaces included sterile plastic, glass, and ceramic, as described in **6.1.6** "Decontamination tests on hard surfaces" paragraph. After spreading, bacteria were left to dry at room temperature for 15 minutes, then phage preparations were diluted in PBS and 50µl were spread uniformly on the artificially contaminated area. The amount of phage solution was set up in order to obtain complete drying in 10 minutes at room temperature. After 1, 3, 6 and 24 hours of incubation, surfaces were sampled by Rodac contact plates containing selective medium for target bacteria and residual CFU where counted after 24h of incubation. **Figure 35** shows phage action on *S. aureus* contamination in ceramic tiles.

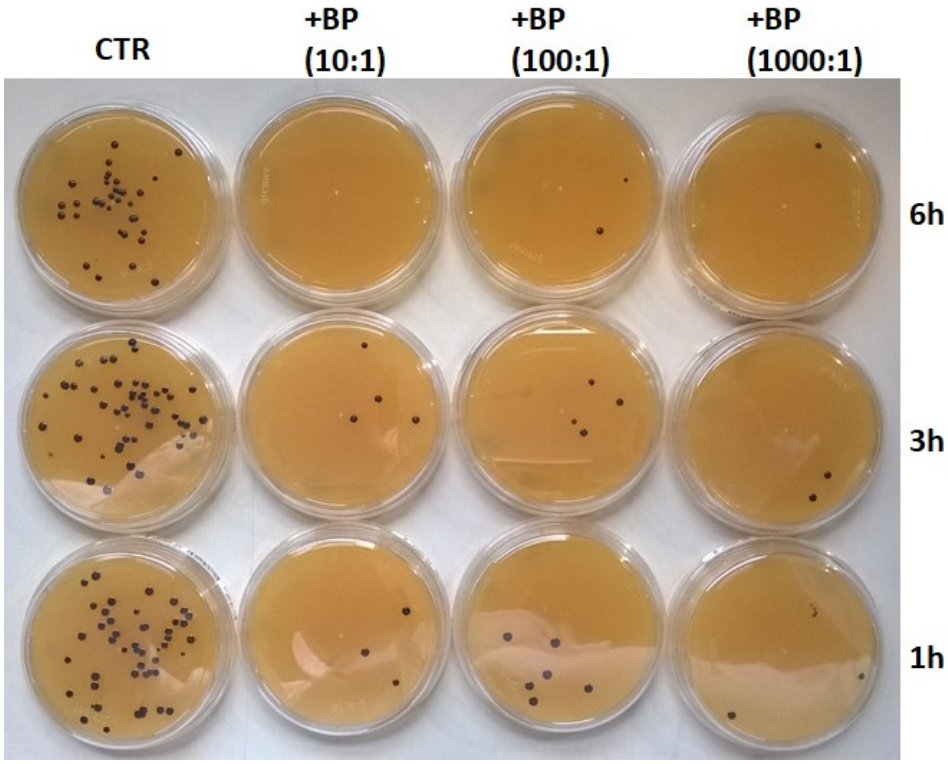


Figure 35. Decontamination tests on hard surfaces. *S. aureus* (10^2 CFU) was spread on ceramic tiles, and allowed to dry. Surfaces were then treated with anti-Staphylococcal phages suspended in PBS at the indicated MOI (10, 100, 1000:1, phage/bacteria ratio). After 1, 3, 6 h residual cells were collected by Baird Parker agar, and CFU enumerated. Results refer only to the following times: 1,3 and 6 h. CTR samples were treated with PBS alone. Pictures refer to anti-*S. aureus* phages.

Superimposable results were obtained with the phages directed against *E. coli* and *P. aeruginosa* (not shown).

Results evidenced the ability of phages to reduce up to 90±8% the bacterial colonies on treated surfaces, even when the bacterial density is relatively low.

In particular, a significant reduction was already detected at 1 hour post treatment at MOI 10:1 (-40±15%, $p<0.05$) compared to controls (D'Accolti et al., 2018).

Phage efficiency increased with increasing MOI, showing statistically significant differences between 10:1 and higher MOIs (100-1000:1) at all times tested ($p<0.01$), whereas no significant difference was observed between 100:1 and 1000:1 MOI. Phage activity increased with time, as almost no survivors were detected after 6 hours, when using MOI 1000:1, and the decrease was maintained in the subsequent 24 hours. By contrast, disinfectant-treated control (CTR+) surfaces showed an evident drop of bacterial cell number within the first hours, followed by new re-growth of bacteria after 24 hours, suggesting a bacteriostatic effect rather than a true bacterial killing (**Figure 36**) (D'Accolti et al., 2018).

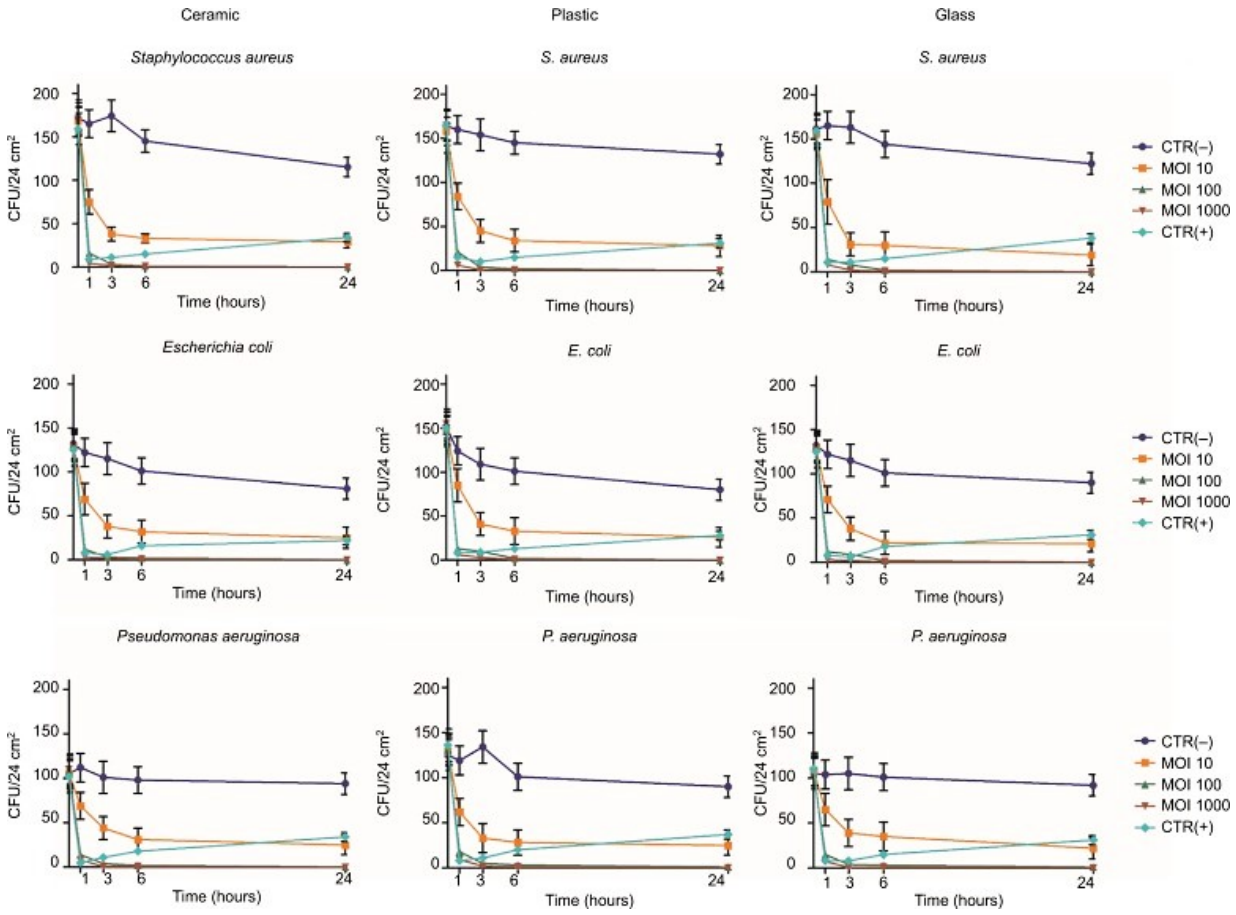


Figure 36. Reduction of bacterial load on different types of hard surfaces (ceramic, plastic, glass) treated with specific phages. Briefly, each ATCC strain (*S. aureus, E.coli, P. aeruginosa)* was spread on sterile surfaces, allowed to dry, and subsequently treated with the specific phages at the indicated MOI. After 1,3,6 and 24 h, residual viable cells were collected by Rodac sampling with specific selective media, and counted after 24 hours of incubation at 37°C. Negative CTR (-) samples were treated with PBS alone. Positive CTR (c+) samples were treated with denatured alcohol. Results represent the mean ± SD of triplicate samples in two independent assays per bacteria target (D'Accolti et al., 2018).

The results were superimposable, independently of the surface type and bacterial species used (*S. aureus, E. coli* and *P. aeruginosa*).

The same decontamination tests were performed on MDR *S. aureus* (SA2-R73), *E. coli* (EC-R60), *P. aeruginosa* (PA-V6) isolates, collected from hospital surfaces. To this aim, phage activity was assessed by using the only MOI 1000:1 (phage/bacteria ratio) on different types of surfaces (ceramic, plastic and glass). Also in this case, results showed a significant reduction of MDR bacteria on phage-treated surfaces, with no significant differences in the percentage of reduction observed in MDR strains compared to ATCC strains, and no differences among the different types of hard surfaces used (**Figure 37**) (D'Accolti et al., 2018).

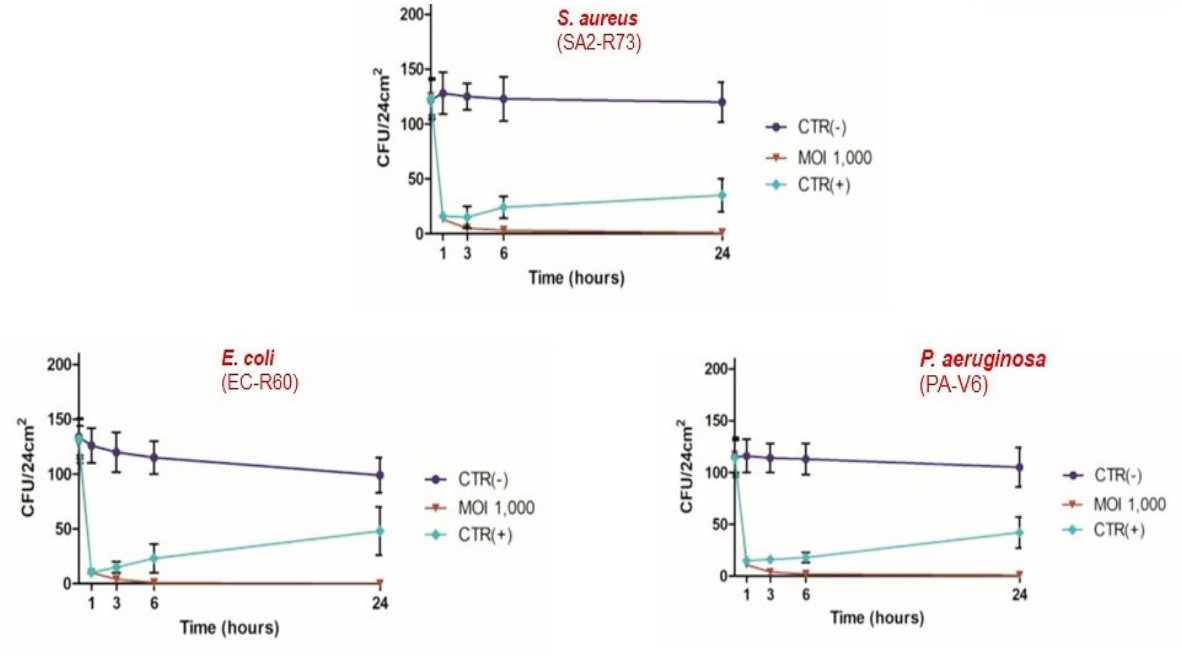


Figure 37. Reduction of MDR hospital isolates on hard surfaces treated with specific phages. Briefly, each bacterial isolate (wild-type *S. aureus, E. coli,* and *P. aeruginosa strains*) were spread on ceramic, plastic, or glass surfaces, allowed to dry, and subsequently treated with the specific phages at 1000:1 MOI. After 1, 3, 6, and 24 hours at room temperature, residual viable cells were measured by Rodac sampling with specific selective media, and subsequent CFU count after 24 hours of incubation at 37°C. Negative CTR (−) samples were treated with PBS alone. Positive CTR (+) samples were treated with denatured alcohol. Results represent the mean of triplicate samples in two independent experiments, for each surface type. As no significant differences were observed between surface types, graphed values represent the mean ± SD of all the measured samples (18 total samples) (D'Accolti et al., 2018).

9.3 Phage stability in probiotic detergent and decontaminating assays

Since one objective of this study was to determine the potential use of phages as decontaminants during routine sanitation procedures in hospital cleanings, the next step was to test phage stability in the eco-sustainable detergent PCHS.

To this aim, concentrated PCHS detergent (pH= 8.4) was prepared at work dilution by diluting it 1:100 in sterile water, as indicated by the manufacturer.

The diluted detergent was then used to suspend phages at 10^7 PFU/ml. Control phage solution was prepared in sterile PBS. Phage stability was measured after 1, 2, 3 and 7 days of incubation at room temperature, by PFU titration on the specific bacterial targets, after removing the *Bacilli* component by centrifugation.

The results, shown in **Figure 38**, evidenced that phages retained their full activity when suspended in PCHS detergent at work dilution (D'Accolti et al., 2018).

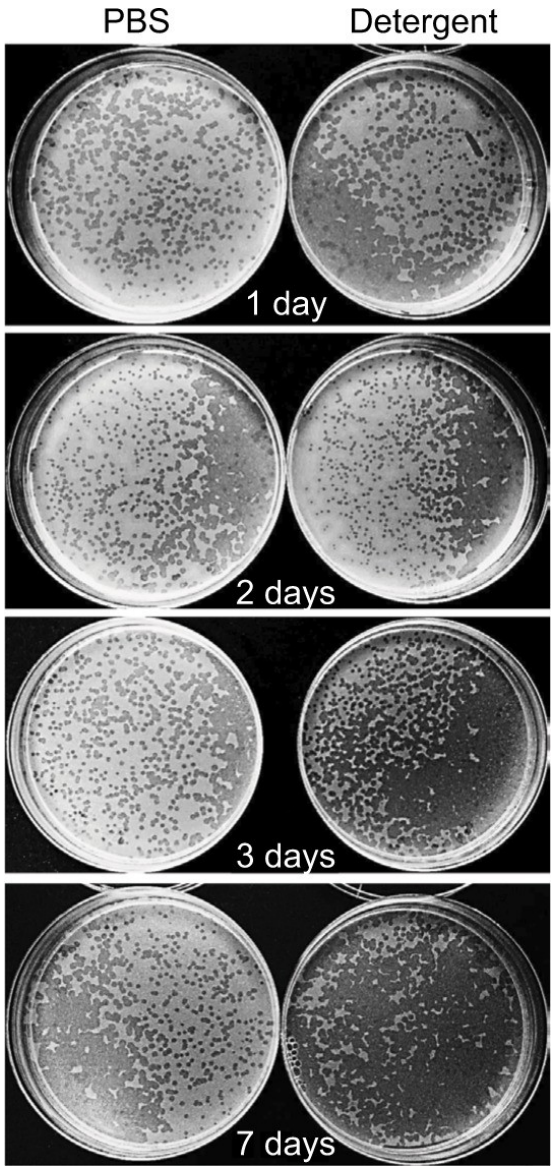


Figure 38. Phage stability in PCHS detergent. Phage stocks were suspended in PBS or in PCHS detergent diluted 1:100 in water as indicated by the manufacturer, and kept at room temperature in closed plastic tubes for 1, 2, 3, or 7 days. After the indicated times, aliquots were collected and titrated by PFU counting on the corresponding ATCC bacterial target. Samples were performed in duplicate. Pictures refer to anti-*E. coli* phages. Superimposable results were obtained with the phages directed against *S. aureus* and *P. aeruginosa* (D'Accolti et al., 2018).

Indeed, both the number and diameter of lysis plaques obtained with phages in PCHS were even larger compared to what observed when phages were suspended in PBS, although differences were not statistically significant (**Table 24**) (D'Accolti et al., 2018).

Table 24. PFU titration of phage preparation in detergent and PBS (D'Accolti et al., 2018)

Bacteriophage titer	*Bacteriophage titer*		
	PBS	***PCHS***	***p***
Staphylococcus aureus	7.7±1.9	8.5±1.3	ns
Escherichia coli	7.6±2.0	8.4±1.8	ns
Pseudomonas aeruginosa	7.8±1.8	8.6±2.0	ns

Results are expressed as mean PFU ± SD x 10^6/ml in triplicate experiments.

The decontamination potential of phages in PCHS (without bacterial component) was then tested *in vitro* by the same described decontamination assays, performed by spreading 100 CFU/24 cm^2 of target bacteria and applying phages at 100 and 1000:1 MOI.

Results showed a significant reduction of target bacteria on treated surfaces, evidencing a full antimicrobial activity even after 7 days in PCHS detergent, with no significant differences between 100 and 1000:1 MOI (**Figure 39**).

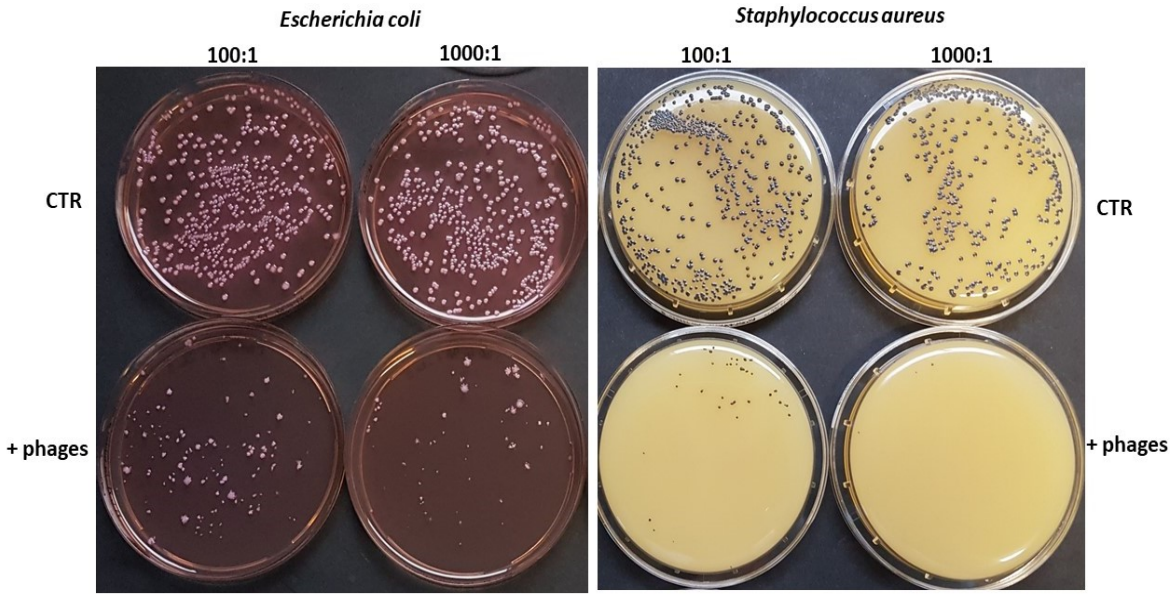


Figure 39. Phage stocks were suspended in filtered PCHS detergent diluted 1:100 in water as indicated by the manufacturer, and kept at room temperature in closed plastic tubes until 7 days. Then decontamination tests were performed by spreading bacterial target on ceramic tiles, treating them with phages at 100 and 1000:1 MOI, followed by sampling with Rodac plates with selective medium. CTR, treatment with only PBS. Pictures refer to anti-*E. coli* and anti-*S. aureus* phages. Superimposable results were obtained with the phages directed against *P. aeruginosa* (not shown).

9.4 Phage/probiotic decontaminating effectiveness *in situ*

Based on results showing phage stability in PCHS detergent, next step was to assess the effectiveness of a combined phage/probiotic detergent in reducing bacterial load on surfaces. To this purpose, we performed decontamination assay *in situ,* by artificially contaminating with *S. aureus* a ceramic sink of a bathroom, as described in **6.2.1** "Probiotic/phage treatment" paragraph.

Briefly, a logarithmic-phase culture of *S. aureus* was uniformly spread on sink surface, allowed to dry for 24 hours and then treated by spraying PCHS detergent with or without addition of 10^5 PFU/ml of anti-staphylococcal phage (MOI 1000:1).

To mimic routine surface cleaning, detergent solution sprayed on the tested surfaces was kept low enough to dry completely in <10 minutes. After treatment, surface contamination was assayed by Rodac plates containing Baird Parker agar after 1 hour and 1, 3 and 15 days. Both *S aureus* and PCHS-*Bacillus* can grow on such selective medium, but their colonies are clearly distinguishable as *S. aureus* form black round colonies surrounded by a clear zone, whereas *Bacillus* form irregular gray-brown colonies.

Results showed that the phage treatment alone reduced in a significant way the staphylococcal CFU at early times post treatment (90± 5 % at 1 and 24 hours after treatment, $p<0.01$), confirming results obtained by *in vitro* assays. However, the reduction was not maintained at later times (15 days), likely due to the loss of intact and therefore infectious phages on surfaces. By contrast, contrarily to what observed with phages alone, probiotics alone started to be active after 3 days, with a maximum reduction of 75 ± 6% at 15 days after application, as expected from previous studies and from their peculiar mechanism of action (competitive exclusion). Notably, when probiotics and phages were used in a combined way, results showed a rapid reduction of *S. aureus* target bacteria (94±4% within 1 hour), followed by the maintenance of CFU reduction even after 15 days, representing the assay time end (99±1%) (**Figure 40**) (D'Accolti et al., 2018).

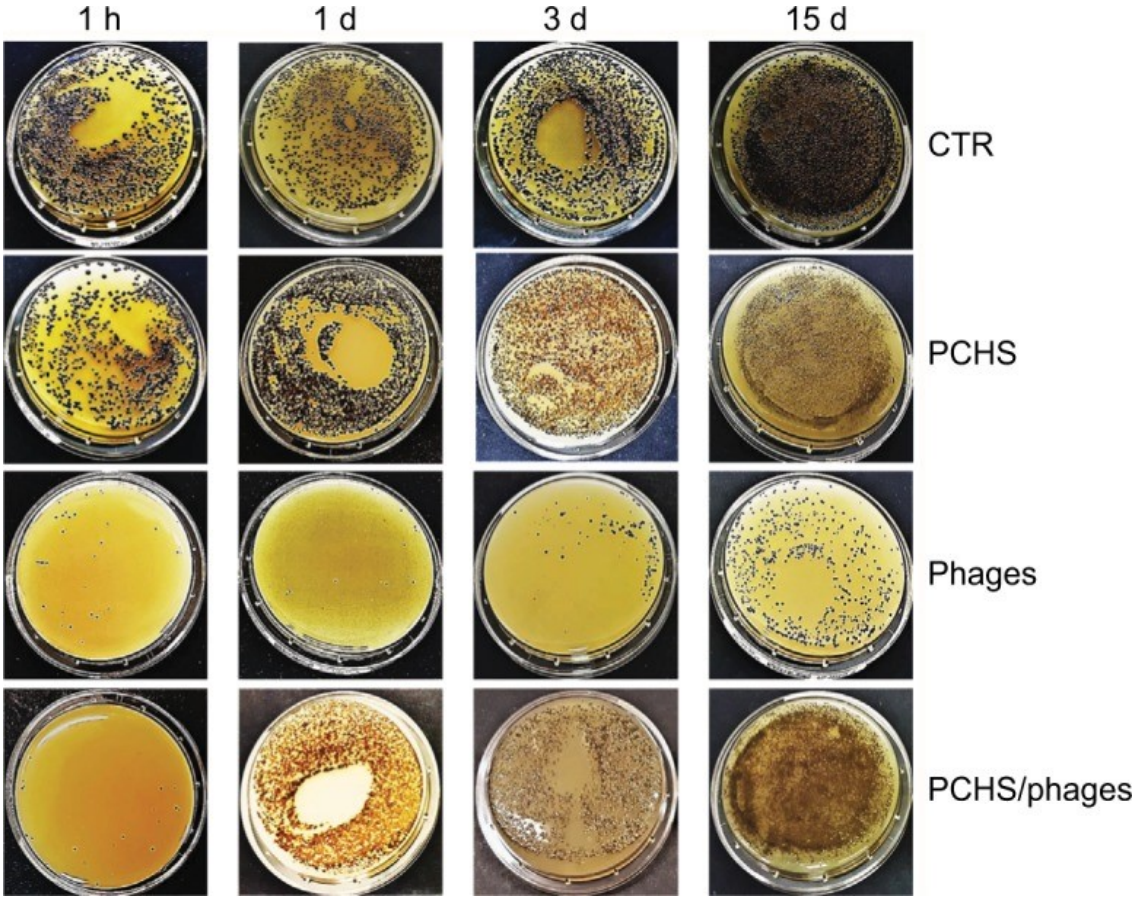


Figure 40. Reduction of *S. aureus* contamination in situ, by a combined phage–probiotic detergent. *S. aureus* were uniformly spread on the surface of a ceramic sink, and treated after 24 hours with water (CTR), probiotic detergent alone (PCHS), anti-staphylococcal phages in PBS alone (Phages), or combined probiotic and phages (PCHS/phages). Phages were used at 1000:1 MOI. After 1 hour, and 1, 3, and 15 days, residual *S. aureus* viable cells were on surfaces were evaluated by CFU count on Baird–Parker Rodac plates. PCHS-*Bacilli* gave rise to gray-brown irregular colonies on Baird–Parker medium, easily distinguishable from the *S. aureus* ones. Results are representative of duplicate samples in three independent experiments (D'Accolti et al., 2018).

Results are schematically summarized in **Figure 41** (D'Accolti et al., 2018).

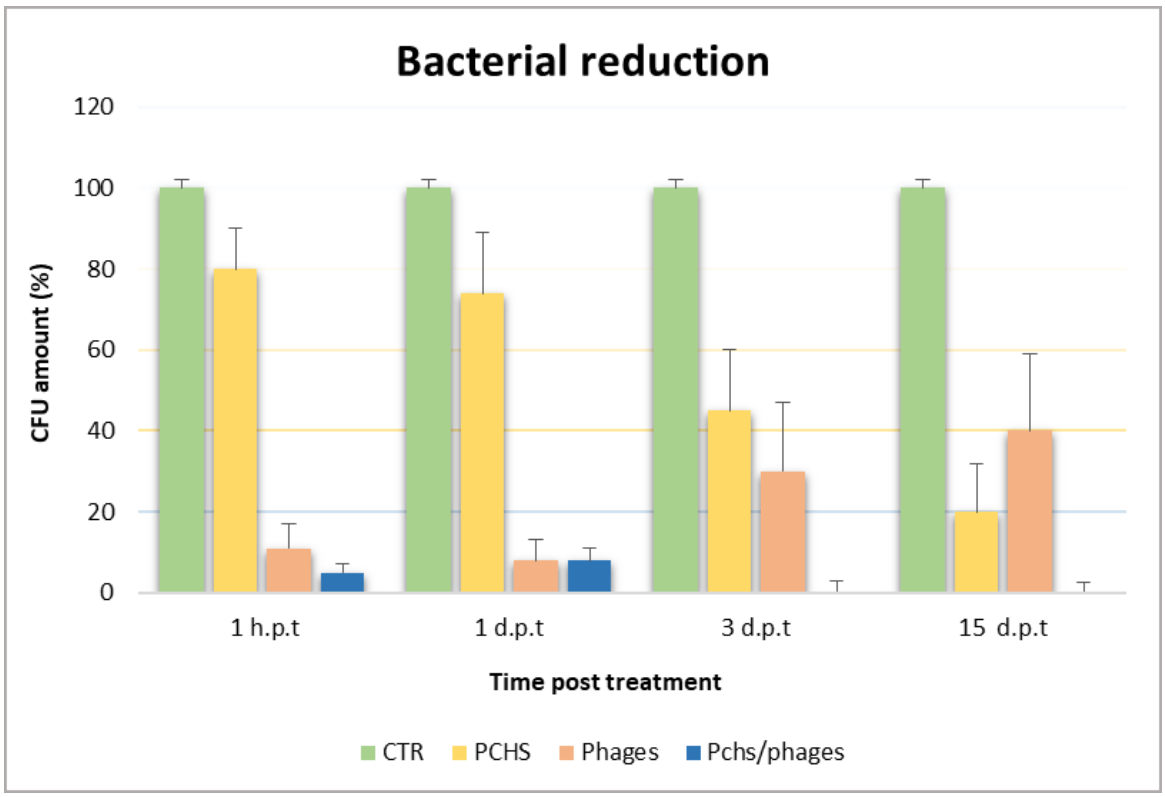


Figure 41. Average reduction of *S. aureus* contamination *in situ*, by a combined phage–probiotic detergent. Results are expressed as mean value ± SD of duplicate samples from three independent experiments. CTR, water; PCHS, probiotic detergent alone; phages, anti-staphylococcal phages in PBS alone; PCHS + phages, probiotic detergent including anti-staphylococcal phages. Modified from D'Accolti et al., 2018.

10. Evaluation of Staphylococcal decontamination on hospital surfaces by a bacteriophage-probiotic sanitation strategy: a monocentric study

Based on results obtained *in vitro* and *in situ*, a monocentric study was performed, aimed to analyze the potential effectiveness and feasibility of a sanitation procedure based on the addition of bacteriophages to the probiotic-based detergent (PCHS system).

The study was performed in a private hospital located in Ferrara (Italy), already using PCHS sanitation, after approval by the local Ethics Committee. As a proof of concept, we focused the analysis on the bathrooms of the Internal Medicine ward and on the Staphylococcal contamination, being the bathrooms the most contaminated areas and the Staphylococci the most prevalent contaminants in such areas, as detected in previous studies (as described in **7.1** "Study design and sanitation procedure" paragraph). Briefly, 8 rooms were enrolled, 4 of which received a combined PCHS-phage sanitation, whereas 4 received only PCHS.

10.1 Characterization of the initial Staphylococcal contamination

Prior to starting the bathroom sanitation, Staphylococcal contamination was quantified and characterized in all enrolled rooms and bathrooms by performing 3 samplings on alternate days, as described in **7.3** "Environmental sampling, culture isolation and species identification" paragraph.

Results obtained by microbiological tests evidenced a mean level of contamination corresponding to 3.7 x 10^4 CFU/m^2 in the bathroom area (range 0–84 210 CFU m^2) and 1.6 x 10^4 CFU/m^2 (range 0–41 684 CFU m^2) in the room area, respectively, confirming the higher contamination level in the bathrooms (D'Accolti et al., 2019b).

Staphylococcus population was then characterized by culture isolation, and individual *Staphylococcus* colonies were identified by Maldi-Tof (AccuPRO-ID; Charles River Lab Europe SaS, Ecully, France). Identification results showed that the majority of isolates were coagulase-negative Staphylococci, whereas only < 10% was represented by *S. aureus*. In detail, 4 coagulase-negative species were the most prevalent: *S. epidermidis, S. haemolyticus, S. cohnii* and *S. simulans.*

Since the phage preparation (obtained from Eliava Institute, Tbilisi, Georgia) was originally directed against *S. aureus* only, all 4 detected species were tested for the susceptibility to "Staphylococcal Bacteriophage" lysis, as reported in **7.2** "Bacteriophages used for treatment" paragraph.

Susceptibility tests were performed by both spot tests and double-layer assays, as described in **7.4** "Host range analysis: double-layer plate assays and spot test" paragraph.

Results indicated that the "Staphylococcal bacteriophage" was able to lyse all the *Staphylococcus* species detected on hospital surfaces, in addition to *S. aureus* (**9.1** "Phage susceptibility of tested bacteria" paragraph), although *S. simulans* was less susceptible, suggesting a broad tropism of "Staphylococcal bacteriophage" against *Staphylococcus* genus and allowing their use for decontamination of bathrooms (**Figure 42**) (D'Accolti et al., 2019b).

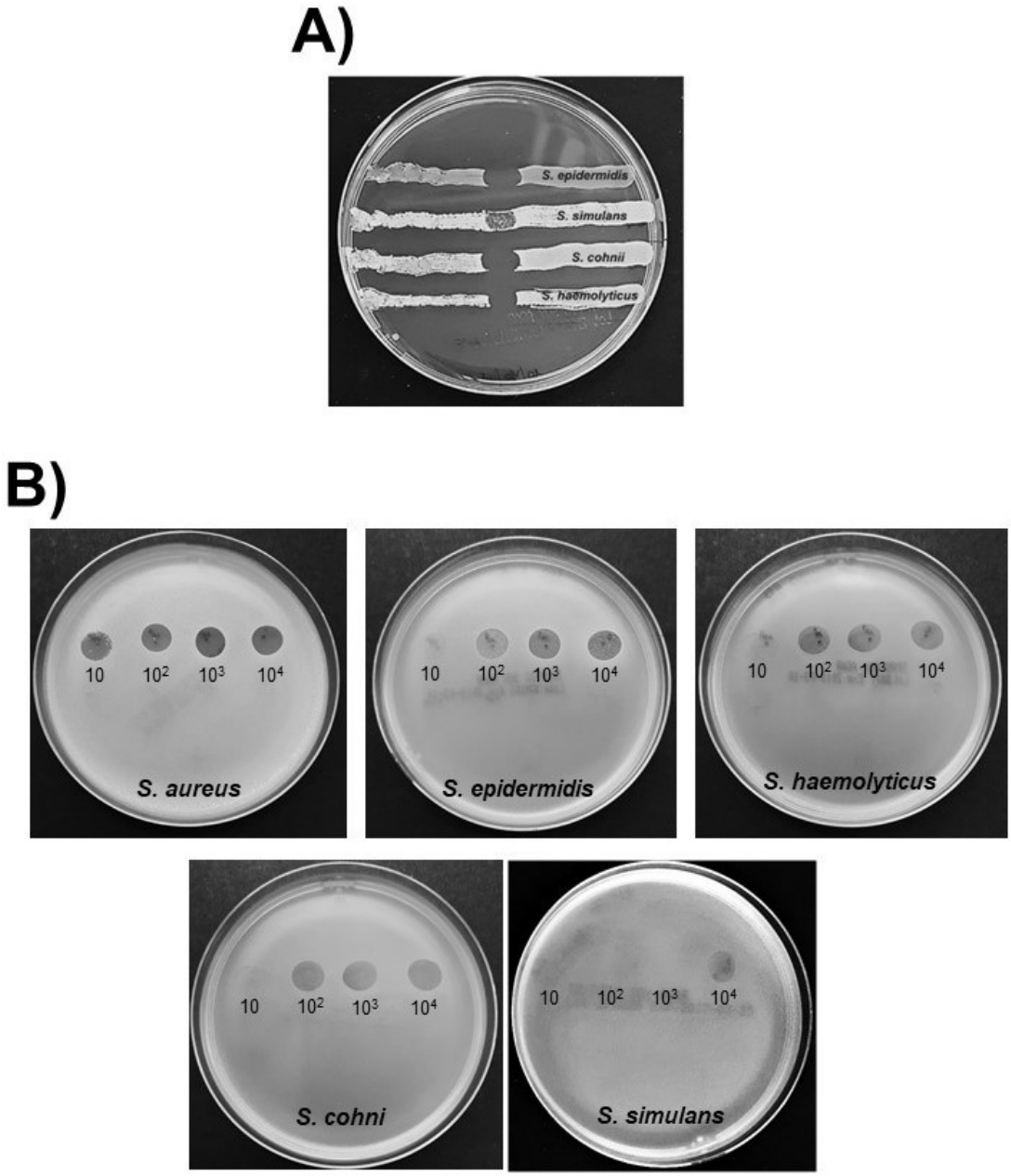


Figure 42. Phage susceptibility test of *Staphylococcus* spp. isolated from hospital surfaces. **A)** Each coagulase-negative Staphylococcus isolate (in order of abundance *S. epidermidis, S. haemolyticus, S. cohni, S. simulans*) was analyzed for susceptibility to 'Staphylococcal bacteriophage' preparation by spot test. **B**) Both *S. aureus* and *non-aureus* isolates were tested by soft agar plate assay for their susceptibility to different concentrations of 'Staphylococcal bacteriophage' preparation, using 10–10^4 PFU per spot (D'Accolti et al., 2019b).

10.2 Phage application and impact on Staphylococcal contamination

Based on the contamination level measured on hospital (3.7×10^4 CFU/m^2 in the bathroom area and 1.6×10^4 CFU/m^2 in the room area), and considering the optimal phage: target ratio on hard surfaces, a multiplicity of infection (MOI) 1000:1 was chosen for surfaces treatment, corresponding to $>4x10^7$ PFU/m^2 and about $2x10^8$ PFU per bathroom (considering a bathroom floor surface of 4.5 m^2). As reported in **7.1** "Study design and sanitation procedure" paragraph, phages were applied by nebulization after suspension in filtered PCHS detergent for the minimum time to assure sufficient contact between phages and target bacteria in aqueous solution (10 minutes, as determined in the *in situ* study). Residual *Staphylococcus* load was assessed by Rodac plates containing selective Baird-Parker medium at alternate days throughout the study period.

Results showed a rapid decrease in *Staphylococcus* spp. on surfaces of bathrooms treated by phage-PCHS sanitation (Intervention group). The reduction was already detectable at day 1 (-87%) and was maintained or further increased throughout the following sampling times, until day 7 (-97%). When the phage treatment was discontinued (days 8-11), the *Staphylococcus* spp. load on surfaces gradually increased, although the CFU level remained lower compared to the original load detected at T0. Following phage reintroduction, a new and more pronounced reduction of *Staphylococcus* spp. was observed on surfaces (days 14, 16 and 18), suggesting that the decrease was actually attributable to phage action. Measured differences were highly statistically significant at all times tested ($p<0.001$), except for the T11 sampling time (**Figure 43**) (D'Accolti et al., 2019b).

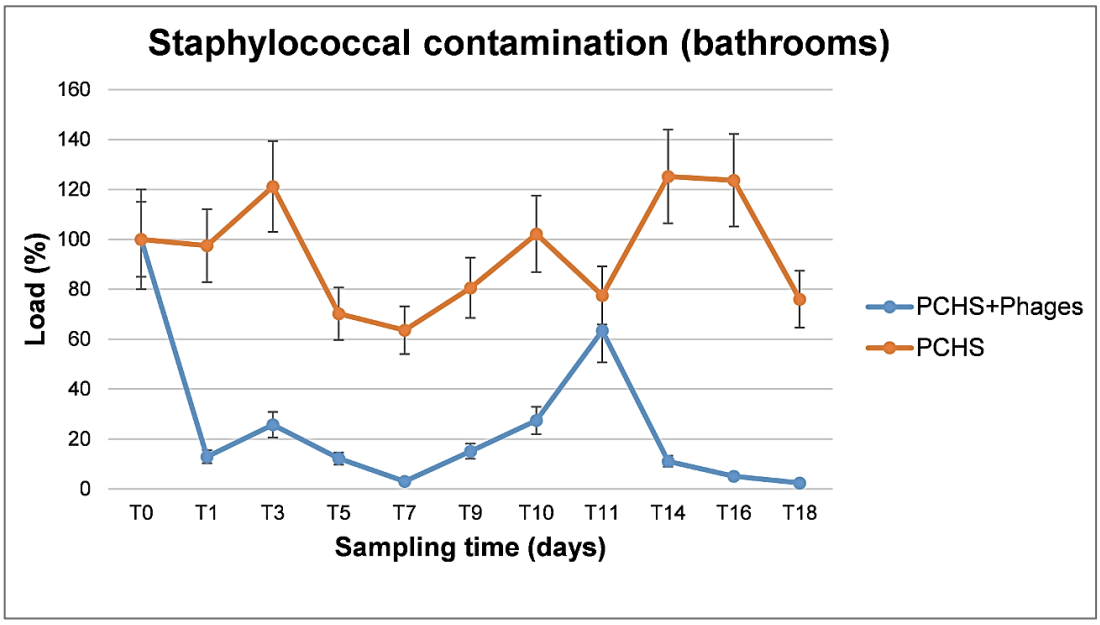


Figure 43. Staphylococcal load in bathrooms' areas. Results are expressed as mean percentage ±SD detected in the Intervention (blue) and Control (orange) groups, referred to the initial load, measured at T0 and representing 100% value (D'Accolti et al., 2019b).

Notably, some Staphylococcal CFU reduction was also observed in the rooms of the Intervention group, (**Figure 44)** although the differences between the Intervention and Control groups were not statistically significant (D'Accolti et al., 2019b).

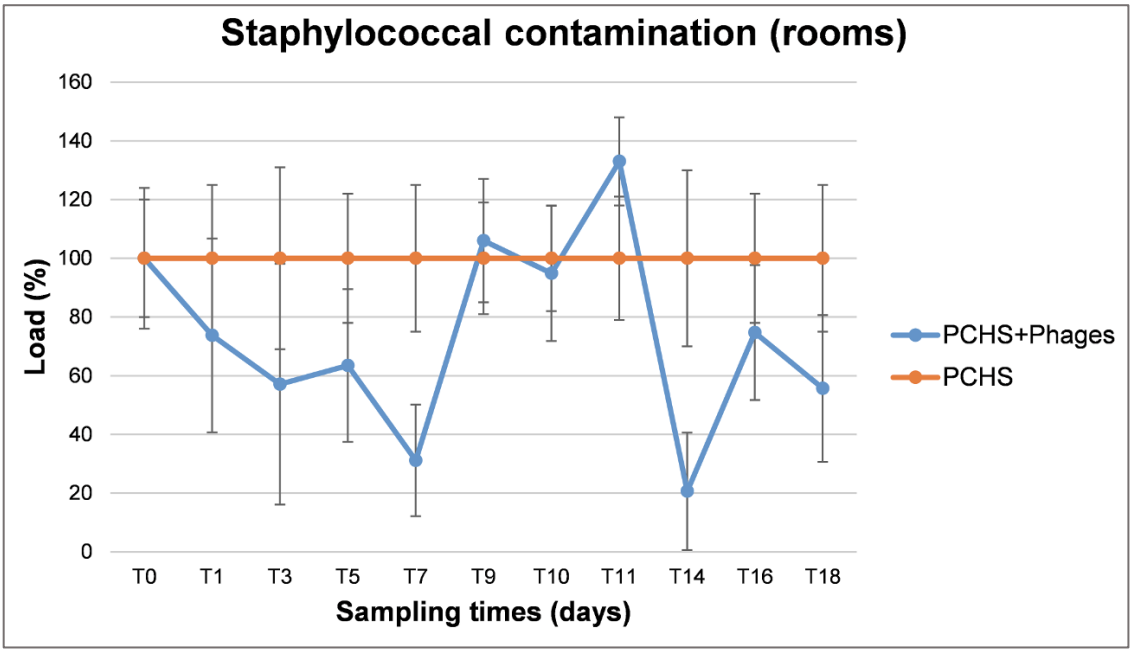


Figure 44. Staphylococcal load in rooms' areas. Results are expressed as mean percentage ±SD detected in the Intervention (blue) and Control (orange) groups, where each control sample times represents 100% value (D'Accolti et al., 2019b).

Notably, the decrease of *Staphylococcus* CFUs on bathroom surfaces was paralleled by the increase of anti-*Staphylococcus* phages on surfaces, as measured by a specifically set up qPCR (*Sb-1* qPCR, described in **7.5.1** "qPCRs: *Sb-1*, *PanB* and *spo0A* qPCRs" paragraph). As shown in **Figure 45**, the phage load was in fact significantly higher at all times tested on surfaces of the bathrooms of the Intervention group compared to the Control group ($p<0.001$), including days 9,10 and 11, when the phage treatment was discontinued, suggesting some persistence of residual phages on surfaces (D'Accolti et al., 2019b)
Interestingly, we detected some phage load increase also in the room area of the Intervention group compared to Control group ($p<0.05$), suggesting that phages were somehow passively transported from bathrooms to adjacent areas.

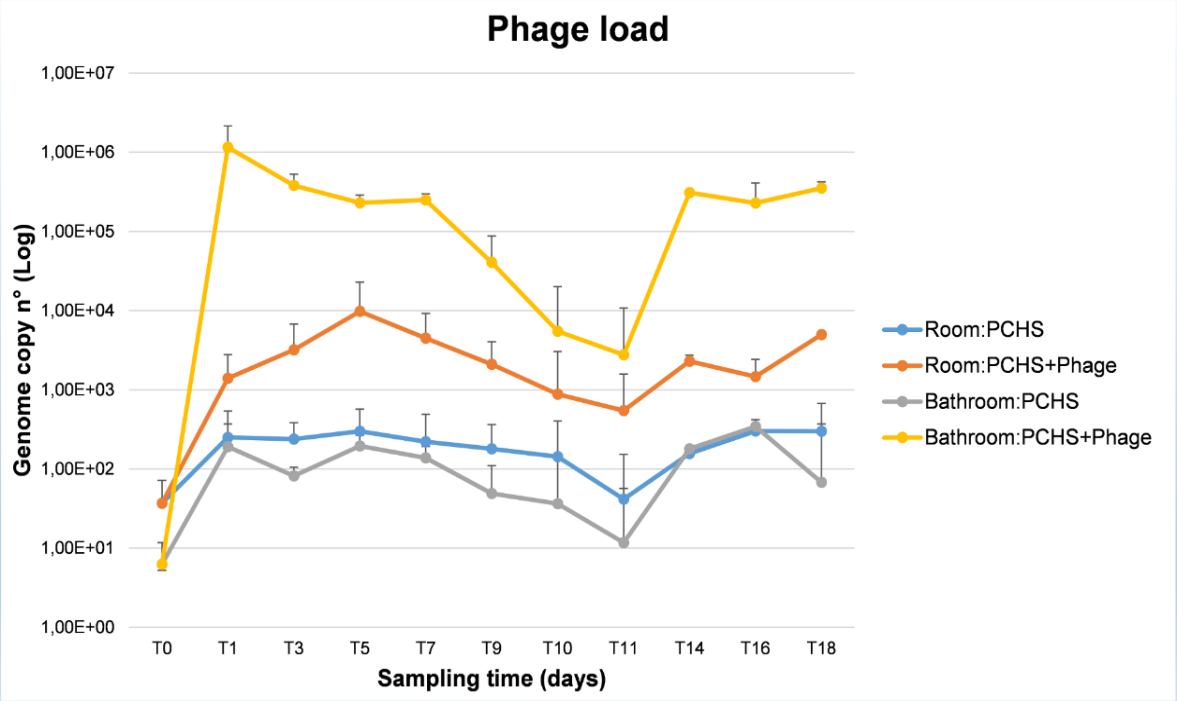


Figure 45. Anti-*Staphylococcus* phage load in enrolled bathrooms and rooms. Bacteriophage amount on treated surfaces was measured by a specific *Sb-1* qPCR performed on collected samples. Results are expressed as mean genome copy number ± SD in rooms and bathrooms of the Intervention (PCHS + Phage) and Control (PCHS) groups (D'Accolti et al., 2019b).

To ascertain whether the decrease in *Staphylococcus* spp. was specific, the microbial population collected from surfaces was analyzed by a customized qPCR microarray able to detect and quantify simultaneously the following microbes: *Staphylococcus aureus, Staphylococcus epidermidis, Enterococcus faecalis, Enterococcus faecium, Escherichia coli, Klebsiella pneumoniae/ Enterobacter, Acinetobacter baumannii, Proteus mirabilis, Pseudomonas aeruginosa, Clostridium perfrigens, Clostridium difficile, Aspergillus*

fumigatus and Candida albicans, as described in **7.5.1.1** "Customized qPCR microarray for microbial characterization" paragraph.

The results, summarized in **Table 25**, confirmed the presence at T0 of high amounts of *Staphylococcus* spp. ($4.1 \times 10^4 \pm 2 \times 10^3$ genomes/m^2) and showed the presence of detectable amounts of *E. faecalis* ($10^2 \pm 7$ genomes/m^2), *E. faecium* ($10^2 \pm 5$ genomes/m^2), *K. pneumonia/Enterobacter* ($1.5 \times 10^3 \pm 8 \times 10^2$ genomes/ m^2) and unidentified mycetes ($2 \times 10^2 \pm 1 \times 10^2$ genomes m^2) (D'Accolti et al., 2019b).

At T18, analysis results showed a decrease only in *Staphylococcus* spp. load, with no variations in all the other analyzed microbes, confirming that the phage action was specifically directed towards *Staphylococcus* spp.

Table 25. Microbial load on bathroom surfaces of the Intervention group (D'Accolti et al., 2019b).

Microbes	T0 (PCHS) (genome copy number)	T18 (PCHS+ phages) (genome copy number)
S. aureus	$9 \times 10^2 \pm 2 \times 10^2$	65 ± 54
S. epidermidis	$4.1 \times 10^4 \pm 2 \times 10^3$	$1.2 \times 10^3 \pm 9 \times 10^2$
E. faecalis	$10^2 \pm 7$	$1.2 \times 10^2 \pm 9$
E. faecium	$10^2 \pm 5$	$0.9 \times 10^2 \pm 11$
E. coli	ND	ND
K. pneumonia/Enterobacter	$1.5 \times 10^3 \pm 8 \times 10^2$	$1.8 \times 10^3 \pm 9 \times 10^2$
A. baumannii	ND	ND
P. mirabilis	ND	ND
P. aeruginosa	ND	ND
C. perfrigens	ND	ND
C. difficile	ND	ND
A. fumigatus	ND	ND
C. albicans	ND	ND
Total bacteria (panB)	$7.2 \times 10^4 \pm 8 \times 10^3$	$6.7 \times 104 \pm 5 \times 103$
Total mycetes (panM)	$6.5 \times 10^2 \pm 3 \times 10^2$	$6.0 \times 102 \pm 4 \times 102$

Results are expressed as mean genome copy number ± SD per m2, measured on bathroom surfaces of the Intervention group at T= 0 and T=18 days. ND, not detected.

Finally, to assess whether the *Staphylococcus* decrease was somehow attributabe to an increased load of PCHS-*Bacillus*, also the PCHS-*Bacillus* load was evaluated on treated surfaces. The analyses were performed by both conventional CFU count on Rodac plates as described in the **7.3** "Environmental sampling, culture isolation and species identification" paragraph, and by using a specific qPCR amplifying *spo0A Bacillus* gene, as described in **7.5.1** "qPCRs: *Sb-1*, *PanB* and *spo0A* qPCRs" paragraph. Both the results obtained by direct CFU counts and specific qPCR showed that PCHS-*Bacillus* amounts were superimposable on surfaces of the bathrooms of the Intervention and Control groups, as well as in room areas, at all times tested during the study (**Figure 46**) (D'Accolti et al., 2019b). These data confirmed that the reduction in *Staphylococcus* spp. on surfaces was associated with bacteriophage application rather than an increase in probiotic Bacilli.

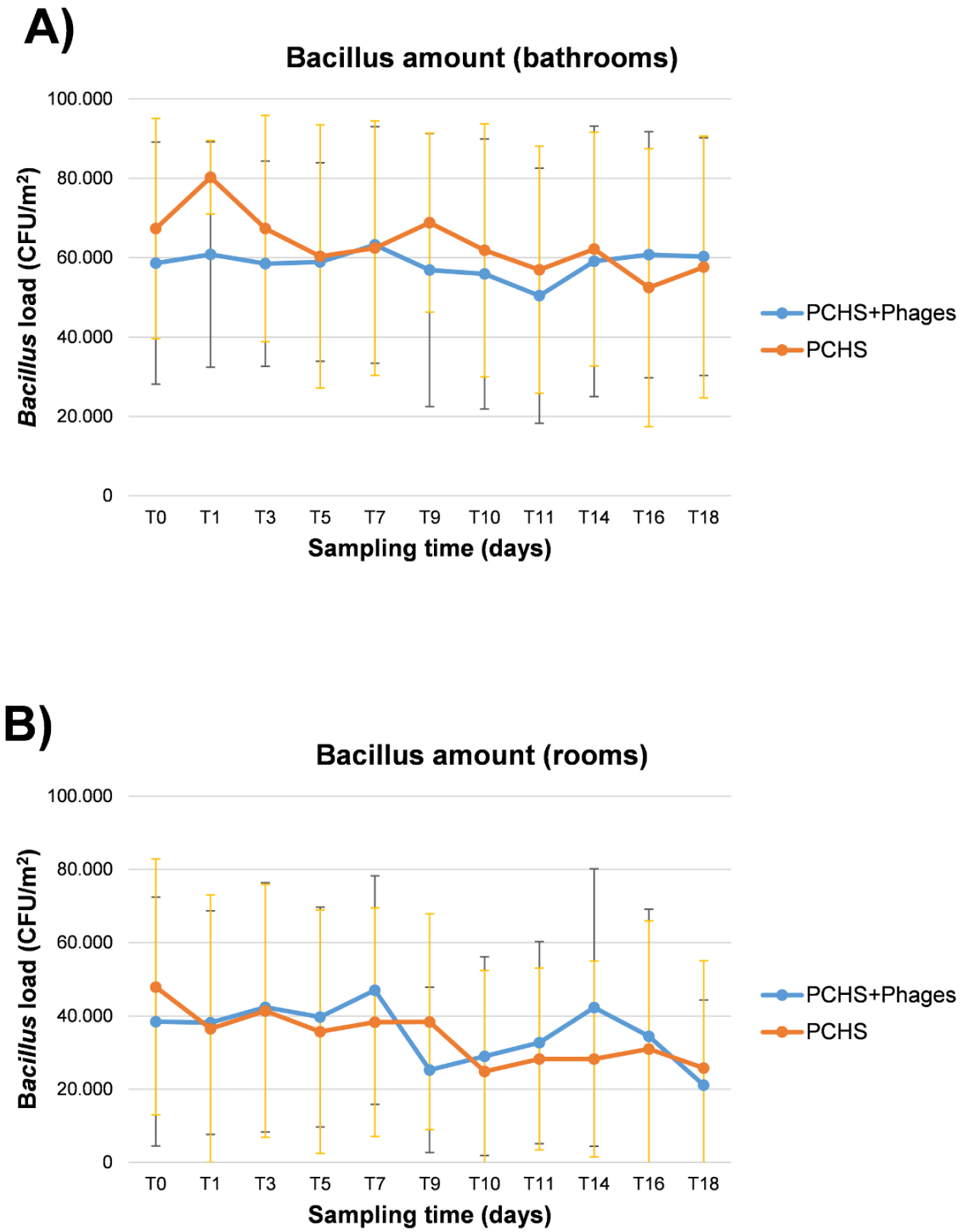


Figure 46. *Bacillus* load in enrolled rooms. The amount of PCHS derived Bacilli on sanitized surfaces was assessed by CFU counts on Rodac plates. **Panel A**. *Bacillus* load in bathrooms. **Panel B**. *Bacillus* load in rooms. Results are expressed as mean CFU counts ± SD per m^2, detected in the Intervention (PCHS + Phages) and Control (PCHS) groups (D'Accolti et al., 2019b).

DISCUSSION

Contamination of hospital surfaces by clinically relevant pathogens represents a major concern in healthcare settings, due to its impact on transmission of healthcare-associated infections (HAIs) and to the continuous growing of drug resistance of HAI-associated pathogens. The frequent recontamination processes, associated with the presence of colonized or infected patients, as well as visiting people and hospital staff, renders the elimination of surface contamination a very difficult task to address.

The so far used chemical-based sanitation shows important limits that should be considered: in addition to a high environmental impact, it has been proved effective for short-term abatement of pathogens, but it cannot prevent recontamination phenomena that usually occurs as fast as 30 minutes after chemical applications (Vandini et al., 2014). More importantly, chemical-based sanitation can contribute to the selection of resistant strains, and resistance may be directed against disinfectants themselves but even against antibiotics, as recently reported for chlorhexidine disinfectant induction of resistance against colistin antibiotic, a last resort drug for treatment of difficult-to-treat infections caused by MDR Gram-negative bacteria (Wand et al., 2017).

Interestingly, the environment health has recently re-thought as the human body health, considering that, rather than trying to eradicate all microbes, it may be more effective replacing bad, potentially pathogenic microorganisms with good ones, in order to restore a positive microbial balance and prevent pathogen colonization (Al-Ghalith and Knights, 2015).

In the search for eco-friendly and efficient sanitizing systems, we studied the application of a probiotic-based sanitation system, named Probiotic Cleaning Hygiene System (PCHS), which consists of using eco-sustainable detergents containing spores of probiotic bacteria belonging to *Bacillus* genus. Namely, three species have been studied (*Bacillus subtilis, Bacillus pumilus* and *Bacillus megaterium*) that are not pathogenic, ubiquitous and with a long history of safe use in human fields.

Following preliminary tests *in vitro*, showing the high antimicrobial activity of PCHS-*Bacillus* against pathogenic bacteria, experiments on field showed that PCHS was able to decrease in a stable way pathogen contamination up to 90% more than chemical sanitation, remodulating surface microbial population and without selecting resistant strains (Vandini et al., 2014; Caselli et al., 2016b).

Based on these first results, the aim of this PhD-project was to determine whether PCHS application could directly impact on HAI incidence, whose decrease generally represents the

final goal of any sanitation procedure in hospital. To this aim, a multicentre, pre-post interventional study was performed for 18 months in the Italian Medicine wards of six Italian public hospital, located in different regions.

The intervention consisted uniquely in the substitution of conventional sanitation with PCHS, maintaining unaltered any other procedure that could influence HAI control.

Results showed that PCHS was associated, in the absence of any other Infection Control and Prevention (ICP) interventions, with a significant reduction ($p<0.0001$) of HAI incidence (-52%). In addition, multivariable analysis, besides confirming the role of commonly HAI-associated risk factors, such as the presence of catheters (OR=2.68 and OR=1.99 for urinary and CVC, respectively), evidenced the use of PCHS as an independent protective factor (OR= 0.44; 95% CI, 0.35-0.54) ($p<000.1$).

The bioburden data confirmed in a very large sample (over 32,000 environmental samples were analyzed) the ability to remodulate hospital surface microbiota, reducing pathogen contamination of about -83% compared to chemical sanitation. These data confirmed the ability of *Bacillus* to displace and replace pre-existing pathogens, preventing their recolonization and spreading. Even more importantly, no selection of drug-resistant strains was associated with PCHS use, but rather resistome analysis showed that PCHS use was associated with a significant decrease (up to 1000 folds) of all the R genes harbored by the surface microbiota, independently of the resistance types originally present in each treated setting ($p_c < 0.01$).

The decrease was particurarly evident for those genes which were highly represented in the pre-PCHS phase, in all the five enrolled hospital, including *mecA* (coding for methicillin resistance), *aad1* (aminoglycoside resistance, often present in *S. aureus*), *OXA-51 group* (β-lactamases, oxacillinases), *ermA-B-C* genes (coding for resistance against macrolides, lincosamide, and streptograminB), *mefA* and *msrA* (both coding for macrolide resistance). These molecular findings were also confirmed at the phenotype level for all the *S. aureus* isolates detected on surfaces of treated hospitals. In fact, antibiogram results showed that PCHS introduction was accompanied by a 61.9%–93.5% reduction of resistant isolates, depending on the antibiotic type, with a global 73.7% decrease of the number of MDR strains.

Interestingly, even less represented resistances were affected by PCHS introduction, such as colistin resistance. Since colistin resistance provided by *mcr-1* plasmid was not included in the qPCR microarray analysis used for the resistome evaluation, we verified its presence by nested PCR performed on detected *Enterobacteriaceae*, showing its presence in 9.2% of all

strains. PCHS implementation was associated with a 77.1% decrease of *mcr-1* positive *Enterobacteriaceae*, and molecular results were confirmed at the phenotypic level by microdilution tests, showing a parallel reduction of MDR strains (77.1%).

In addition, as to the safety concern of using a probiotic-based sanitation, both microbiological and molecular monitoring performed in the enrolled setting confirmed the safety of use of PCHS observed in previous trials, confirming the genetic stability of the PCHS-*Bacillus* despite the continuous contact with pathogenic bacteria on surfaces, and the absence of any infectious risk correlated to the use of PCHS probiotics in hospital setting.

Overall, collected results indicate that an environmental intervention based on microbiota remodulation can have a significant effect, limiting in a significant way the spread of resistant pathogens and reducing the onset of HAIs. This suggests that an "ecological" approach might be successfully included among the current available tools for infection prevention and control (IPC).

Additionally, results show the usefulness of molecular techniques in investigating environmental bioburden and its resistance characteristics.

Conventional culture-based microbiology, in fact, shows unavoidable limitations, which are linked to a need for cultural isolation, culture time, biochemical identification, etc.

Furthermore, some microorganisms are particurarly difficult to grow, need specific culture media and particular growth conditions (specific temperature and time of incubation, or anaerobiosis conditions), or grow with different efficiency, thus rendering very time-consuming and complex the analysis of a whole microbial population. Last, culture-based techniques only detect searched and cultivable microbes.

By contrast, an efficient monitoring system in hospitals should be able to provide rapid and precise information on the microbial population examined, leading to a detailed characterization of the environmental bioburden in real time. Toward this aim, molecular methods, which are based on the DNA technologies, can consistently help in overcoming such limitations, providing simultaneous analysis of high numbers of different parameters, and thus helping to precisely define the microbial populations from both a qualitative and quantitative point of view. In fact, one of the main advantages of molecular assays consist in their high rapidity, sensitivity and specificity, thanks to which even a very low number of targets can be efficiently detected in few hours.

This aspect is particularly useful when it is needed to evidence very rapidly even minimal variations in the composition of a complex microbial population, allowing itself to be extremely powerful to monitor surface bioburden, as well as clinical isolates. Both

qualitative and quantitative real time PCR, as well as qPCR microarrays, have been proven to be effective tools toward this aim. In particular, we applied PCR and qPCR assays to identify and quantify *Bacillus* strains, to amplify and quantify the total bacterial or mycetes load, and to detect and quantify specific pathogens or resistance genes, allowing a detailed monitoring of the antibiotic-resistance profile (resistome) of the entire population in a timely way, compared to conventional antibiograms performed on individual isolates. We strongly believe that these data might be important for the development of future guidelines for hospital cleaning directed to prevention strategies to reduce infectious diseases onset, likely applicable not only in healthcare settings.

However, coming back to the effectiveness of the probiotic-based sanitation, being mainly based on competitive antagonism, the system is not able to counteract specifically certain pathogens, which instead would be a desirable feature when it is needed the elimination of a specific pathogen, for example in controlling specific outbreaks. Furthermore, the system is quite slow, as several weeks are needed to achieve the maximum inhibition of pathogens growth on treated surfaces. For these reasons, PCHS can not be considered as a rapid mean for the elimination of specifically targets, but rather as a preventive and stabilizer system.

By contrast, this type of action may be highly desirable in particular situations than can occur in hospitals, for example when a room is occupied by an infected and/or colonized patient. In fact, it is known that the risk to acquire a specific nosocomial pathogen increases when a patient is admitted in a room previously occupied by an infected or colonized patients by that specific infectious agent, due to the high persistence of pathogens in the environment (Huang et al., 2006).

For these reasons, we investigated the possibility to improve the probiotic-based system by the use of lytic bacteriophages, which are viruses that infect only prokaryotic cells, characterized by specificity and rapidity of action against specific bacteria, thus potentially usable in a targeted way.

Thanks to these features, bacteriophages have been for example already proved effective against foodborne pathogen, and are successfully applied for food and food processing surfaces treatment (Lang, 2006).

However, most of the studies reported in the literature are not applicable for routine hospital sanitation, since prolonged contact between phages and target bacteria in aqueous solution is generally needed (Abuladze et al., 2008), that is not compatible with daily sanitation protocols and inpatients presence in the room.

Furthermore, high bacterial densities, favouring the encounter between phages and target bacteria, were used (Jensen et al., 2015), but this is not consistent with the bacterial densities that are normally found on hard-surfaces in hospital (3-5 logs lower). This implies that, to be predictive, *in vitro* tests should be performed using bacterial amounts similar to those detectable on hospital surfaces.

Based on these observations, the aims of the second part of the present PhD project was to evaluate the feasibility of a combined phage/probiotic sanitation in routine sanitation protocols.

To this purpose, *in vitro* and *in situ* tests were performed to assess the effectiveness of phage application in decontamination of hard surfaces contaminated at low density with common HAI-associated pathogens, including MDR hospital isolates.

Namely, *S. aureus*, *E. coli* and *P. aeruginosa* were used as target bacteria testing different hard surfaces (ceramic, plastic and glass), as these surfaces typologies are often present in hospital rooms and environment.

Target bacteria were seeded at 100 CFU/24cm^2, corresponding to 4x10^4 CFU/m^2 in order to mimic bacterial densities comparable to those found o hospital surfaces, based on our previous studies (Vandini et al., 2014; Caselli et al., 2016b).

In addition, we kept the contact time between phages and bacterial target as low as possible, using phage volumes drying in a maximum time of 10 minutes, in order to imitate what is done during routine sanitation procedures.

The results showed that phages are able to decrease the amount of HAI-associated pathogens in all the types of hard surfaces tested, without any significant difference between bacteria strains and surface types, indicating that phages are active in removing pathogen levels similar to those detected on field on hospital surfaces, even when they are MDR.

Notably, phages maintained their full activity when suspended in PCHS detergent at work dilution. Results showed an even greater activity of phages in PCHS, compared to that observed when phages were suspended in PBS. These data may suggest that the chemicals contained in the PCHS detergent, beside being not aggressive toward phage structure, might somehow stabilize phages at room temperature, or favour the contact between phages and bacterial target, or facilitate the entrance/action of phages in the bacterial cell.

Consistently, *in situ* assays showed that the combined probiotic and bacteriophages application resulted in a stronger decontaminating activity, compared to individual probiotic and phage components. In fact, phage activity was detectable within 1 hour, but it was gradually lost after 3 days, whereas probiotics alone started to be active after 3-7 days.

By contrast, the combined phage-probiotic treatment induce a rapid and stable decrease of target bacteria until 15 days.

Based on these data, the combined system was finally assessed on field, to verify the feasibility and the effectiveness of a routine phage application added to PCHS sanitation. To this purpose a monocentric study was conducted in a hospital routinely sanitized since 2014 by PCHS. As a proof of concept, we tested the combined system in the bathrooms of the Internal Medicine ward and against Staphylococcal contamination, being bathrooms the most contaminated areas of the hospital environment, and Staphylococci the most prevalent type of microbial contamination.

Prior to application on field, the phage preparation against *Staphylococcus* spp. (a concentrated "Staphylococcal bacteriophage" preparation specially produced for us by Eliava Institute, Georgia) was tested against all the *Staphylococcus* species detected in the environment of the enrolled hospital. Briefly, *Staphylococcus* species identification showed that most of contaminant isolated from surfaces were coagulase-negative *Staphylococci*, with *S. aureus* representing less than 10% of the total *Staphylococci*. The 4 prevalent species were *S. epidermidis*, *S. haemolyticus*, *S. cohnii* and *S. simulans*, and all of them were efficiently lysed by the "Staphylococcal bacteriophage" preparation, showing that such phages had a broad host tropism. Following susceptibility tests, the application on field was performed, enrolling 8 rooms which were randomly subdivided in two groups: the Intervention group (receiving sanitation of bathrooms by combined phage-PCHS application) and the Control group (receiving only PCHS sanitation).

Phages were applied through nebulization by using an atomizer, after suspension in filtered PCHS detergent, for the minimum time to guarantee the persistence of solution on surfaces (10 min, sufficient for allowing the contact between phages and target bacteria in aqueous solution as determined in previous studies (D'Accolti et al., 2018)).

This also allowed also minimization of discomfort for patients, limiting the time during which the bathroom was not available, and thus allowing a daily treatment.

Following phage introduction, an evident decrease of *Staphylococcus* contamination on treated surfaces was observed. In particular, up to 97% less Staphylococci were detected compared to what was obtained by using PCHS sanitation alone. Notably, phage action was immediately detectable after 24 hours and was gradually lost when discontinuing the treatment. As expected, the action was specifically directed towards target bacteria (*Staphylococcus* spp.), since no variations were observed for other contaminating species

(Gram-negative bacteria or mycetes) and it was actually due to phage application and not to the eventual increase of PCHS-*Bacillus*.

Interestingly, some effect was also observed in the rooms having their bathrooms treated with phages (rooms of the Intervention group), suggesting that phages were somehow also passively transported to contiguous areas, likely by persons walking and/or touching phage-treated surfaces. Alternatively, the diminished Staphylococcal contamination of the bathroom might have had a positive impact on the level of room contamination as well.

Interestingly, the potential use of phages as decontaminating agents in the hospital environment was recently investigated in Intensive Care Units (ICUs) against Acinetobacter spp. (Ho et al., 2016), in addition to chemical-based conventional disinfection performed at the patient discharge. Briefly, in that context, a single treatment with anti- *Acinetobacter* phages reduced the occurrence of HAIs caused by such bacteria, suggesting that phages might be used effectively to reduce specific pathogens in hospital rooms, although the aerosol phage application used in the study was only compatible with sporadic use, when the room was empty (terminal cleaning).

Instead, the results collected in the present projects suggest that a biological sanitation including phage usage might be performed not only for sporadic treatment of empty rooms after patient discharge, but also for routine sanitation of specific areas in the hospital wards, thus helping to prevent the persistence of high loads of common surface pathogens, and consequently diminishing the risk of contracting infections associated with those pathogens.

As PCHS alone was proven to be associated with the reduction in HAIs (Caselli et al., 2018; Caselli et al., 2019) the use of a more effective sanitation strategy consisting in combined phage and probiotic usage might further reduce the infection rate, allowing additional significant improvements in patients' outcome and cost savings in terms of HAI management. Our results, therefore, opens the way to future research aimed at evaluation of the impact of a combined probiotic phage strategy on specific nosocomial infections.

Although not observed in our experimental studies, a potential limitation of phage application might consist in the onset of phage resistance in treated bacteria.

For *S. aureus*, previous *in vitro* reports showed the appearance of phage resistance with a very low frequency (1.3×10^{-8}) (Capparelli et al., 2007), suggesting that it may be a quite infrequent event. In addition, generally phage resistance was observed in phage therapy models (Oechslin, 2018), where the density of actively proliferating bacteria is very high. By contrast, bacterial density is much lower on hospital surfaces, and bacterial increase is

mainly due to recontamination phenomena (facilitated by continuous spread of pathogens by inpatients and staff), rather than to proliferation of contaminating microbial species, thus rendering even more unlikely the onset of phage resistance. However, future studies should be addressed to analyze any potential risk to develop resistance to phages in the pathogens contaminating treated surfaces.

Overall, being that phages are completely safe for humans, so much so that they are used for therapeutic purposes, our results suggest that phage-based systems might be considered as a part of prevention and control strategies, to prevent HAIs and to counteract specific outbreaks and AMR spread. This, together with the stabilizing potential of probiotics, might open new ways to modulate microbiome, not only in the environment but also as potential preventing and therapeutical treatments in vegetables, animals, and humans.

FUTURE PERSPECTIVES

The obtained results open the way to new interesting studies aimed to improve human health and render safer the environment.

Future researches will include:

i) to understand the mechanism/s of action of probiotics and bacteriophages in decreasing and remodulating the microbiota contaminating hospital surfaces, by exploring in detail the synergism that occurs between them,

ii) to isolate and study the individual essential components of such biological action to eventually produce new more powerful sanitizing formulations,

iii) to verify the action of phages and/or probiotics on the dry biofilms that often contaminate hospital surfaces, by evaluating their potential ability to prevent and/or remove them, overcoming limitations related to the low effectiveness of routine cleaning and disinfection practices which are currently used in hospitals,

iv) to enlarge the research to other fields potentially treatable with these systems (such as livestocks, agriculture, etc), to meet the needing of a “One Health” approach to the human health, aimed to achieving optimal health outcome recognizing the real interconnection between animals, people, plants and their shared environment.

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