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The burden of healthcare-associated infections and antibiotic-resistance: investigation of novel strategies to reduce bacterial load in healthcare settings

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Clarifications

- Most of the data presented in this study have already been reviewed and published in a scientific journal, in particular in one work. The corresponding reference is reported below:
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ABSTRACT

Introduction

Hospital-acquired infections (HAIs) represent an important clinical and economic burden, with pathogens such as *Escherichia coli*, *Acinetobacter baumannii*, and *Clostridium difficile* contributing to high rates of morbidity and mortality. Traditional cleaning methods often result as insufficient, especially in high-risk hospital environments: there is, in fact, an increasing need to identify eco-sustainable detergents as alternatives to conventional chemical disinfectants, particularly in healthcare settings. At the same time, advances in nanotechnology have enabled the development of nanoparticle-based antimicrobials with strong bactericidal properties. The aim of this thesis project is to investigate innovative strategies to enhance surface decontamination, to reduce infection risk. Firstly, we evaluated the effectiveness of the SMEG BPW1260 bedpan washer-disinfector, a device designed for the thermal disinfection of human waste containers. Afterwards, we aimed at assessing the efficacy of a new methyl-ester sulphonate ecological detergent in combination with silver-derivatized magnetic nanoparticles against bacteria commonly found in hospital infections. Finally, we focused on the infection risk associated with items commonly used to transport patients inside the hospitals, such as wheelchairs: to this aim, we tested the SAFE-HUG Wheelchair Cover, a disposable non-woven barrier designed to minimize patient exposure to potentially contaminated wheelchairs.

Materials and methods

To assess residual contamination of human waste containers after the washing process with the SMEG BPW1260 bedpan washer-disinfector, molecular techniques such as spectrophotometry, cell counting and DNA quantification were employed. Similarly, the efficacy of the wheelchair cover in reducing bacteria transmission was evaluated through the same methods. The methyl-ester sulphonate detergent and the magnetic nanoparticles were then tested on 30 *Escherichia coli* and 30 *Acinetobacter baumannii* strains, which exhibited various resistance profiles, assessing their effects through minimum inhibitory concentration (MIC_{50} and MIC_{90}) and minimum bactericidal concentration (MBC_{50} and MBC_{90}) tests. Two types of nanoparticles were tested: silver-derivatized magnetic nanoparticles (MNPs) and silver-derivatized biomimetic magnetic nanoparticles (BMNPs).

Results

The results of colony-forming units (CFUs) count before and after the thermal disinfection treatment demonstrated a reduction of *Clostridium difficile* and *Escherichia coli* contamination by >99.9% (>3 log reduction). The significative decrease in bacterial load was further confirmed through molecular techniques: in particular, *Clostridium difficile* showed a reduction of roughly 89% in both cell count (cells/ml) and optical density (OD); in the case of *Escherichia coli*, a reduction of approximately 82% in OD was observed, with an even more marked decrease in cell count, reaching almost 99.3%. DNA quantification resulted below detectable levels for both microorganisms. Additionally, we lowered power consumption by 45% when compared to standard protocols by optimizing the disinfection cycle's energy efficiency, maintaining unchanged the efficacy in reducing bacterial contamination.

The methyl-ester sulphonate detergent showed its strongest effect in lowering detectable contamination when used in combination with nanoparticles, exhibiting both inhibitory and bactericidal activity; in particular, the two types of silver-derivatized magnetic nanoparticles showed comparable antimicrobial efficacy.

Finally, the significative barrier effect of the wheelchair cover was demonstrated by optical density and bacterial DNA quantification, which resulted undetectable after both one and 24 hours.

Conclusion

Overall, the approaches presented in this project - thermal disinfection, eco-friendly detergent enriched with nanoparticles, and protective transport barriers – are proposed as effective solutions to the urgent need for efficacious and sustainable infection control in healthcare facilities. These findings demonstrate the potential of these systems to counteract microbial contamination while minimizing environmental impact, offering promising solutions for the future of infection prevention in healthcare settings.

INTRODUCTION

1. Healthcare-associated infections

Hospital-acquired infections (HAIs), also referred to as healthcare-associated infections (HCAIs), are defined by the Centre for Disease Control and Prevention (CDC) as "localized or systemic conditions resulting from an adverse reaction to the presence of an infectious agent(s) or its toxin(s). There must be no evidence that the infection was present or incubating at the time of admission to the acute care setting."

Firstly called "nosocomial infections", referring mostly to infections linked with admission to an acute-care hospital, HCAIs currently comprise a wider group of infections, that can develop in various healthcare facilities such as long-term care, family medicine clinics, home and ambulatory care [1]. These infections are not present or incubating in the moment of hospital admission, but appear at least 48 h after admission or within 30 days after having received health care [2], [3]. Representing a relevant clinical and economic burden for healthcare systems worldwide, HCAIs are among the most common types of adverse events affecting hospitalized patients, together with adverse drug events and surgical complications [4], [5], [6], [7], [8].

HCAIs lead to increased healthcare costs, extended hospital stays and increased morbidity and mortality, as they affect millions of patients every year: sepsis and sepsis-related deaths, primarily caused by diarrheal diseases, register 9.2 to 15 million cases annually, while lower respiratory infections were reported in 2017 to account for 1.8 to 2.8 million cases annually [2].

A study conducted in 2016 on HCAIs burden in Europe shows that 2,609,911 new patients were identified as having HCAIs annually in the European Union and European Economic Area, and that at least one in 20 infections acquired by these hospitalized patients was preventable; this study highlighted, in addition, the broad spectrum of resistance of *Klebsiella pneumoniae* and the *Acinetobacter* species, indicating the problem of the lack of new antimicrobials [9].

According to the CDC, approximately 1 in 31 hospitalized patients develop HCAIs [10], and 9-20% of these infections are present in the ICUs [11]. Although HCAIs represent a considerable issue worldwide, their distribution is not uniform globally, as the highest incidence rates are generally found in low- and middle-income countries due to disparities in infection control procedures, healthcare infrastructure, and resource availability [12]. In fact, according to WHO, roughly 7% of hospitalized patients in developed countries and at least 10% of patients in in developing countries contract one or more HCAIs while they are in hospital [13]. Furthermore, it is estimated that the burden of HCAIs in Africa is double than in developed regions [14], considering also that 22.1% of

HCAIs in sub-Saharan Africa, for instance, regards neonatal populations [15]. A 2024 review reports that the overall prevalence of HCAIs in this part of the African continent is 12.9% [13], while a prevalence rate of 3.2% was reported in USA [11], 6.5% in Europe [16] and 9% in South-East Asia [17]. These disparities are attributable to several factors, such as weak infection prevention and control programs, overcrowding in hospitals and deficiencies in both hand hygiene and infrastructure [18], [19], [20], other than poverty [21], a high rate of maternal and child malnutrition favouring infections [13], poor access to clean water and sanitation [22]. Notably, the sub-Saharan African region accounts for more than 70% of global HIV infections [23], and the immunocompromising state caused by this virus probably exacerbates the risk of acquiring other infections [24]. These marked differences among different continents and countries in the world is reflected also on the economic resources invested to face HCAIs worldwide: according to a 2019 WHO report, while in low-income countries the expenditure in healthcare in 2017 corresponded on average to $41 per person, in high-income countries it was more than 70-fold higher, namely $2937 [25].

In addition, it was estimated that in Europe HCAIs cause 16 million extension days of hospital stay and 37,000 attributable deaths, and also that at least 2.6 million cases of HAIs occur annually in long-term care facilities, and 4.1 million in acute-care hospitals [26]. In the USA around 99,000 deaths were attributed to HCAIs in 2002, and associated costs were approximately $6.5 billion in 2004 [27]. In 2019, 8,874 (7.4%) patients staying in an ICU for more than two days reported at least one ICU-acquired HCAI [13]. In 2020, the CDC estimated that 5% of all of USA hospital admissions result in a HCAI, leading to 722,000 infections and 75,000 deaths per year, as well as $28-33 billion in excess costs [11].

2. Types of healthcare-associated infections

Although HCAIs are considered the most common adverse event endangering patients' safety worldwide [28], [29], [30], it is stated by the World health organization (WHO) that the public attention is often focused on HCAIs only in case of epidemics [1]. In particular, intensive care unit (ICU) patients are markedly susceptible to HCAIs, as they are often very critically ill and immunocompromised, and this has led to estimate around 0.5 million of HCAIs diagnosis every year just in ICUs. Impaired immune response is though a characteristic that can facilitate the spread of HCAIs, involving patients susceptible to common infections. Infections usually occur due to cross-contamination between patients and health workers [1], and this is increased by the sharing of spaces and medical equipment, particularly in the context of multidrug-resistant organisms and pandemics [31]. The most frequent cases of HCAIs are represented by infections at surgery sites (SSIs), as well as the ones linked to implants and prostheses: the latter include central line-associated bloodstream infections (CLABSIs), catheter-associated urinary tract infections (CAUTIs), and ventilator-associated pneumonia (VAP) [32], [1].

A study carried out in 183 hospitals in United States found that out of 11,282 patients, 452 acquired at least one HCAIs, corresponding to 4%; among these, 21.8% were pneumonia, 21.8% SSIs, and 17.1% gastrointestinal infections. Device-associated infections (CLABSI, CAUTI and VAP) represented 25.6% of these HCAIs, being also the traditional focus of programs aimed at preventing HCAIs [33].

2.1 Central line-associated bloodstream infections (CLABSIs)

The majority of CLABSI cases usually occur in inpatient wards and in outpatient haemodialysis centres [34]. This kind of infections is associated with consistent health care costs, as well as high mortality and morbidity, despite a wide variation in reported infection rates, namely from 20% to 62.5%, in emerging economies [35]. It was reported in a study that CLABSIs were caused by both Gram-negative (39.2%) and Gram-positive (33.2%), but also *Candida* spp. microorganisms (27.6%); the occurrence was revealed to be 3.93 per 1000 central-catheter days, and eight days were required to develop the infection after the insertion of the central-line catheter [36]. An investigation of incidence of nosocomial infections, conducted in Shanghai General Hospital, found that arteriovenous catheterization and use of antifungal drug are factors that significantly influence the diffusion of CLABSIs among elderly patients [2].

2.2 Surgery site infections (SSIs)

Named also "wound infections", SSIs are infections that may rise up to 30–90 days after surgery in patients receiving an organ, group of cells, or device and affecting both the incisional site and deeper tissues around the surgery location [1]. They are associated with significant morbidity, high death rates and a significant increase in healthcare costs, and they represent the most frequent complication in postoperative surgical patients [37], [38]. Surgery interventions showing the highest risk are the orthopaedic, cardiac and intra-abdominal ones, with a percentage comprised between 2% and 36% of patients who may develop SSIs [39], [40], [41]. Consequences of SSIs can be shock and severe sepsis, which can lead to patients to prolong also for many days their hospital stay, being transferred to an ICU [35], [42], [43], [44]. Central venous catheters, lungs and surgical sites represent the most common locations of this type of HCAI [1]. The incidence rate of infection after cardiac surgery was reported to be between 5% and 21.7%; they are often linked to multiple organ failure and prolonged hospital stays, with consequent increased mortality rates [45], [46]. Another kind of particularly harmful SSIs are the orthopaedic ones, due to the difficulty of getting rid of the infection at the bones and joints level [47]. A higher percentage of Gram-positive isolates than Gram-negative ones was found in a study which analysed SSIs after orthopaedic surgery, discovering also that the majority of these isolates was resistant to cefuroxime, usually employed in the management of this type of infections [48]. Some of the statistically significant risk factors that can influence the incidence of SSIs include surgical wound contamination potentials, patients' clinical conditions, type and length of surgery, but also the movement and number of staff and the structural features of the operating theatre, as well as diabetes mellitus, smoking, the absence of antibiotic prophylaxis, and a history of previous surgery [48], [49], [50]. Furthermore, other than arteriovenous catheterization, operation and surgical incision for contamination, also hepatopathy and coma were associated with SSI risk [2]. Other types of surgery that were reported to give rise to SSIs, in percentages comprised between 11.3% and 36.9%, are gastrectomy, colorectal surgery, hepatectomy and pancreaticoduodenectomy [51]. In 2019, nearly 20,000 SSI were reported from a total of over 1.2 million surgical procedures in 13 European countries, among more than 2,500 hospitals that were part of the surveillance network [10].

2.3 Catheter-associated urinary tract infections (CAUTIs)

CAUTIs are usually benign infections, nevertheless they can lead to increased mortality in some patients, as they may hold potentially pathogenic virulent bacteria without showing any symptom [52], [53]. CAUTIs represent the highest percentage of HCAIs (40%) [39], [54], [55], with biofilm formation representing one of the major elements connected to the development of CAUTIs: microbes result to be protected from both antimicrobials and host defence mechanisms by the biofilm that lies on both the extraluminal and intraluminal portal catheter surface [56], [57], [58], [59]. Several factors have been identified as risk factors for CAUTIs through multivariate analysis, among which longer duration of the catheter, female sex, older age, diabetes mellitus, the absence of systemic antibiotics, catheter insertion outside the operating room, and a breach in the closed system of catheter drainage [52], [60]. Urinary catheterization and implant and diabetes were found to be significant influencing factors in another study too, together with a smoking history longer than ten years [2]. Moreover, the patients exposed to a higher risk are the ones having long-term indwelling catheters, who are more probably likely to contract CAUTIs-associated fever than short-term catheter using patients; they show also a higher risk to host pathogenic microorganisms and other urinary tract diseases than those without catheters [61]. However, healthcare-associated urinary tract infections rarely lead to death, although the probability is higher for elderly patients.

2.4 Ventilator-associated pneumonia (VAP)

Post-surgery pneumonia is a complication developed by a high number of patients, and it can be both hospital-acquired pneumonia (developing 48–72 hours after admission) and VAP (developing 48–72 hours after endotracheal intubation) [62]. Its incidence of morbidity and mortality is very high, being one of the most common consequences of all types of surgery [63]. Pneumonia affects more than one-quarter of patients in ICUs; it was found that VAP affects between 9% and 27% of patients with assisted ventilation, and that 86% of HCAIs are associated with motorized automatic ventilation and VAP, with an average of 2-3 days usually required to contract VAP after endotracheal intubation and mechanical ventilation [64], [65], [66]. The microorganisms causing this pneumoniae are often identified: according to a research conducted in an Indian hospital, for instance, the uppermost were non-fermentative Gram-negative bacilli [67]. Patients may also develop HCAIs affecting lower respiratory tract other than pneumonia (LRTIOP): also in this case, they often need artificial ventilation, with a much higher prevalence compared to patients carrying other HCAIs [68].

3. **Most common causative pathogens and associated antibiotic-resistance**

HCAIs represent a considerable burden which is tightly connected to another growing concern worldwide, namely the antibiotic resistance. According to a 2020 American study, it was estimated that 2.8 million antibiotic resistant infections occur every year in US alone, which in turn are the cause of 35,000 deaths annually and are associated with increased morbidity, mortality and healthcare costs. Although Gram-positive bacteria, such as MRSA and *Clostridium difficile*, were previously the main subjects of antibiotic-resistance global concern, more recently drug-resistant Gram-negative bacteria have gained attention worldwide [69].

Accordingly, a lot of pathogens can cause HCAIs, and among the most common we find *Staphylococcus aureus* (including methicillin-resistant *S. aureus*, MRSA), *Clostridium difficile*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, various multidrug-resistant organisms (MDROs), and also microorganisms different from bacteria, such as fungal and viral agents which are frequently implicated in HCAIs [33]. However, it has been demonstrated that approximately 12-17 pathogenic microorganisms are responsible for 80% - 87% of HCAIs, in particular *Enterococcus* species (e.g., *Enterococcus faecalis, Enterococcus faecium*), *A. baumannii*, *Enterobacter* species, *Proteus* species, *Escherichia coli*, *Candida* species (e.g., *albicans*, *glabrata*), *S. aureus*, coagulase-negative *Staphylococci*, *P. aeruginosa*, *Klebsiella pneumoniae* and *K. oxytoca*, *Bacteroides* species, Yeast NOS; among these ones, 16% - 20% include multidrug-resistant bacteria, such as MRSA, carbapenem-resistant *P. aeruginosa*, extended-spectrum cephalosporin-resistant *K. pneumoniae*/*K. oxytoca*, *E. coli*, *A. baumannii*, vancomycin-resistant *E. faecium* and *Enterobacter* species. Of these pathogens, the predominant antimicrobial resistance phenotypes were observed among Gram-negative microorganisms, and, in parallel, also among the pathogens isolated from device-associated HCAIs [70], [71], [72]. In the most recent of these studies, differently from other bacterial phenotypes, it has been shown an important increase in resistance profiles of *E. coli*, especially against fluoroquinolones [72]. Another study investigated the resistance profiles of *Acinetobacter, Burkholderia spp.* and *Pseudomonas spp.* isolates, reporting their total or superior to 92% resistance to cephalosporins and fluoroquinolones. On the other hand, carbapenems, which are widely used in ICUs, were shown to be more efficacious against *Burkholderia* spp., which had 20% resistance, while the resistance percentages demonstrated by *Acinetobacter* spp. and *Pseudomonas* spp. were, respectively, 86.4% and 62.5% [32]. Another study focused on *Acinetobacter* spp. demonstrated their high (76.99%–92.01%) resistance to most antimicrobials; however, 30% of the isolates were shown to be susceptible [73], suggesting that, as the pathogens vary depending on the country, the same is true for the resistance patterns [1]. Carbapenems showed an 80% efficacy against *E. coli* in another study, although these bacteria presented several other

resistances. In the same work, *Enterobacteriaceae* community demonstrated a total resistance to third-generation cephalosporins, and over 80% of the *Klebsiella* spp. community were resistant to ciprofloxacin, gentamicin, piperacillin, tazobactam, and imipenem. Accordingly, *Citrobacter* spp., which represents a minor cause of HCAIs, showed resistance to cephalosporins, fluoroquinolones, and aminoglycosides [74].

Staphylococcus aureus was the most frequent microorganism detected in a 2012 study investigating the prevalence of HCAIs in acute-care hospitals in Florida [75]. This bacterium, together with *Pseudomonas aeruginosa*, was the main causative agent of HCAIs (mainly undetermined clinical sepsis and pneumonia) in a 2017 study carried out in Singapore; in addition, this work reported an important resistance to carbapenems by the *Acinetobacter* species and *P. aeruginosa* [76]. These two pathogens, together with *Klebsiella* species, were found to be the most common microorganisms in a systematic review about HCAIs in Southeast Asian countries [17]. Gentamicin was reported to be highly effective on both Gram-positive and Gram-negative microorganisms in a study conducted in Ghana in 2012, despite other six antibiotics among 12 tested which resulted to be ineffective [77]. In US, the ESKAPE group (*Enterococcus faecium* and *S. aureus* as Gram-positive species, *K. pneumoniae, A. baumannii, P. aeruginosa*, and *Enterobacter* spp. as Gram-negative ones) has been pointed out as the major responsible of HCAIs [78], [79], [80].

S. aureus, both the methicillin-sensitive and the MRSA, was shown to be resistant to ciprofloxacin, clindamycin, trimethoprim/sulfamethoxazole, erythromycin, gentamicin, and tetracycline in a work published in 2016 [81].

Plazomicin, one of the most recent aminoglycosides, demonstrated more effectiveness against *A. baumannii* than other antibiotics (gentamicin, tobramycin, and amikacin) [82], and also against some microorganisms of the extended-spectrum beta-lactamase (ESBL) group, such as *Enterobacter* spp., *Escherichia coli*, and *K. pneumoniae* [83]; moreover, it demonstrated a better safety profile than other drugs, even with high and multiple doses [84].

A research conducted in an Indian hospital in 2016 found the following as prevalent pathogens causing VAP: *Pseudomonas, Klebsiella, Enterococci, S. aureus* (among which MRSA), some of them characterized by an upward trend in resistance to various antibiotics [67]. Some of these species were found also in the isolates of VAP patients at Chonnam National University Hospital in South Korea, with a strong prevalence of *S. aureus* (44%), of which some MRSA strains, followed by *A. baumannii* (30%), *P. aeruginosa* (12%), *Stenotrophomonas maltophilia* (7%), *K. pneumoniae* (6%, of which 67% was ESBL-positive), and *Serratia marcescens* (2%) [85].

Some of these pathogens were found among the most common also in a study focused on the incidence of SSIs in orthopaedic patients, including *Staphylococcus* species (of which some MRSA,

29.11%), *Acinetobacter* species (21.5%), *Pseudomonas* species (18.9%), and *Enterococcus* species (17.7%) [86]. SSIs connected to abdominal surgery are often caused by coagulase-negative *Staphylococci*, *Enterococcus* spp., *E. coli* and, again, *S. aureus* [38]: the latter has been considered one of the main causes of HCAIs for over a hundred years. Initially susceptible to penicillin, the abuse of this antibiotic lead to *S. aureus* resistance by producing β-lactamase enzyme, and this has led to the development of alternative penicillin-like antibiotics such as methicillin, oxacillin, cloxacillin, and flucloxacillin [87]. However, MRSA started to appear more than fifty years ago [88], and nowadays these pathogens represent one of the more threatening microorganisms causing HCAIs in US and Europe, due to their potential resistance to multiple antimicrobials [89], [90], [91].

All these data highlight that the increasing prevalence of multidrug and antibiotic-resistant pathogens make treatment more challenging, pointing out the urgent necessity for improved infection prevention and control strategies [69], [92].

4. *Clostridium difficile*

Clostridium difficile, also known as *Clostridioides difficile,* is a Gram-positive, rod-like, obligate anaerobic, spore-forming bacterium [93]. It is widely found in soil and in the gastrointestinal tract of young animals and humans, without being harmful [94]. It can exist in both a vegetative and spore form; like many other similar species, it can produce toxins that facilitate its virulent properties.

C. difficile had been first described in 1935, as part of the faecal microbiota of healthy newborns [95]. It can be found in up to 15% of adults' intestinal microbiota, and it can be isolated in 80% of toddlers' faeces samples [96].

Since the end of 1970s, cytotoxin presence was detected in stool specimens of pseudomembranous colitis patients [97], [98], and in 1978 the confirmation of the association between *C. difficile* infection and antibiotic-associated pseudomembranous colitis (PMC) was provided [99], [100]. Nevertheless, *C. difficile* did not represent an important concern until the end of the 20th century, as the mortality rates associated with its infection were not significant until then, but later some factors, such as the extensive use of antimicrobials and the emergence of highly virulent *C. difficile* isolates, have led to an upsurge in terms of frequency and severity of *C. difficile* infections (CDI) [101].

C. difficile is considered the main cause of antibiotic-related diarrhea in several healthcare settings, especially the rehabilitative ones and elderly centres [102]. Antimicrobials can, in fact, disrupt the gut microbiota, favouring *C. difficile* growth in the digestive tract, conferring it a selective advantage [103], [104]. CDI can vary from mild diharrea to more serious infections; the clinical manifestations and the severity strongly depend on patients' characteristics, but in general the common symptoms include diarrhea, abdominal pain, vomiting, fatigue, and loss of appetite. Life-threating clinical features that are typical of a severe infection include PMC, colon perforation, septic shock and kidney failure, that can lead to death [105], [96]. CDI transmission happens via the oral-faecal route [105]. It can survive for long periods on contaminated surfaces, and this facilitates its transmission through both symptomatic and asymptomatic carriers, and not only in healthcare settings; in fact, *C. difficile* has been associated with the increase of community-acquired cases of colitis [93].

In addition, its ability to form spores makes *C. difficile* particularly resistant to cleaning agents, alcohol-based disinfectants and antimicrobials, facilitating its transmission in healthcare settings [102], [105]. Being the spores highly resistant to environmental conditions, *C. difficile* can survive in this form in an oxygenated environment outside the host [105] and also when exposed to the acidic gastric juices in the stomach: in this way, spores survive and turn into vegetative cells once they get to the intestine [106].

The primary mediators of inflammation caused by *C. difficile* infections are clostridial toxins: toxin A (TcdA) is an enterotoxin, and toxin B (TcdB) is cytotoxic. Both affect mucous membranes of the

colon, and it was shown that tcdB is the major virulence factor in *C. difficile* infections [104], [107], [108]. The toxins lead to a complex cascade of host cellular responses that cause diarrhea, inflammation, and tissue necrosis; however, it is reported that certain *C. difficile* strains are non-pathogenic and lack toxin genes [100]. Other virulence determinants include colonization and adherence factors, such as surface proteins, flagella, and fimbriae, biofilm formation and bacterial spread factors (namely, proteolytic enzymes) [104], [109].

The emergence of CDI is often associated with antibiotic use: despite being able to survive to antimicrobial therapy due to its capacity to form spores, it has showed antibiotic resistance against broad-spectrum antimicrobials such as aminoglycosides, tetracyclines, erythromycin, clindamycin, penicillin, cephalosporins, and fluoroquinolones [110]. On the other hand, fidaxomicin and vancomycin seem to remain effective against *C. difficile* [111], [112], [113], even if it has been shown that some isolates have decreased susceptibility to vancomycin and metronidazole [114], [115]; this can be enhanced, for instance, by the formation of biofilm [116]. In case of failure of these two antibiotics, fidaxomicin and rifamycin have been recommended as alternative antibiotic strategies to treat CDI [117], but the latter has already been reported to be ineffective against several isolates due to high levels of resistance by some studies [118].

C. difficile is among the most common causes of nosocomial infections in numerous countries. It has been estimated that almost 500,000 patients contract a HCAI by this bacterium, with 29,000 attributable deaths every year in the United States [119], [120]. In a survey published in 2014, involving 183 US hospitals with 11,282 patients, it has been reported that the most common microorganism causing HCAIs was *C. difficile* [33]. The European CDC has stated that around 124,000 patients in the European Union develop CDI each year [105]. Furthermore, *C. difficile* epidemiology is changing: even though it initially appeared as a HCAI, currently one out of three of CDIs seem to be community-acquired infections [120].

5. *Escherichia coli*

Escherichia coli is a Gram-negative bacterium that plays a significant role in both scientific research and public health. It is facultative anaerobic, rod-shaped, not sporogenous, belonging to the *Enterobacterales* order; it may have a capsule and motility abilities, thanks to its flagella. It can survive for extended times in the environment, also because of its capacity to grow in a wide range of temperature (15-45°C) [121]. *E. coli* is naturally present in the intestinal microbiota of humans and warm-blooded animals, where it performs essential functions in the digestive system.

Due to its well-characterized genetics and ease of laboratory cultivation, *E. coli* has been extensively studied and has become a model organism in molecular biology and biotechnology. It has been instrumental in advancing our understanding of basic cellular processes, such as DNA replication, transcription, and translation. *E. coli* also serves as a host to produce various recombinant proteins and pharmaceuticals [122].

While most strains of *E. coli* are harmless and even beneficial, other strains can cause illness in humans [123]: in particular, some of these have developed pathogenetic mechanisms that may lead to severe pathologies [124], [125]. These pathogenic *E. coli* strains are responsible for a range of infections, including urinary tract infections, gastrointestinal infections, and even severe conditions like haemolytic uremic syndrome (HUS).

E. coli has been recently classified on the basis of its pathogenetic profile, taking into account specifically the underlying diseases and the corresponding virulence factors [126], which are genes encoding for adherence, colonization, invasion, cell surface molecules, secretion, transport, siderophore formation for iron acquisition, toxins, lipopolysaccharides, polysaccharide capsules, mobile genetic elements and the ability to manipulate the host innate immune response [127], [128]. Overall, these pathogenic *E. coli* variants are classified into two main categories, namely intestinal pathogenic *E. coli* (IPEC), which cause intestinal infections, and extra-intestinal pathogenic *E. coli* (ExPEC), which lead instead to the development of systemic infections [123], [129]. The latter are pointed out as the most common Gram-negative human pathogens, globally causing more than two million of deaths every year. Infections caused by these E. coli strains include urinary tract infections (UTI), hospital-acquired pneumonia, sepsis, surgical site infection, cholecystitis, cholangitis, peritonitis, cellulitis, osteomyelitis and arthritis [128], and also neonatal meningitis [130].

Accordingly, in the context of HCAIs, these bacteria are highly widespread and are responsible of numerous infections, including infections of the gastrointestinal tract, wound infections, bloodstream infections (bacteraemia), and urinary tract infections [131]. Among these, the latter represent an important challenge in healthcare facilities [132]: in fact, urinary tract infections (UTIs) originate from microbial colonization of the urinary tract, and this can cause significant complications, such as

urethritis, pyelonephritis, epididymitis, cystitis and prostatitis, potentially developing to bloodstream infections [133]. Uropathogens are involved, as many other microorganisms, in the increasing prevalence of antimicrobial resistance, and this further complicates the clinical and economic issues linked with UTIs [134]; among these, 75% of uncomplicated UTIs are caused by uropathogenic *E. coli*, as well as 65% of complicated ones [135]. On the other hand, UPEC strains are also the primary causes of community-acquired UTIs worldwide: it is estimated that 40% of women and 12% of men have experienced one or more symptomatic UTI during their lifetime, with 27-48% of women suffering from recurrent UTIs [136], [137], [138].

In 2014, the WHO has reported *E. coli* in a list of the top nine microorganisms of international concern causing the most common infections in different settings, such as the community, the healthcare facilities, or transmitted through the food chain [127]. This is also connected to the antibiotic-resistance problem: in fact, *E. coli*, especially the commensal strains hosted by the intestinal tract of humans and warm-blooded animals, have been shown to play a key role in the spreading of antibiotic-resistance genes [139], [140], as well as it probably happened with the acquisition of virulence-associated determinants [141], [142].

E. coli developed resistance traits since a long time, such as genes encoding for β-lactamases, extended-spectrum β-lactamases and carbapenemases [143], [144], [145], [146]. Among the most common antibiotics used to counteract community and hospital *E. coli* infections cephalosporins, fluoroquinolones, and trimethoprim-sulfamethoxazole can be found, other than β-lactams, but several epidemiological surveys conducted in Europe and America have indicated that 20–45% of ExPEC isolates were resistant to these first-line antibiotics [143], [147], [148], [149].

Moreover, strains producing these kinds of β-lactamases often show resistance traits against other antibiotics: for instance, strains expressing a new type of metallo β-lactamase (MBL), named New Delhi metallo-β-lactamase-1 (NDM-1), can be treated with other antibiotics such as colistin, tigecycline, and fosfomycin [150]. However, this may lead to the development of resistance against these agents too [151]; besides, they are often broadly resistant to many drug classes and antibiotics, such as aminoglycosides and fluoroquinolones, drastically reducing the treatment options [152].

6. *Acinetobacter baumannii*

Acinetobacter baumannii (*A. baumannii*) is a Gram-negative, aerobic, non-motile bacterium. It is one of the six pathogens belonging to the ESKAPE group, characterized by multidrug resistance and virulent properties [153], [154], [155]. Both in communities and livestock have emerged several reservoirs of MDR *A. baumanni* strains, which cause dangerous outbreaks [156], [157]. Infections caused by *A. baumannii* comprise wound, skin and soft tissue infections, urinary tract infections, and they can lead to bacteraemia, endocarditis, meningitis and pneumonia. Due to its ability to penetrate through skin and airway defects, the risk to get infected by *A. baumanni* is higher for hospitalized patients, in particular for the ones admitted to ICUs [158], [159], [160]: VAP is, in fact, the most frequent HCAIs caused by *A. baumanni*, that affects mainly patients staying in ICU and breathing through the ventilator [161], [162]. Moreover, it has been shown that there is a strong association between burn infection and *A. baumanni*, especially among military personnel [163]. Central nervous system infections may also be caused by *A. baumanni*, and these can be counteracted through colistin antibiotics [164]. In this context, multi-drug resistance (MDR) and low penetration of several antibiotics represent a considerable issue connected with *A. baumanni* infections [165], [166], [167], [168]. It is characterized by a strong capacity to survive for long periods in the environment, facilitating the spread of pathogenic MDR strains; this has been also enhanced by an excessive usage of β-Lactam antibiotics, which has led to the emergence of resistant strains [169], [170], [171]. Specifically, some strains have emerged as resistant to broad-spectrum β-lactams antibiotics, including carboxy-pencillins and the third generation of cephalosporins. Fluoroquinolones and aminoglycosides are antibiotics against which some resistant strains have emerged, too [172], [160]. Carbapenems and colistin became so the antibiotics of choice to treat *A. baumanni* infections, but currently several resistant strains have been found against these antibiotics too, limiting their efficacy [169], [170], [171]. Other than antibiotics, resistance to antimicrobials and biocides, including antiseptic and disinfectant substances, leads to the emergence of MDR strains and to their spread in hospitals [173]: concerning *A. baumanni*, a study showed that the usage of povidone-iodine increased the resistance of the bacterium by 18.5%, although other antiseptics and disinfectants have been shown to be effective against 81 strains [174]. *A. baumanni* pathogenicity and infections mechanisms are mediated by its attachment to epithelial cells and the production of enzymes and toxins [175]: the bacterium colonizes human skin and bronchial epithelial cells, as well as inert surfaces, and this ability leads to infection and to its propagation in the environment, respectively [176]. *A. baumanni* attachment and invasion of eukaryotic cells cause their apoptosis and, in the case of epithelial cells, this may reduce the mucosal surface, facilitating the penetration toward deep tissue [177], [178], [179], [180]. In addition, *A. baumannii* infections are characterized by the secretion of endotoxins

and a moderate stimulation of the inflammatory response, and this helps the bacteria to overcome the immune system defence, facilitating the spread of the infection [177], [178], [179], [180]. It has also been demonstrated that the ability to constitute biofilms, that characterizes some *A. baumannii* strains, plays an important role in the interactions both with the host cells and the medical devices surfaces, and its formation is mainly mediated by the production of exopolysaccharides and pilus structures [175], [181], [182], [183], [184], [185]. Strains producing the biofilm demonstrated a higher survival rate than non-biofilm forming ones [186]: some strains have in fact been observed to be able to survive on the hospital bed rails and in wet environments. Lipopolysaccharide layer, found on the outside of the biofilm surfaces, has the function to protect bacteria from alterations in environmental humidity, increasing also antibiotics resistance [187].

Finally, *A. baumannii* infections are associated with an increase in iron deficiency, due to its ability to produce siderophores that, in turn, acquire ferric ions; the most important is acinetobactin, which has also been associated with its virulence [188], [189].

7. Pathogens transmission in healthcare settings

Pathogens transmission in healthcare settings can happen both through direct contact with contaminated surfaces and via healthcare workers (HCWs), as reported by a review investigating the potential contribution of healthcare personnel to the spread of *C. difficile* infections.

In this work, which took into consideration 11 studies regarding HCWs' hand carriage, it is reported that between zero and 59% of HCWs' hands were found contaminated with *C. difficile* after caring for a patient with CDI. This high variability among the results obtained by the different studies is probably due to the differences in the study design, sampling timing and culturing methods used, and context and observance of gloving and hand hygiene. In addition, the review reported two cases of *C. difficile* outbreaks involving HCWs and six series of cases of contagion from patients to the personnel [190]. HCWs may enter in contact and contaminate their hands also through routine practices normally considered at low-risk, such as taking patients' temperature, and examining or feeding them [191].

Conversely, patient-to-patient infection transmission can take place either by environmental or medical devices contamination [192], [193]: notably, a prior room occupant carrying a CDI represent a risk factor for CDI [191], [194], [195], [196]. Gloves, which represent one of the principal protection barriers recommended by the guidelines, are often insufficient to prevent contamination: a study demonstrated, in fact, that contamination of skin or clothing after glove and gown removal occurs in 46% of cases [197]. Guidelines about hands hygiene to prevent, specifically, the diffusion of *C. difficile* infection may be highly variable: while the Society for Healthcare Epidemiology of America indicates alcohol-based handrub in routine practices and handwashing with soap and water only during outbreaks or in hyperendemic settings [195], in Europe just soap and water hands hygiene is suggested as normal practice [198], because spores are alcohol-resistant [199], [200]. Indeed, soap and water handwashing have been suggested as a preventive measure also for patients and visitors, other than HCWs [201], [202], [203].

A study investigating the potential sources of transmission of HCAIs within the hospital quantified the average number of microorganisms on working surfaces, door handles and taps, and they found that the door handles hosted the highest number of microbes, while the highest variance of pathogens was on hospital floors. Within these isolates, 46.14% of microorganisms were disease-producing, where the most common were *S. aureus* and *Bacillus subtilis* [77].

An additional issue related to HCAIs spread involves the hospital waste, especially the contaminated surgical one, as it often represents a reservoir for pathogenic virulent microorganisms: 20%–25% of the waste produced by health care outlets is supposed to be potentially HCAIs-causing, therefore its proper handling and disposal needs to be addressed [204], [205].

Overall, all these data highlight the importance of the knowledge and the compliance with the prevention measures, which may in turn be improved, by both healthcare personnel and patients themselves; this should start from very simple but crucial routine practices, such as the correct handwashing procedures [206], [207], [190].

8. Current strategies for cleaning and disinfection

Environmental cleaning in healthcare settings is usually based on chemical disinfectants, although several studies have demonstrated that manual cleaning alone is not effective enough to reduce contamination to safe levels [208]. Cleaning protocols for infection prevention, in fact, still face some challenges: these disinfectants (chlorine-based agents, quaternary ammonium compounds and alcohols), for instance, may favour the raise of environmental pollution and antimicrobial resistance [209], [210]. Cross-contamination risks in shared spaces and medical equipment, particularly in the context of pandemics and multidrug-resistant pathogens, are other elements that further need additional attention, as well as the necessary efforts in maintaining effective and sustainable hygiene standards, especially in environments dedicated to vulnerable or mobility-impaired patients. These problems underline how urgently effective, innovative and ecologically responsible disinfection strategies are needed [31].

Of great importance, regarding cleaning and disinfection procedures in healthcare settings, are medical devices and surgical instruments. All the endoscopies and invasive medical procedures that involve the contact between a medical device with patients' sterile tissue or mucous membranes carry the risk of infection by pathogenic microorganisms, when proper disinfection or sterilization procedures fail [211], [212]. Therefore, correct disinfection and sterilization practices are fundamental to avoid transmission of infectious microorganisms to patients; however, it has been reported by many studies that the compliance with the relative guidelines is often not achieved, leading to outbreaks and patient exposures [213], [214], [215], [216]. The level of disinfection or sterilization of the medical device depends on its use; therefore, they are usually subdivided into the following categories: critical, if they are intended to contact sterile tissue, such as surgical instruments and catheters; semicritical, if they are used for mucous membranes, such as endoscopes; non critical, if they touch just intact skin, like stethoscopes, bedpans, patient furniture. These three categories of objects require respectively sterilization, high-level disinfection, or low-level disinfection.

Critical items need to be sterilized from both vegetative microorganisms and bacterial spores. Sterilization process usually requires steam or, if heat-sensitive, the objects can be treated with ethylene oxide (ETO), hydrogen peroxide (HP) gas plasma, vaporized HP, HP vapor (HPV) plus

ozone, or by liquid chemical sterilants, after a previous cleaning step to eliminate organic and inorganic material. Differently from semicritical items, critical ones have rarely been associated with infection transmission [217]. On the other hand, semicritical items allow small quantities of spores to be present, because intact mucous membranes are generally resistant to infections caused by bacterial spores, while susceptible to vegetative microorganisms. Semicritical items are subjected to high-level disinfection through chemical disinfectants, such as glutaraldehyde, HP, ortho-phthalaldehyde (OPA), peracetic acid with HP, and chlorine. Usually, this kind of items is subjected to reprocessing procedures; however, semicritical equipment has been associated with reprocessing errors. Furthermore, an association between semicritical items and more than 100 infection outbreaks was found; in particular, endoscopes, which are widely used tools both for diagnosis and therapeutics, were found to be the most frequent reusable medical devices associated with health care–associated outbreaks [214], [215], [216]. This may happen because, even if HLD is supposed to eliminate all microorganisms, a few spores can survive on the instruments. In some cases, the reason is that healthcare personnel do not always strictly follow the reprocessing recommendations [213]; however, it has also been shown that reprocessing guidelines may be insufficient to guarantee effective decontamination [218]. In fact, some outbreaks occurred although the guidelines were followed correctly, specifically MDR bacterial infections caused mainly by carbapenem-resistant *Enterobacteriaceae* associated with the usage of duodenoscopes [211], [212], [219]: the reasons, in this case, can be linked to the complex design of these devices, and to the high amount of bacteria that duodenoscopes are contaminated with and their tendency to form biofilms, intrinsically complex to eradicate [210], [220].

Noncritical items are often decontaminated in the same site of usage, without the necessity to be transported to a central processing area. Ideally, coming in contact just with intact skin, noncritical items don't carry any risk of transmission of infectious pathogens [221]; nevertheless, some objects can potentially play a role in secondary transmission, contacting medical equipment or healthcare workers' hands [222]. Low-level disinfectant commonly used for noncritical items include alcohol, sodium hypochlorite, HP, quaternary ammonium compounds [210].

Besides medical devices, patients' rooms, including all the objects and surfaces, should be decontaminated: better environmental cleaning has, in fact, been associated with decreased contamination levels of VRE, MRSA and *C. difficile* [223], [224], [225]. Improved cleaning thoroughness have thus demonstrated an efficacy; however, in healthcare environments several surfaces in the rooms remain only partially cleaned, so potentially contaminated. To face this problem, no-touch systems have been introduced, such as UV light and HPV/mist [226], with the aim to supplement standard cleaning and disinfection. In particular, UV light has shown effectiveness in

eliminating bacteria such as MRSA, VRE, *Acinetobacter baumannii* and *C. difficile*, both in its vegetative and spores form [227], [228], [229]; accordingly, HP systems are used for rooms decontamination and demonstrated efficacy in eliminating infectious bacteria such as MRSA, *Mycobacterium tuberculosis*, *Serratia*, *C. difficile* spores, *Clostridium botulinum* spores [210]. Both these two strategies have the capacity to yield a significative reduction in vegetative bacteria, as well as *C. difficile* spores, differently from low-level disinfectants that have scarce activity against spore-forming bacteria [230]; UV light and HPV are thus effective methods to reduce contamination and, in turn, HCAIs [231].

Another kind of no-touch system in the context of healthcare environments disinfection is represented by automated disinfection technologies, like bedpan washer-disinfectors (BWDs). These devices streamline the decontamination and cleaning process of human waste containers directly in the ward, avoiding the passage in a central processing area [31]. They are widely used, although their validation currently lacks for the need of biological indicators in the European Standard EN ISO 15883-3 [232] and, even if performed, conventional culture-mediated microbiological techniques for pathogen identification are often time-consuming and not suitable to detect non-culturable microorganisms [233]. Taken into account all these issues that healthcare structures still face, new alternatives to improve infection prevention need to be implemented, such as the validation of already used disinfectants, the characterization of novel ones, and the development of effective strategies for bacterial detection and eradication [31].

AIM OF THE THESIS

The increasing need for innovative solutions to the burden of HCAIs, tightly connected with the growing problem of antibiotic-resistance and to the insufficiency of the currently adopted methods to prevent infections, led us to address some of these concerns by studying three strategies.
At first, we evaluated the efficacy of a thermal disinfector bedpan washer in lowering contamination by infectious pathogens commonly causative of HCAIs, namely *Escherichia coli* and *Clostridium difficile*. In parallel, we investigated the environmental sustainability of the process by monitoring its energy consumption, looking for a protocol aimed simultaneously at achieving a significative disinfection level through an optimized energy-efficiency workflow.
Accordingly, we subsequently addressed the sustainability and environmental challenges related to standard cleaning methods, which are mainly based on high resource consumption and the massive use of chemical agents: this contributes to environmental pollution and antimicrobial resistance [234], [235]. To face these issues, we developed a novel cleaning system based on circular economy principles, in particular we formulated a detergent starting from waste cooking oil to yield methyl-ester sulphonate surfactants. Its effectiveness in reducing bacterial load was firstly evaluated by rinsing contaminated surfaces and measuring bacterial reduction; then, the detergent was complexed with nanoassemblies, based on silver alternatively bound to magnetic nanoparticles (MNPs) or biomimetic magnetic nanoparticles (BMNPs), to boost its bactericidal effect.
Furthermore, we proposed a new alternative to prevent infections spread through wheelchairs. These items are widely used for intra-hospital transport of patients and, when contaminated, they can pose a serious infection risk, especially for the most vulnerable ones (e.g., post-operative and immunocompromised patients). However, cleaning after each use is crucial, yet time-consuming and not always sufficiently accurate; so, considering that a solution may be focusing on preventing pathogens transmission risk, we evaluated the effectiveness of a disposable non-woven fabric named "SAFE-HUG Wheelchair Cover", that works as a protective barrier between the wheelchair and the patient, to prevent cross-contamination.
Together, these approaches address both the environmental sustainability and the effectiveness needed for pathogens decontamination strategies, proposing innovative solutions to improve infection prevention in hospital settings.

MATERIALS AND METHODS

1. Bacterial cell cultures

Bacterial strains were thawed and then incubated for 24 hours, under agitation, at 37°C in specific culture media: *C. difficile* (NCTC 11204 strain; National Collection of Type Cultures, London, UK) was cultured in Fluid Thioglycolate Medium (Merck, Rahway, NJ, USA) supplemented with 1% vitamin K1 (Fluka, Buchs, Switzerland), under anoxic conditions (Oxoid™ AnaeroJar™ containing an Oxoid™ AnaeroGen™ Compact Sachet) (Oxoid Ltd., Basingstoke, UK). Conversely, to grow *E. coli* (ATCC 25922D strain; American Type Culture Collection, Manassas, VA, USA) we used LB medium, composed of 4% bactotryptone (Thermo Fisher Scientific, Waltham, MA, USA), 2% Bactoyeast extract (Thermo Fisher Scientific, Waltham, MA, USA), and 4% NaCl (Sigma-Aldrich Corporation, St. Louis, MO, USA).

Cell counting through a Bürker chamber (Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany) was performed for both *C. difficile* and *E. coli* suspensions, after an incubation of 24 h. The counting was carried out with an optical microscope at 40× magnification. Both the bacterial suspensions were successively diluted with their respective culture broth to get the desired concentration suitable for the experiments; specifically, the final bacterial concentration used in the experiments was 10^6 cells per 100 μL.

2. Preparation of bacterial and RAMS solution

The Representative Artificial Mucosal Substrate (RAMS) solution utilized in this study was adapted from standardized test soils described in the context of ISO/TS 15883-5. This technical specification expresses microbiological test procedures to assess the cleaning efficacy of mechanical processes related to medical devices [31].

Each powdered component was dissolved in Phosphate-Buffered Saline (PBS) (Merck KGaA, Darmstadt, Germany) at the following concentrations: 0.6% bovine serum albumin (BSA) (Sigma-Aldrich Corporation, St. Louis, MO, USA; catalog No. A7030), 1% mucin from bovine submaxillary glands (Sigma-Aldrich Corporation, St. Louis, MO, USA; catalog No. M3895), and 3% maize starch (commercially available food-grade maize starch was used in this study), to prepare the RAMS solution. Afterwards, it was used to dilute bacterial suspensions 1:1, yielding a final bacterial concentration of 10^7 cells/mL. The mixture was then homogenized by vortexing to guarantee uniform distribution.

3. Contamination of pans, drying of spots and control mix solution collection

For this experimental phase, the pans used were stainless steel trays commonly found in clinical settings for the collection of faecal samples and biological fluids.

These devices were preventively cleaned using a washer-disinfector, before each experiment. The cleaning program comprised an alkaline detergent-based wash phase at 65°C, then a neutralization phase with acid, and finally a thermal disinfection phase at 93°C for 1 min ($A_0 = 600$). After this process, the pans were subjected to an additional cleaning step through 90% ethanol, to further ensure effective decontamination.

Subsequently, the pans were contaminated by putting eight spots of bacterial suspension, combined with RAMS, on every pan. We strategically placed the spots according to the following scheme: two spots in the centre and six spots on the border of each pan. Since each spot had a volume of 100 µL, the respective concentration resulted in 10^6 cells per spot. Two pans were treated concurrently in every cycle, and the process was repeated at least three times to guarantee sufficient data for statistical analysis. To dry the spots, the pans were then introduced in a static incubator for 30 min at 37°C, ensuring the bacterial suspension adhered to the surface of the pans. Afterwards, environmental sampling swabs (Neogen® Quick Swab) (Neogen Corporation, Lansing, MI, USA; Manufacturer Part No. 700002007) were employed to collect the deposited material from half of the spots. Successively, the leftover spots were collected from the pans following the washing process with the SMEG Bedpan Washer.

4. SMEG BPW1260 Bedpan Washer

The bedpan washing process was composed by the following phases. At first, the device accomplished two cycles comprising a cold water-based pre-wash phase, followed by another pre-wash step using hot water. After that, a washing phase was carried out with hot water and the BPW1260 detergent (10 mL/L); this passage was repeated four times. Then, the device performed for three times a rinsing step with hot water, followed by a rinsing phase with limescale remover and hot water. The entire procedure was finally completed with a thermal disinfection phase based on the A_0 parameter.

Notably, the SMEG Bedpan Washer BPW1260 offers several wash programs - short, medium, and intensive - each composed of different washing and thermal disinfection phases with different A_0 values set:

$$A_0 = \tau * 10^{\left(\frac{T-80}{10}\right)}$$

The effectiveness of the thermal disinfection process is directly proportional to its duration in seconds, where in this formula t = time (s); T = temperature (°C).

The maximum thermal disinfection A_0 value that this device unit can achieve is 6000. Thus, tests were carried out at $A_0 = 600$ or $A_0 = 6000$. Pr 08-IdProg. 708, a program designed specifically for thermal disinfection of sanitary bedpans and urine bottles, was set to confirm the efficacy of the bacteria reduction. A calibrated power meter connected to the disinfection apparatus was used to track the energy consumed during the cycles of disinfection.

This device monitored and recorded the total electrical energy (in kWh) spent throughout each treatment cycle. Measurements started at the beginning of the cycle and concluded upon completion, allowing a precise detection of energy usage for both $A_0$6000 and $A_0$600 programs. This permitted energy efficiency comparison between treatments under consistent operational settings.

5. **Analysis of the bacterial load after disinfection**

The efficiency of the disinfection carried out by the bedpan washer was assessed by analysing the residual bacteria on the pans after the washing cycles. Bacterial population was determined by the following methods.

- **Bacterial incubation and colony-forming units count (CFU/mL)**

Using the aforementioned swabs, residual bacteria were collected from the surfaces after the treatment. Vortexing each test surface in 10 mL of sterile phosphate-buffered saline (PBS) (Thermo Fisher Scientific Inc., Waltham, MA, USA, catalog No. J61196.AP) for 1 minute allow the samples to be homogenized. Selective agar plates were used to plate serial dilutions: in particular, *E. coli* underwent aerobic incubation at 37°C for 24 h, in MacConkey Agar (Thermo Scientific™ Oxoid™ Waltham, MA, USA; catalog No. CM0007B), while *C. difficile* was incubated under anaerobic conditions at 37°C for 48 h using Columbia Blood Agar (Thermo Scientific™, Thermo Fisher Scientific Inc.; Catalog No. CM0331T). Colony-forming units (CFU) were counted and expressed as CFU/mL.

- **Bacterial analysis by optical density (OD600 nm)**

The optical density of the bacterial suspension, at the specific wavelength of 600 nm (OD600 nm), was assessed with a spectrophotometer (Eppendorf Biophotometer #6131) (Eppendorf AG, 22,331 Hamburg, Germany), with proper cuvettes. In order to determine baseline absorbance values without thermal disinfection (control samples), and to assess the potential variations in bacterial content, the

measurements were carried out in the control samples as well as following the thermal disinfection procedure.

Each bacterial suspension analysed was placed in a clear cuvette, to measure the absorbance value at a wavelength of 600 nm. This approach was aimed at comparing the bacterial suspension densities before and after the washing process, to better establish the effectiveness of the treatment on bacterial viability and to measure any potential reduction in bacterial concentration thanks to the disinfection process.

- **Cell count by Bürker chamber**

The bacterial suspension samples were then placed into a counting chamber composed of grids of known volume for enumeration. The chamber enables for the visualization and manual counting of individual bacterial cells within the grid squares. The bacterial control samples, collected before the washing steps, were diluted 1:100 prior to counting, to ensure the cells to be sufficiently spaced for accurate enumeration; conversely, the samples collected after the thermal disinfection were counted without any dilution. Once placed in the chamber, each sample was observed with a microscope in brightfield, at magnification of 40×, to count the number of cells within a defined grid area.

- **DNA quantification**

DNA was isolated from the samples through a column-based extraction procedure, by utilizing Qiagen DNA Kit (Cat. No./ID: 51104, Hilden, Germany) following the manufacturer specifications. Successively, using the OD260 measurement as the standard for quantification, the DNA concentration was quantified with a Qubit fluorometer and expressed in ng/µL.

6. Eco-friendly detergent formulation

Using waste sunflower cooking oil as starting material, the sulfonated methyl-esters-based eco-friendly detergent was prepared. The oil was first filtered and then subjected to transesterification and sulfonation to yield the detergent. Many trials were carried out by altering methanol-to-oil molar ratio, temperature and reaction times, as waste cooking oil samples are characterized by a high variability. Moreover, the Resi Levi Permadani et al. protocol [236] was slightly varied by optimizing the amount of KOH, acting as a basic catalyst, to 1.3% *w*/*w* based on the oil weight.

Waste cooking oil (WCO) was initially heated to 60°C, 70°C or 80°C, depending on the experimental settings. Methyl esters were produced from methanol in various molar ratios (1:6, 1:9, and 1:12). KOH was incorporated to the preheated oil, after being dissolved in methanol and heated to 30°C.

After varying reaction times (30, 60, and 120 min), the mixture was cooled to allow phase separation between the trans-esterified product and the glycerol by-product; then, to remove the latter, a separatory funnel was employed. An additional washing step, using water at 30°C, was performed to eliminate excessive soap. GC-MS analysis was finally carried out to confirm that methyl esters had formed in the final product.
To analyse the composition of the esterified product, an ISQ 7000 single quadrupole GC-MS system (Thermo Scientific™, Waltham, MA, USA), equipped with a capillary column (HP Innowax, 30 m × 0.25 mm (I.D) × 0.25 µm), was employed, using helium as carrier gas.
An approximative volume of 1 µL of the solution was injected into the column, with an injection temperature was 260°C; the oven was initially set at a temperature of 40°C, which was then progressively increased to 195°C at a rate of 80 °C/min. Finally, it was raised to 225°C at a rate of 5°C/min and held for 60 min. Before being analysed, samples were then diluted 1:1000 with hexane.
Subsequently, after the methyl-ester formation was confirmed, the sulfonation reaction was carried out by adding 1% (*w*/*v*) $NaHSO_3$ to the esterified product. The reaction was conducted at 80°C for 2 h under stirring (8000 rpm). Finally, the correct formation of sulfonated methyl esters was tested using FTIR spectroscopy. The chemical structure of the methyl ester sulphonate (MES) detergent was characterized through Attenuated Total Reflectance (ATR) Fourier Transform Infrared (FTIR) spectroscopy, utilizing the Thermo Scientific™ Nicolet™ iS50 spectrometer (Thermo Scientific™, Waltham, MA, USA). To enhance signal quality and resolution, up to 128 scans of spectral data were obtained within the 4000–400 cm−1 range.
The ATR configuration enabled for direct analysis of the liquid sample, offering an effective method to identify the functional groups included in the eco-friendly detergent.
The formulated detergent was then utilized for nanofluid preparation and successive testing.

7. Isolation of bacterial strains from environmental sources and identification of antibiotic-resistance genes

Several strains of *E. coli* and *A. baumannii* were isolated from wastewater sources, sampled in different cities in Romania. Other *E. coli* strains were isolated instead from urine cultures of residents of an elderly care facility. The isolation method consisted in filtering the wastewater samples, using nitrocellulose filters with pores having a diameter of 0.45 µm. The filters were then inoculated on selective culture media, to detect the different bacteria contained in each water sample. On the other hand, the remaining *E. coli* strains were isolated from urine samples inoculated on CLED (Cysteine Lactose Electrolyte Deficient) agar. All the different strains were then identified through MALDI-ToF spectrometry.

Successively, after DNA extraction, the whole genomes of part of the bacterial strains isolated from wastewater samples were sequenced, in order to detect genes conferring them antibiotic-resistance profiles. Specifically, DNA was isolated via a DNeasy UltraClean Microbial Kit (Qiagen, Germany). Library preparation was performed via the Nextera DNA Flex Library Prep Kit, followed by sequencing on the Illumina MiSeq platform (V3, 600 cycles). The raw reads were assembled via the Shovill v1.1.0 pipeline, and antibiotic resistance genes were annotated via the ABRicate tool, with NCBI database. Molecular typing, to assess the genetic lineage of each strain, was performed using the MLST (Multi-Locus Sequence Typing) tool.
For *E. coli* strains isolated from urine samples, some genes conferring antibiotic-resistance were identified, after DNA extraction as previously described, through polymerase-chain reaction (PCR). Accordingly, the strains were subjected to antibiotic-susceptibility testing, which consisted in putting an antibiotic-soaked disc in a Petri on which every tested strain was grown separately. After a 24 h incubation at 37°C, the diameter of the inhibition zone around the antibiotic disc was measured and, according to this value, each strain was assessed to be susceptible or resistant to the antibiotics tested.

8. Detergent antimicrobial efficacy testing

E. coli cells (10^7 cells/mL) were put on Petri dishes and subjected to washing treatments, to assess the antimicrobial efficacy of the methyl-ester sulphonate detergent. The bacterial suspension was positioned on the surface of the Petri dish with spots of 100 µL each. The washing procedure consisted of three consecutive rinses with either the detergent solution or distilled water, each followed by gentle agitation to guarantee proper contact. Afterwards, the residual material (respectively before and after the treatment) was collected with swabs, and the bacterial load was finally measured by direct cell counting and by DNA quantification, as previously described.

To further test the efficacy of the detergent, the washing tests were performed after contaminating sterile steel rings with bacterial suspensions, washed then either with water (as a control) or the detergent, in a similar way to the washing procedures on the steel pans performed with the thermo-disinfector bedpan washer.

- Bacterial suspensions and detergent preparation

Bacterial suspensions were prepared with a concentration similar to 0,5 McFarland (correspondent to 10^8 cells/mL) and then diluted 1:10 with physiological water. These suspensions were finally diluted 1:1 with the RAMS solution.
The detergent was diluted with PBS to a final concentration of 10%.

- **Washing procedures**

The steel rings were contaminated putting one spot of the bacterial suspension mixed with RAMS on each, with a concentration of $0.5x10^7$ cells/mL. After the spots got dried, half of the steel rings were positioned on Petri dishes containing CHROMagar^{TM} Orientation medium (Kanto Chemical Co., Tokyo, Japan), according to the following scheme:

- *Unbrushed:* steel rings not cleaned
- *H_2O brush:* steel rings brushed with a swab soaked in sterile water
- *Detergent brush:* steel rings brushed with a swab soaked with the detergent
- *Mixing wash - H_2O:* steel rings left for 40 minutes to wash in a rotating tube, containing sterile water
- *Mixing wash – detergent:* steel rings left for 40 minutes to wash in a rotating tube, containing the methyl-ester sulphonate detergent

The rings were removed after 30 minutes, and then the plates were kept for 24 hours in the incubator at 37°C. The colonies which were grown in each condition were then counted.

The other steel rings were treated with the same procedure as before, but they were successively putted in a tube containing tryptic soy broth (TSB) (Sigma-Aldrich Corporation, St. Louis, MO, USA), and incubated for 24 hours at 37°C. This was performed for a qualitative evaluation, checking bacterial growth according to the turbidity of the medium.

9. Nanofluid formulation

Two types of magnetic nanoparticles, in particular biomimetic magnetic nanoparticles (BMNPs) and magnetic nanoparticles (MNPs), were synthesized and conjugated with silver. The addition of silver was performed to provide the formulated eco-friendly detergent with antimicrobial activity, in order to obtain a nanofluid comprehensive of both detergent and bactericidal properties.

MNPs and MamC-mediated BMNPs used in this work were synthesized according to Jabalera et al. [237]. Briefly, MamC was cloned, expressed and purified according to Peigneux et al. [238], [239]. MamC was purified under denaturing conditions and subsequently dialyzed to eliminate urea, to enable the proper refolding of the protein. To avoid possible oxidation, magnetite precipitation, either in the presence of MamC (BMNPs) or in the absence of any protein (MNPs), was carried out for 1 month in a closed system at 25°C and 1 atm of total pressure inside an anaerobic chamber (COY chamber). A permanent magnet was used to collect the precipitates, which were then washed with deoxygenated water. Nanoassemblies of Ag electrostatically bound to BMNPs (Ag-BMNPs) or to MNPs (Ag-MNPs) were obtained by exploiting the negative charge of both nanoparticles when resuspended in HEPES buffer (pH = 7.4); their isoelectric point (pI) was, in fact, 4.4 for BMNPs, and

7 for MNPs [240]. To immobilize the silver ions on both surfaces, Ag_2SO_4 solution containing 4 mg of Ag_2SO_4 in HEPES buffer was combined with 5 mg of BMNPs or MNPs, following resuspension and disaggregation in HEPES buffer (50 mM, pH 7.4). Through electrostatic affinity, the positively charged silver ions interacted with both kinds of nanoparticles. The nanoassemblies were recovered using a permanent magnet after being rotated for 24 hours at room temperature.

The nanoassemblies underwent to three washing cycles with the same HEPES buffer; afterwards, their magnetic recovery was performed, and the nanoassemblies were used for the further experiments. The bond between the silver ions and the nanoparticles was validated by comparing the ζ-potential of Ag-BMNPs, BMNPs, Ag-MNPs and MNPs at pH 7.4 through dynamic light scattering (DLS), using the Nano ZetaSizer ZS ZEN3600 (Malvern Instruments, Malvern, Worcestershire, UK) [241]. The size was further evaluated for both naked and silver-coupled particles utilizing nanoparticle tracking analysis (NTA) with a NanoSight NS300 (Malvern Panalytical, Malvern, UK). To this aim, before analysis samples were sonicated for 5 min with a bath sonicator and then diluted 50-fold in Milli-Q water. Three 60 seconds runs (1498 frames total) were performed at camera level 11 and a detection threshold of 3–5. The Stokes–Einstein equation [242] was employed to automatically calculate the hydrodynamic diameters and particle concentrations (particles/mL) from the Brownian motion of the particles, as reported below:

$$R_h = \frac{k_B T}{6\pi\eta D}$$

being R_h the hydrodynamic radius (m), k_B the Boltzmann Constant (≈1.38×10^{-23} J/K), T the temperature (K), η the solvent viscosity (kg/m s), and D the diffusion coefficient (m^2/s).

10. Antibacterial activity of the methyl-ester sulphonate detergent and nanofluid compounds

Thirty strains of *E. coli* and thirty strains of *A. baumannii*, selected from the ones isolated from the elderly-care facility residents or wastewater sources previously mentioned, were used to test the methyl-ester sulphonate detergent and the silver nanoparticles, separately or in combination with each other. Six type-strain microorganisms' samples, among the most frequently involved in HCAIs, were tested for their susceptibility to the detergent and the nanoparticles, too. Specifically, we analysed five pathogenic bacteria (*Enterococcus faecalis*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Enterobacter aerogenes*, *Klebsiella pneumoniae*) and one fungus (*Candida albicans*).

The detergent was diluted with PBS to a final 10% concentration. Ag-BMNPs and Ag-MNPs, at initial concentration of 5 mg/mL, were firstly washed with PBS; then, they were diluted 1:10 with either PBS or with the 10% concentrated detergent. The effects of the detergent and the nanoparticles were

evaluated by means of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) tests. MIC_{50} and MIC_{90} represent the minimum inhibitory concentrations of a determined substance required to inhibit the growth of 50% and 90% of the tested bacterial strains, respectively. Similarly, MBC_{50} and MBC_{90} indicate the minimum bactericidal concentrations required to kill 50% and 90% of the strains. The bacterial suspensions were diluted 1:10 with Mueller-Hinton broth (MHB; Thermo Scientific™ Oxoid™, Waltham, MA, USA; catalog No. CM0405B) after being adjusted to a density of 0.5 McFarland (corresponding to 10^8 cells/mL). MIC test was performed in 96-well plates, putting in each well 75 μL of MHB mixed, in the first column of each plate, with 75 μL of either the 10% detergent, the nanoparticles diluted in PBS, or the nanoparticles diluted with the detergent. Then, 1:2 serial dilutions of these mixes were performed in the plates. After that, 75 μL of the bacterial dilution was applied to each well. After a 24-hour incubation period at 37°C, the MIC was determined by examining the growth of bacteria in each well.

Moreover, after the incubation, by plating on agar Petri dishes 5 μL of the content of each well of the MIC test plates in which bacterial growth was not observed, MBC was performed; the first well in which there was evidence of growth for each bacterial strain was also tested. MBC was then measured after 24 h of incubation at 37°C.

11. Wheelchair cover

EASYLINKED S.r.l. (20122 Milan, Italy) provided the SAFE-HUG PRO Wheelchair Cover. For this investigation, a commercial version of the cover was used, which has been tested and certified according to EN 14126:2004 and ISO 22610:2006 [31], to evaluate the resistance of protective textiles to bacterial penetration under moist and mechanical conditions.

A controlled in vitro test was carried out using *E. coli* as the test organism, incubating it at 37°C under growth-promoting conditions, aiming at simulating a worst-case scenario for bacterial proliferation, in order to evaluate its barrier efficiency against microbial penetration.

A sample of the wheelchair cover was placed in direct contact with a total of 5×10^7 cells of *E. coli* on a sterile Petri dish.

To determine if any microorganisms had penetrated the tested barrier, the side of the cover that had not come in direct contact with the bacteria was examined after one and 24 hours of incubation, respectively.

The three complementary methods previously described were then employed to determine bacterial penetration: cell counts to assess viable bacterial cells, Optical Density (OD600) measurements to estimate bacterial presence, and DNA extraction to detect and quantify the bacterial genetic material.

12. Statistical analysis

Student's *t*-test was used to analyse the data and to compare the differences between the control and experimental groups. The *t*-test was applied to determine whether samples before and after thermal disinfection showed significant differences in the measured values, such as absorbance or bacterial concentration. SPSS for Windows, version 22.0 (SPSS Inc., Chicago, IL, USA, 2013) was employed for all statistical analyses. A p-value < 0.05 was considered statistically significant. The results are presented as the mean ± standard deviation (SD) of at least three independent experiments.

RESULTS

1. Efficacy of SMEG BPW1260 bedpan washer-disinfector in reducing bacterial contamination

To assess the efficacy of the washer-disinfector (*Figure 1A*) in reducing bacterial load on bedpans, we initially contaminated them with specific spots composed by a mixture of RAMS and bacterial suspension. Specifically, the bacterial/RAMS mixture was applied on eight spots along the border, and two spots in the centre of each bedpan (*Figure 1B*); the bedpans were then dried at 37°C for 30 minutes. After drying, five of the ten spots corresponding to the "Pre" value (A: before disinfector cycles) were collected, representing the baseline samples (i.e., the level of contamination prior to treatment). The remaining five spots were recovered after thermal disinfection with SMEG Bedpan Washer. To evaluate *Clostridium difficile* contamination reduction after washing, we employed the Colony Forming Unit (CFU/mL) counting method, along with spectrophotometry, direct cell counting, and DNA quantification. The evaluations were performed at least three times, treating two different pans simultaneously. Using the $A_0$6000 program of the thermo-disinfector, we did not observe any colony growth of *Clostridium difficile*. Therefore, we further investigated whether thermal disinfection could also be effective with the $A_0$600 program, taking advantage of a lower energy consume. As shown in *Figure 1C*, also with this lower-energy program, we did not detect the growth of any *Clostridium difficile* colony after thermal disinfection.

Figure 1. *(A) SMEG BPW1260 bedpan washer-disinfector.*

(B) The bedpan image showing 5 of the 10 spots corresponding to the Pre value ((A): before disinfector cycles) and the remaining 5 spots corresponding to the Post value ((B): after thermal disinfection).

(C) CFU/mL before (PRE) and after (POST)

Thus, we evaluated the ability of the bedpan washer to reduce the contamination level of *C. difficile*, as well as *E. coli*, through spectrophotometry, direct cell counting, and DNA quantification after thermal disinfection. The absorbance (OD600 nm) of *C. difficile* and *E. coli* suspensions was measured using a spectrophotometer in the samples before and after thermal disinfection, with each bacterial strain analysed separately. Besides absorbance measurements, bacterial cells counts were performed using a Bürker counting chamber to determine the bacterial concentration in the samples. As shown in *Figure 2*, we observed a significant reduction in absorbance (*Figure 2A, 2B*) and a decrease in bacterial cells count (*Figure 2C, 2D*) after thermal disinfection for both bacterial strains.

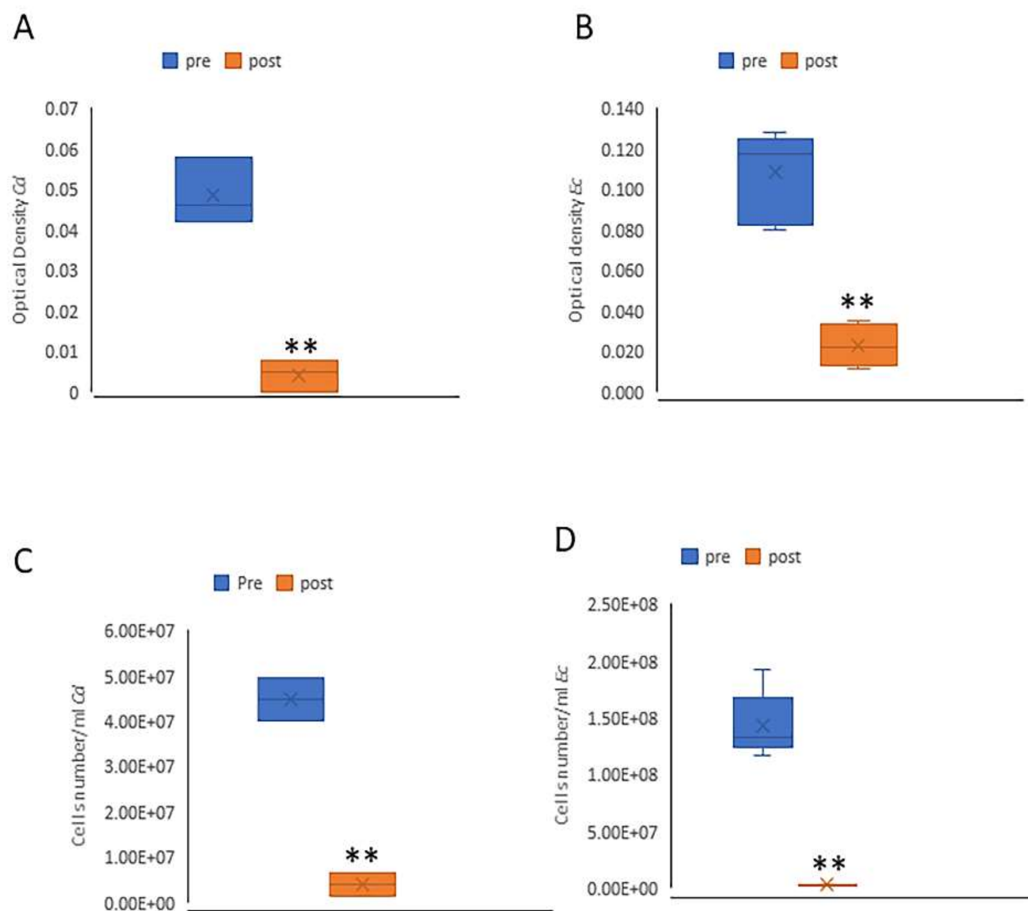


Figure 2. *Graph shows the absorbance values before (blue) and after (orange) thermal disinfection for C. difficile (A) and E. coli (B). This decrease in bacterial population was also confirmed by the bacterial cells counts obtained through the counting chamber method for C. difficile (C) and E. coli (D). ** ($p < 0.005$).*

The amount of DNA present in the bacterial samples was quantified by measuring the optical density (OD) at 260 nm, which is commonly used to determine nucleic acid concentration, with a Qubit spectrophotometer. The results were expressed as DNA concentration (ng/µL). Measurements were taken both before and after thermal disinfection to determine any potential reduction in DNA levels

through the washing treatment. A significant reduction in DNA levels was observed following thermal disinfection for both *C. difficile* and *E. coli* strains (*Figure 3*), indicating that the treatment effectively compromised the bacterial DNA. These results are consistent with the 600 nm absorbance and bacterial cells count data, collectively providing strong evidence of the effectiveness of thermal disinfection in reducing bacterial viability and genetic material.

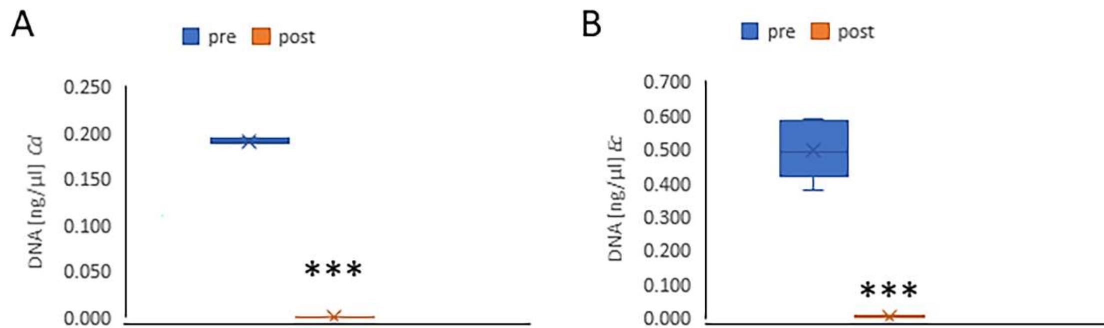


Figure 3. *The graph shows the concentration of extracted DNA before (blue) and after (orange) thermal disinfection. The reduction in DNA levels following thermal disinfection was statistically significant for both Clostridium difficile (A) and Escherichia coli (B). *** p<0.0005.*

2. Energy efficiency and bacterial load reduction

The results obtained with the $A_0$6000 and $A_0$600 thermo-disinfection programs, as previously reported, both demonstrated a significant decrease in bacterial load, highlighting the effectiveness of both parameters in the disinfection process. As shown in the table below (*Table 1*), the $A_0$600 treatment resulted in a considerable improvement in energy efficiency compared to $A_0$6000. Besides reaching effective disinfection level, the $A_0$600 cycle consumed only 0.42 kWh of energy, compared to the 0.77 kWh required by the $A_0$6000 cycle: this means a reduction of 45% in energy consumption, which leads, in turn, to a shorter cycle time, specifically 28 min instead of 35 min. This improvement in energy efficiency points out the better performance of the $A_0$600 treatment without compromising disinfection effectiveness.

Table 1. *Steam condensation, cooling, and energy consumption analysis.*

			Steam condensation and cooling	*400 V three-phase*	
SHORT NAME	**ID PROG**	**A_0**	**T target [°C]**	**ENERGY (KWh)**	**CYCLE TIME (min)**
Pr 08	708	6000	55	0,77	35
Pr 08	708	600	55	0,42	28

3. Synthesis of the methyl esters

The chemical process carried out in this experimental phase consisted in the conversion of waste sunflower cooking oil into a biodegradable detergent through a two-step reaction: transesterification followed by sulfonation. This approach is noteworthy not only for its alignment with the increasing demand for environmentally friendly cleaning agents, but also for its sustainable utilization of waste products. During transesterification, triglycerides derived from sunflower waste cooking oil (SWCO) react with methanol catalysed by a potassium hydroxide, yielding fatty acid methyl esters and, as a by-product, glycerol [243]. Sulfonation is a chemical reaction that adds a sulfonic acid group ($–SO_3H$) to an organic compound, typically through reagents such as sulfur trioxide (SO_3) or oleum, which is a mixture of sulfur trioxide and sulfuric acid [244]. In this study, sodium bisulfite ($NaHSO_3$) was employed as the sulfonating agent due to its milder reactivity, which allows for greater control over the reaction and reduces the risk of over-sulfonation or unwanted side reactions. For this reason, it results particularly suitable for modifying sensitive organic compounds such as fatty acids. The sulfonation process increases the polarity and water solubility of the resulting molecules, making them particularly suitable for applications in detergents and surfactants. The transesterification process allowed for the formation of two phases, separated in the reaction vessel: one containing the esterified compounds and the other carrying the non-useful mixture (*Figure 4*).

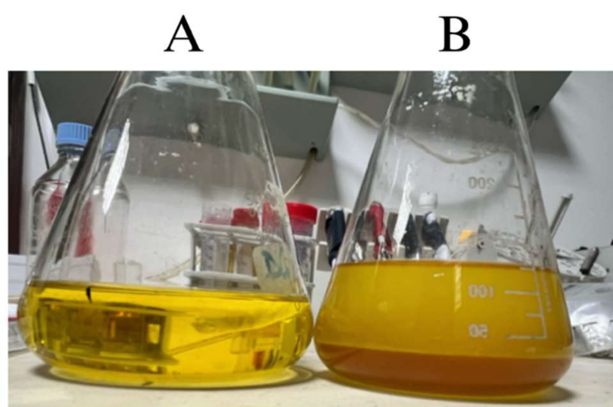


Figure 4. *Waste cooking oil pre- (A) and post-transesterification (B), where phase separation between esters (yellow) and glycerol (brown) was observed.*

The filtered waste cooking oil did not exhibit ester groups, as shown in *Figure 5*. Several volatile and semi-volatile compounds, typical of thermo-oxidative degradation products, formed during repeated use of sunflower oil for cooking are shown in the GC-MS chromatogram: in particular, peak “a" corresponds to 2-Propanone, methylhydrazone, a derivative of acetone — a common secondary product of lipid peroxidation and β-scission of oxidized unsaturated fatty acids; peaks “b” (1-methyl-2-propenylhydrazine) and “c” (Azetidine) are small nitrogen-containing heterocycles commonly formed by reactions between reactive carbonyl compounds (e.g., aldehydes and ketones), generated during triglyceride breakdown, and amino compounds, deriving from food materials such as batters,

meat, or vegetable proteins. As a result, their presence is indicative of used, rather than fresh, cooking oil. Peak "d" is identified as 2-Ethylthiirane, a sulfur-containing volatile compound likely produced through thermal degradation of sulphur amino acids (such as cysteine or methionine), frequently found in fried foods; lasty, peak "e", namely Ethyl tetracosanoate, is a long-chain waxy fatty acid ethyl ester (C_{24}), formed through fatty acid esterification or ester–exchange reactions involving high-molecular-weight components under prolonged heating conditions [245], [246].

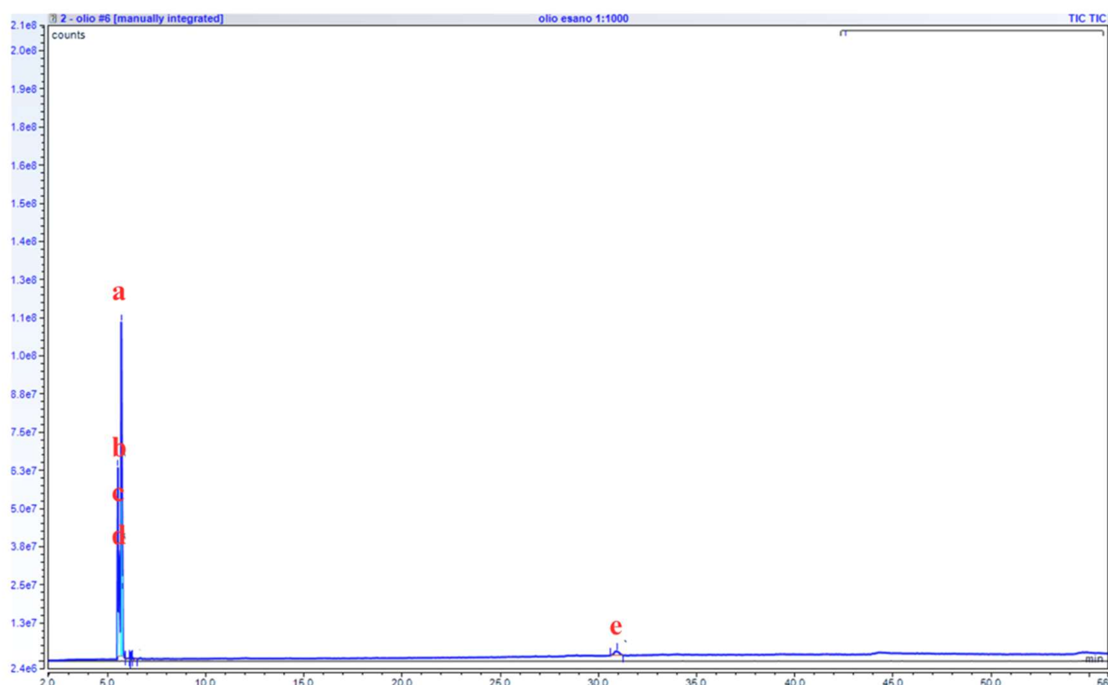


Figure 5. GC-MS chromatogram of waste cooking oil before being subjected to the transesterification process. (2-Propanone, methylhydrazone (a), 1-Methyl-2-propenylhydrazine (b), Azetidine (c), 2-Ethylthiirane (d), Ethyl tetracosanoate (e)).

The reaction performed at 60°C for 30 minutes with a methanol-to-oil molar ratio of 1:9 demonstrated the best phase separation between the ester products and the glycerol by-product, out of all the trials carried out. When the basic catalyst was raised to 1.3% w/w, this indicated full transesterification.
These results are comparable to those published by Resi Levi Permadani et al. [236].
The chromatogram in *Figure 6* illustrates the abundance of methyl esters, with 9,12-Octadecadienoic acid (Z,Z)-methyl ester as the major component (63.2%), and 28.2% of 9-octadecenoic acid (Z)-methyl ester. The complete methyl ester composition is provided in *Table 2*.

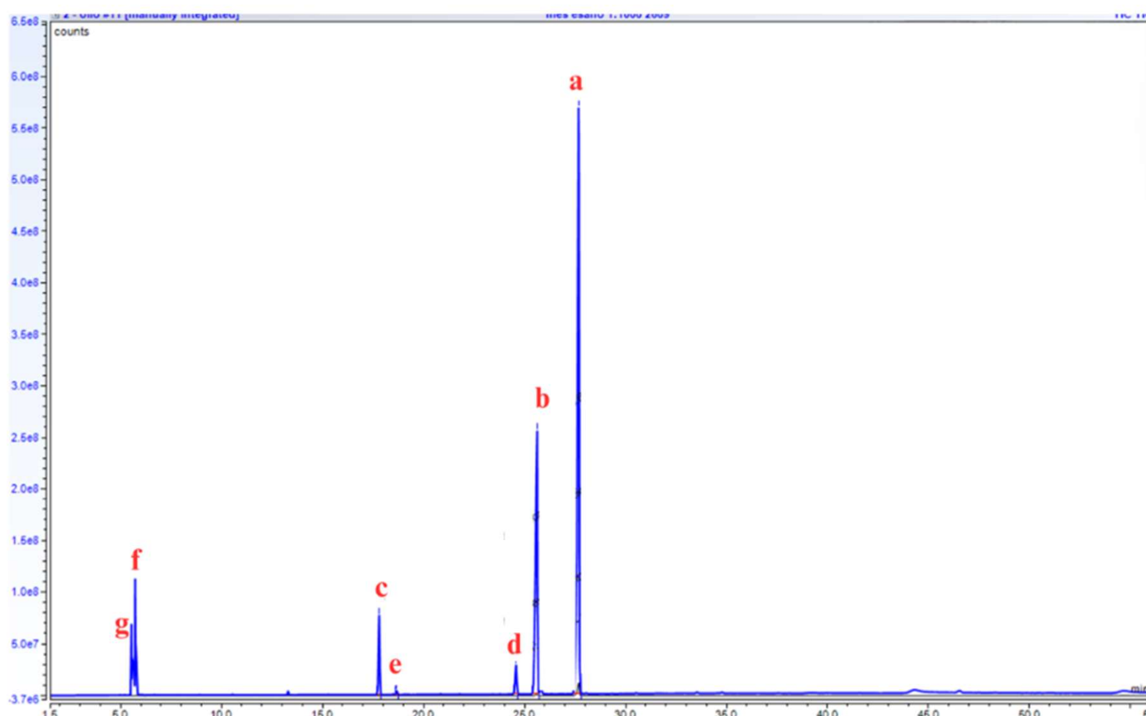


Figure 6. GC-MS chromatogram of waste cooking oil after the transesterification reaction, showing the detection of 5 different methyl ester compounds (9,12-Octadecadienoic acid (Z,Z)-methyl ester (a), 9-octadecenoic acid (Z)-methyl ester (b), hexadecanoic acid methyl ester (c), methyl stearate (d), 9-hexadecenoic acid methyl ester (e)), and the presence of residual 2-propanone, methylhydrazone (f), and azetidine (g).

Methyl ester composition	Percentage (%)
9,12-octadecadienoic acid (Z, Z)	63.2
9-octadecenoic acid (Z)	28.2
Hexadecanoic acid	5.7
Methyl stearate	2.7
9-hexadecenoic acid (Z)	0.2

Table 2. Methyl ester composition after transesterification, calculated from peak areas in the GC-MS chromatogram.

4. Methyl Ester Sulfonation

The product after the sulfonation chemical reaction was analysed by FTIR, to determine its composition (*Figure 7*). Two peaks at 2853 and 2924 cm^{-1} identify the aliphatic groups of the fatty acid chains. The presence of a strong peak at 1740 cm^{-1} indicates the carbonyl (C=O) group [236], [247], [248].

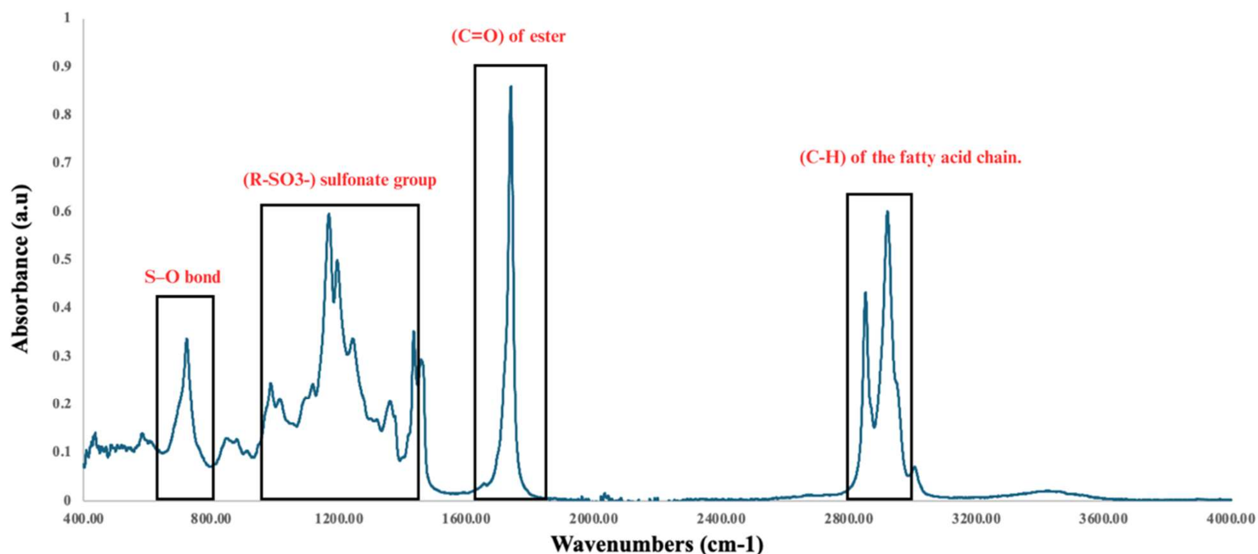


Figure 7. FTIR spectrum of the methyl ester sulfonate showing both sulfonate groups (R-SO3−) and ester (C=O) groups, as well as sulfonic (S-O) and fatty acid chain (C-H).

The absorption bands in the 1015–1361 cm^{-1} region are typical of the stretching vibrations of the sulfonate groups ($R\text{-}SO_3^-$) [236], [248]. Moreover, no characteristic band was observed around 3500 cm^{-1}, indicating the absence of alcoholic or water residues.

Therefore, according to the FTIR spectrum, the methyl esters resulted to be sulfonated, confirming the successful preparation of a transparent MES-based detergent (*Figure 8*).

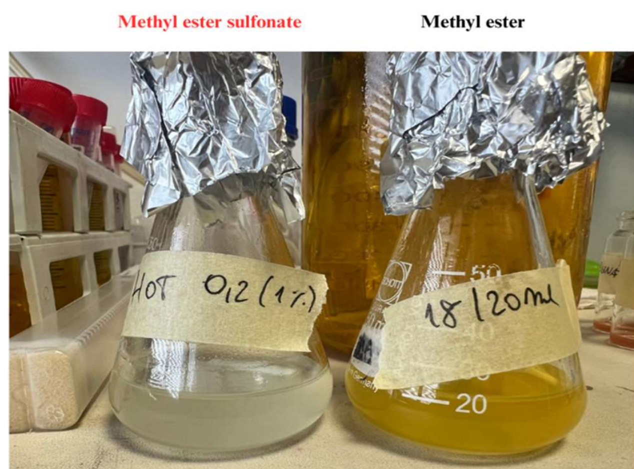


Figure 8. The transparent product indicates the methyl ester sulfonate (left flask), while the methyl ester before sulfonation appears yellow (right flask).

5. **Characterization of bacterial strains isolated from environmental sources**

The bacterial strains identified through MALDI-ToF spectrometry, from the samples isolated either from wastewater sources or the urine samples of an elderly-care facility residents, were further subjected to DNA extraction.

The *Table 3* reports the results of the PCR, aimed at identifying the antibiotic-resistance genes, and the results of the antibiotic-susceptibility tests performed on the strains isolated from urine samples (encoded with "URO" prefix).

Sample	**AR gene**	**Target antibiotic**	**Other resistances**
URO 1	TEM	Cephalosporins, penicillins	Amoxicillin/clavulanic acid, cefepim, tobramycin
URO 2	/	/	Amoxicillin/clavulanic acid, ciprofloxacin
URO 3	CTXM	Cephalosporins	Amoxicillin/clavulanic acid, ciprofloxacin
URO 4	/	/	Amoxicillin/clavulanic acid, cefepim, tobramycin, amikacyn, ciprofloxacyn
URO 5	SHV	Cephalosporins, penicillins	/
URO 6	OXA 48	Penicillins, carbapenems	Ceftadizim, cefepim
URO 7	TEM	Cephalosporins, penicillins	/
URO 8	SHV	Cephalosporins, penicillins	Amoxicillin/clavulanic acid, cefepim, tobramycin

Table 3. *Antibiotic-resistance genes (AR genes), identified through PCR, along with their respective antibiotic targets for URO samples. The other resistances refer to the antibiotics against which the strains resulted resistant after the antibiotic-susceptibility tests.*

Conversely, the genomes of part of the strains isolated from wastewater samples were sequenced, to detect antibiotic-resistance genes. The ones selected for the successive experiments, in particular 22 *E. coli* and 30 *A. baumannii* strains, are listed in the tables below, with their associated sequence types and antibiotic-resistance genes.

Sample	AR genes	Target antibiotic	ST
19040 CE1	aac(6')-Ib_1	AMIKACIN/TOBRAMYCIN_1	635
	aph(3')-VIa	AMIKACIN/KANAMYCIN_1	635
	blaEC-15	BETA-LACTAM_1	635
	blaKPC-2	CARBAPENEM_1	635
	blaOXA-16	CEFEPIME/CEFIXIME/CEFOTAXIME/CEFTAZIDIME/PIPERACILLIN_1	635
19040 OE1	aadA2	SPECTINOMYCIN/STREPTOMYCIN_1	10
	aph(3'')-Ib	STREPTOMYCIN_1	10
	blaEC	BETA-LACTAM_1	10
	blaOXA-48	BETA-LACTAM_1	10
	blaTEM-1	BETA-LACTAM_1	10
	dfrA12	TRIMETHOPRIM_1	10
	floR	CHLORAMPHENICOL/FLORFENICOL_1	10
	merC	ORGANOMERCURY_1	10
	sul2	SULFONAMIDE_1	10
19042 CE4	aadA2	SPECTINOMYCIN/STREPTOMYCIN_1	410
	blaDHA-1	CEPHALOSPORIN_1	410
	blaEC-15	BETA-LACTAM_1	410
	blaNDM-5	CARBAPENEM_1	410
	blaTEM-1	BETA-LACTAM_1	410
	ble-MBL	BLEOMYCIN_1	410
	dfrA12	TRIMETHOPRIM_1	410
	mph(A)	MACROLIDE_1	410
	qnrB4	QUINOLONE_1	410
	rmtB1	AMINOGLYCOSIDE_1	410
	sul1	SULFONAMIDE_1	410
	sul2	SULFONAMIDE_1	410
	tet(A)	TETRACYCLINE_1	410
19044 CE5	aadA2	SPECTINOMYCIN/STREPTOMYCIN_1	410
	blaDHA-1	CEPHALOSPORIN_1	410
	blaEC-15	BETA-LACTAM_1	410
	blaNDM-5	CARBAPENEM_1	410
	blaTEM-1	BETA-LACTAM_1	410
	ble-MBL	BLEOMYCIN_1	410
	dfrA12	TRIMETHOPRIM_1	410
	mph(A)	MACROLIDE_1	410
	qnrB4	QUINOLONE_1	410
	rmtB1	AMINOGLYCOSIDE_1	410
	sul1	SULFONAMIDE_1	410
	sul2	SULFONAMIDE_1	410
	tet(A)	TETRACYCLINE_1	410
19044 OE5	aac(6')-Ib_1	AMIKACIN/TOBRAMYCIN_1	258
	aph(3')-Ia	KANAMYCIN_1	258
	blaKPC-2	CARBAPENEM_1	258
	blaOXA-9	BETA-LACTAM_1	258
	blaTEM-1	BETA-LACTAM_1	258
	emrD	EFFLUX_1	258
19048 OE3	aadA1	STREPTOMYCIN_1	940
	blaEC-18	BETA-LACTAM_1	940
	blaOXA-48	BETA-LACTAM_1	940
	dfrA1	TRIMETHOPRIM_1	940
	sat2_gen	STREPTOTHRICIN_1	940
	tet(B)	TETRACYCLINE_1	940
19049 EE3	blaCTX-M-15	CEPHALOSPORIN_1	278
	blaEC-13	BETA-LACTAM_1	278
19051 EE1	blaCTX-M-15	CEPHALOSPORIN_1	2332
	blaEC-15	BETA-LACTAM_1	2332
	blaTEM-1	BETA-LACTAM_1	2332
	dfrA7	TRIMETHOPRIM_1	2332
	merC	ORGANOMERCURY_1	2332

Table 4.

For 22 E. coli strains, the antibiotic-resistance genes (AR genes), identified after sequencing, along with their respective antibiotic targets are reported.

The sequence type (ST), detected with MLST, is also associated to each strain.

Sample	AR genes	Target antibiotic	ST
19074 CE5	aadA2	SPECTINOMYCIN/STREPTOMYCIN_1	405
	blaEC-8	BETA-LACTAM_1	405
	blaNDM-5	CARBAPENEM_1	405
	blaTEM-1	BETA-LACTAM_1	405
	ble-MBL	BLEOMYCIN_1	405
	dfrA12	TRIMETHOPRIM_1	405
	qepA4	QUINOLONE_1	405
	tet(B)	TETRACYCLINE_1	405
19074 OE5	aadA1	STREPTOMYCIN_1	38
	blaCTX-M-14	CEPHALOSPORIN_1	38
	blaEC-8	BETA-LACTAM_1	38
	blaOXA-48	BETA-LACTAM_1	38
	blaTEM-32	BETA-LACTAM_1	38
	dfrA1	TRIMETHOPRIM_1	38
	sat2_gen	STREPTOTHRICIN_1	38
20014 OE2	blaEC	BETA-LACTAM_1	10
	blaOXA-48	BETA-LACTAM_1	10
20015 OE1	blaEC	BETA-LACTAM_1	10
	blaOXA-48	BETA-LACTAM_1	10
20025 CE1	aadA2	SPECTINOMYCIN/STREPTOMYCIN_1	1882
	blaEC-13	BETA-LACTAM_1	1882
	blaNDM-5	CARBAPENEM_1	1882
	blaTEM-1	BETA-LACTAM_1	1882
	ble-MBL	BLEOMYCIN_1	1882
	dfrA12	TRIMETHOPRIM_1	1882
	erm(B)	MACROLIDE_1	1882
	mph(A)	MACROLIDE_1	1882
	rmtB1	AMINOGLYCOSIDE_1	1882
20025 CE6	blaEC-15	BETA-LACTAM_1	607
	blaKPC-2	CARBAPENEM_1	607
20026 OE1	aph(3")-Ib	STREPTOMYCIN_1	2562
	blaEC-8	BETA-LACTAM_1	2562
	blaOXA-48	BETA-LACTAM_1	2562
	blaTEM-1	BETA-LACTAM_1	2562
	mph(A)	MACROLIDE_1	2562
	tet(B)	TETRACYCLINE_1	2562
20074 CE1	blaEC-15	BETA-LACTAM_1	167
	blaKPC-2	CARBAPENEM_1	167
	blaOXA-9	BETA-LACTAM_1	167
	catA1	CHLORAMPHENICOL_1	167
	dfrA12	TRIMETHOPRIM_1	167
	dfrA5	TRIMETHOPRIM_1	167
	floR	CHLORAMPHENICOL/FLORFENICOL_1	167
	fosA4	FOSFOMYCIN_1	167
	sul2	SULFONAMIDE_1	167
20074 CE6	aadA2	SPECTINOMYCIN/STREPTOMYCIN_1	167
	blaEC-15	BETA-LACTAM_1	167
	blaKPC-2	CARBAPENEM_1	167
	blaOXA-9	BETA-LACTAM_1	167
	catA1	CHLORAMPHENICOL_1	167
	dfrA12	TRIMETHOPRIM_1	167
	dfrA5	TRIMETHOPRIM_1	167
	floR	CHLORAMPHENICOL/FLORFENICOL_1	167
	fosA4	FOSFOMYCIN_1	167
	sul2	SULFONAMIDE_1	167
19029OE1	-	-	-
19034EE1	-	-	-
20010CE2	-	-	-
19052EE2	-	-	-
19078OE3	-	-	-

Sample	AR genes	Target antibiotic	ST
19003 CNE1	abaF	FOSFOMYCIN_1	2
	ant(3'')-IIa	STREPTOMYCIN/SPECTINOMYCIN_1	2
	aph(3'')-Ib	STREPTOMYCIN_1	2
	aph(6)-Id	STREPTOMYCIN_1	2
	armA	GENTAMICIN_1	2
	blaADC-73	CEPHALOSPORIN_1	2
	blaOXA-23	BETA-LACTAM_1	2
	blaOXA-66	BETA-LACTAM_1	2
	catA1	CHLORAMPHENICOL_1	2
	mph(E)	MACROLIDE_1	2
	msr(E)	MACROLIDE_1	2
	tet(B)	TETRACYCLINE_1	2
20084 CNE2	aac(3)-Ia	GENTAMICIN_1	2
	aacA16	AMINOGLYCOSIDE_1	2
	abaF	FOSFOMYCIN_1	2
	ant(3'')-IIa	STREPTOMYCIN/SPECTINOMYCIN_1	2
	aph(3')-Ia	KANAMYCIN_1	2
	aph(6)-Id	STREPTOMYCIN_1	2
	blaADC-30	CEPHALOSPORIN_1	2
	blaOXA-66	BETA-LACTAM_1	2
	blaOXA-72	BETA-LACTAM_1	2
	blaTEM-1	BETA-LACTAM_1	2
	sul2	SULFONAMIDE_1	2
	tet(B)	TETRACYCLINE_1	2
20085 CNE5	aac(3)-Ia	GENTAMICIN_1	2
	aadA1	STREPTOMYCIN_1	2
	abaF	FOSFOMYCIN_1	2
	ant(3'')-IIa	STREPTOMYCIN/SPECTINOMYCIN_1	2
	aph(3')-VIb	AMIKACIN/KANAMYCIN_1	2
	blaADC-11	CEPHALOSPORIN_1	2
	blaOXA-66	BETA-LACTAM_1	2
	blaOXA-72	BETA-LACTAM_1	2
	blaPER-1	CEPHALOSPORIN_1	2
22002-CA5	abaF	FOSFOMYCIN_1	578
	ant(3'')-IIa	STREPTOMYCIN/SPECTINOMYCIN_1	578
	blaADC-99	CEPHALOSPORIN_1	578
	blaOXA-65	BETA-LACTAM_1	578
22003-CNE3	abaF	FOSFOMYCIN_1	2
	ant(3'')-IIa	STREPTOMYCIN/SPECTINOMYCIN_1	2
	aph(3'')-Ib	STREPTOMYCIN_1	2
	aph(6)-Id	STREPTOMYCIN_1	2
	blaADC-30	CEPHALOSPORIN_1	2
	blaOXA-66	BETA-LACTAM_1	2
	blaOXA-72	BETA-LACTAM_1	2
	sul2	SULFONAMIDE_1	2
	tet(B)	TETRACYCLINE_1	2
22003-ENE2	aac(3)-IIe	GENTAMICIN_1	79
	abaF	FOSFOMYCIN_1	79
	ant(3'')-IIa	STREPTOMYCIN/SPECTINOMYCIN_1	79
	aph(3')-VIa	AMIKACIN/KANAMYCIN_1	79
	aph(6)-Id	STREPTOMYCIN_1	79
	blaADC-5	CEPHALOSPORIN_1	79
	blaOXA-23	BETA-LACTAM_1	79
	blaOXA-65	BETA-LACTAM_1	79
	blaTEM-1	BETA-LACTAM_1	79
	sul2	SULFONAMIDE_1	79
22005-CA3	aac(3)-Ia	GENTAMICIN_1	2
	abaF	FOSFOMYCIN_1	2
	ant(3'')-IIa	STREPTOMYCIN/SPECTINOMYCIN_1	2
	aph(6)-Id	STREPTOMYCIN_1	2
	blaADC-30	CEPHALOSPORIN_1	2
	blaOXA-66	BETA-LACTAM_1	2
	tet(B)	TETRACYCLINE_1	2

Table 5.

For 30 A. baumannii strains, the antibiotic-resistance genes (AR genes), identified after sequencing, along with their respective antibiotic targets are reported.

The sequence type (ST), detected with MLST, is also associated to each strain.

Sample	AR genes	Target antibiotic	ST
22005-ENE1	abaF	FOSFOMYCIN_1	2
	ant(3'')-IIa	STREPTOMYCIN/SPECTINOMYCIN_1	2
	aph(3'')-Ib	STREPTOMYCIN_1	2
	aph(6)-Id	STREPTOMYCIN_1	2
	blaADC-30	CEPHALOSPORIN_1	2
	blaOXA-66	BETA-LACTAM_1	2
	tet(B)	TETRACYCLINE_1	2
22005-ENE4	aac(3)-Ia	GENTAMICIN_1	2
	abaF	FOSFOMYCIN_1	2
	ant(3'')-IIa	STREPTOMYCIN/SPECTINOMYCIN_1	2
	aph(6)-Id	STREPTOMYCIN_1	2
	blaADC-30	CEPHALOSPORIN_1	2
	blaOXA-66	BETA-LACTAM_1	2
	tet(B)	TETRACYCLINE_1	2
22006-CA2	ant(3'')-IIa	STREPTOMYCIN/SPECTINOMYCIN_1	1111
	blaADC-80	CEPHALOSPORIN_1	1111
	blaOXA-91	BETA-LACTAM_1	1111
22007-CA4	ant(3'')-IIa	STREPTOMYCIN/SPECTINOMYCIN_1	-
	blaADC-32	CEPHALOSPORIN_1	-
	blaOXA-64	BETA-LACTAM_1	-
22013-CA5	abaF	FOSFOMYCIN_1	-
	blaADC-158	CEPHALOSPORIN_1	-
	blaOXA-715	BETA-LACTAM_1	-
22013-ENE4	aac(3)-IIe	GENTAMICIN_1	903
	abaF	FOSFOMYCIN_1	903
	ant(3'')-IIa	STREPTOMYCIN/SPECTINOMYCIN_1	903
	aph(3'')-Ib	STREPTOMYCIN_1	903
	aph(3')-VIa	AMIKACIN/KANAMYCIN_1	903
	blaOXA-23	BETA-LACTAM_1	903
	blaOXA-65	BETA-LACTAM_1	903
	blaTEM-1	BETA-LACTAM_1	903
	sul2	SULFONAMIDE_1	903
22014-CA2	abaF	FOSFOMYCIN_1	2
	ant(3'')-IIa	STREPTOMYCIN/SPECTINOMYCIN_1	2
	aph(6)-Id	STREPTOMYCIN_1	2
	blaADC-73	CEPHALOSPORIN_1	2
	blaOXA-23	BETA-LACTAM_1	2
	blaOXA-66	BETA-LACTAM_1	2
	blaOXA-72	BETA-LACTAM_1	2
	catA1	CHLORAMPHENICOL_1	2
	tet(B)	TETRACYCLINE_1	2
21036ONE3	-	-	-
21039CNE5	-	-	-
22025ENE4	-	-	-
22026CNE1	-	-	-
22015CNE3	-	-	-
21035CNE6	-	-	-
21036CNE4	-	-	-
21036ENE2	-	-	-
22026ENE2	-	-	-
20085CNE3	-	-	-
20028ENE2	-	-	-
20028CNE6	-	-	-
19003CNE1	-	-	-
20018ENE4	-	-	-
20018CNE3	-	-	-
20015ENE6	-	-	-

6. **Detergent efficacy evaluation against bacterial growth**

To evaluate the antimicrobial efficacy of the methyl-ester sulphonate detergent, *E. coli* cells were plated on Petri dishes and subjected to washing treatments using distilled water or the detergent solution. Bacterial load was then quantified. The data (*Table 6*) demonstrate a significant reduction in bacterial load after treatment with the methyl-ester sulphonate detergent, showing no detectable viable cells or bacterial DNA ($p < 0.001$). Washing with distilled water also led to a significant reduction in cell counts and DNA levels, although to a lesser extent ($p < 0.01$ and $p < 0.05$, respectively). These results demonstrate the effectiveness of the detergent in reducing bacterial contamination.

Analysis Method	Treatment	Pre-treatment	Post-treatment	Significance
Cell counting	Distilled water	$(2.41 \pm 0.84) \times 10^7$ cells/mL	$(3.84 \pm 0.72) \times 10^5$ cells/mL	$p < 0.01$
	MES detergent	$(2.40 \pm 0.71) \times 10^7$ cells/mL	Not detectable	$p < 0.001$
DNA extraction	Distilled water	0.272 ± 0.35 ng/µL	0.140 ± 0.04 ng/µL	$p < 0.05$
	MES detergent	$(7 \pm 1.35) \times 10^4$ ng/µL	Not detectable	$p < 0.001$

Table 6. *Antimicrobial efficacy of the methyl-ester sulphonate detergent.*

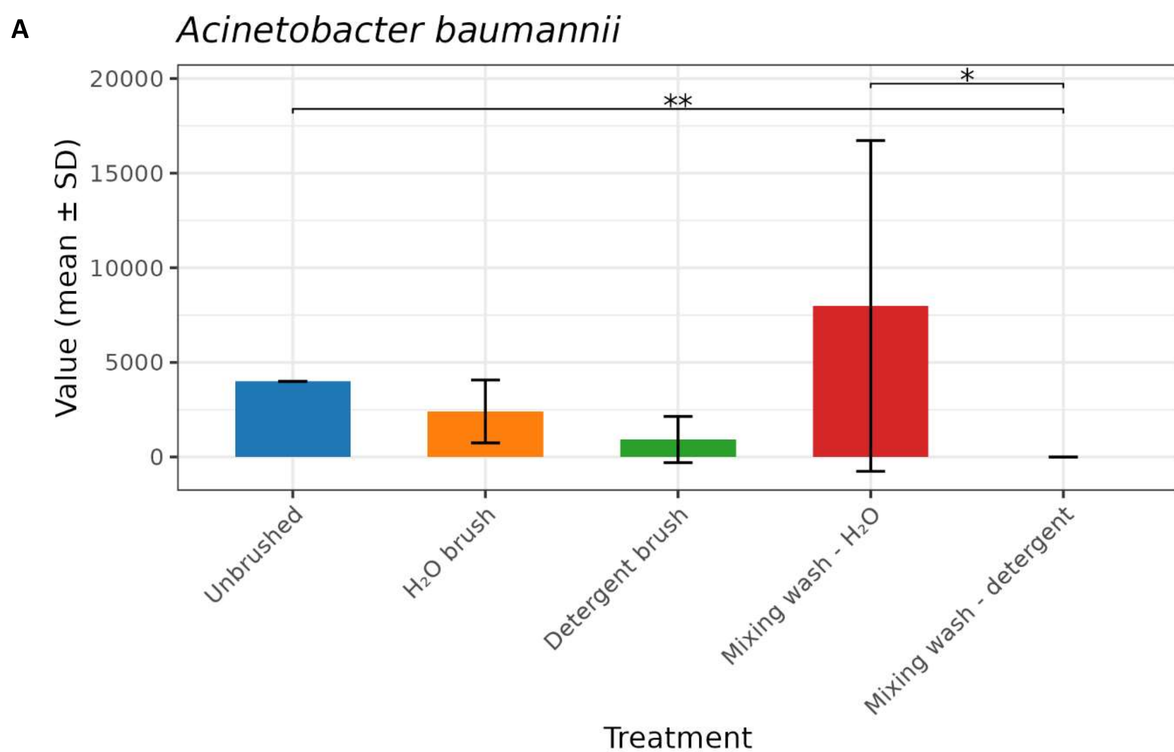
The detergent was further tested on five strains of *A. baumannii* and five strains of *C. difficile*, and on six strains of *E. coli*, using the steel rings. After contamination with the respective bacterial suspensions, the rings were washed either with sterile water or with the detergent, by brushing with a swab or by soaking the ring directly into the water or the detergent. After the treatment, each ring (two for each condition, plus one positive control in duplicate) was put for 30 minutes in contact with the growth medium or in the liquid broth, and incubated at 37°C for 24 hours. The colonies observed were then counted, to calculate the colony-forming units for each bacterial strain in each condition. The results (*Figure 9*) are expressed as CFU/ml.

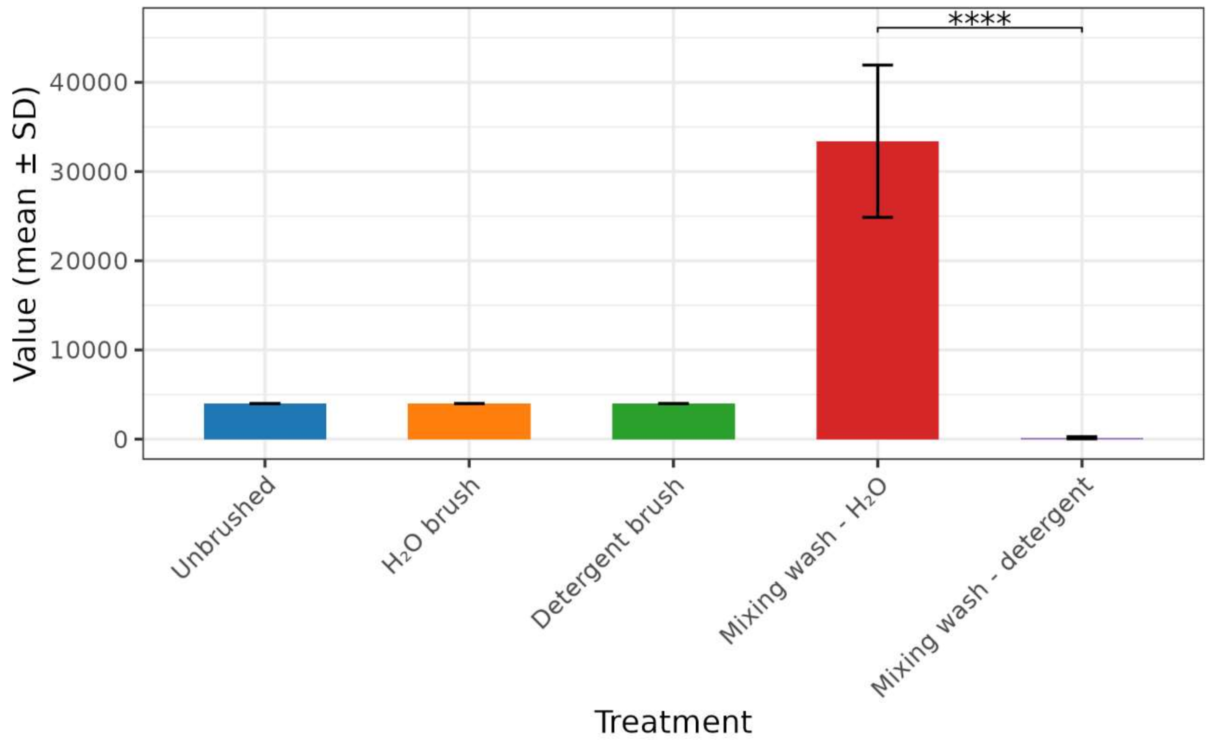
For *A. baumannii*, the treatment through which the steel ring was immersed in the detergent for 40 minutes (mixing wash – detergent) was significatively more effective than the same treatment with water (mixing wash – H_2O), and also significatively different than the positive control (unbrushed).

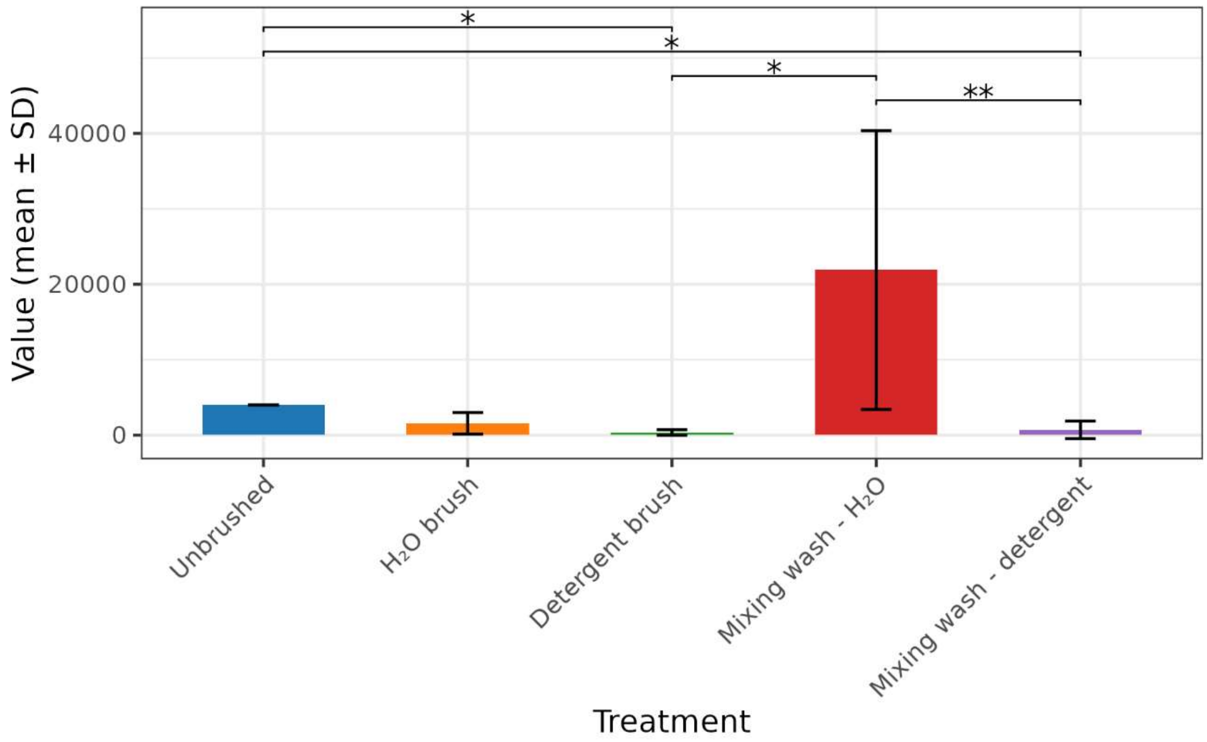
The same was observed treating *E. coli*; moreover, in this case, brushing the contaminated steel rings with a swab soaked with the detergent resulted significatively different than the "mixing wash" treatment with water and than the positive control.

Finally, for *C. difficile* a significative difference was only observed between the “mixing wash” treatment with the detergent or with water.
These results were further corroborated by the evidences given by incubating the steel rings, after the same treatments, at 37°C for 24 hours in TSB medium: in fact, when we did not observe any colony grown in the solid media, in parallel we noticed that the liquid medium remained clear after the same treatment, indicating no bacterial growth.

Figure 9. *Mean value of CFU/ml for each treatment against A. baumannii (A), C. difficile (B) and E. coli (C). The mixing wash treatment with the detergent resulted significative for each bacterium. Notably, for every A. baumannii strain no colony was detected after this treatment.*




B
Clostridium difficile
****
Value (mean ± SD)
40000
30000
20000
10000
0
Unbrushed
H2O brush
Detergent brush
Mixing wash - H2O
Mixing wash - detergent
Treatment


C
Escherichia coli
*
*
*
**
Value (mean ± SD)
40000
20000
0
Unbrushed
H2O brush
Detergent brush
Mixing wash - H2O
Mixing wash - detergent
Treatment

7. Particle's characterization

The immobilization of silver ions was performed on two different types of nanoparticles, BMNPs and MNPs, taking advantage of the pI and establishing an electrostatic bond between the surface of the nanoparticles and the silver cation. ζ-potential values after the coupling showed changes in the surface of the nanoassemblies compared to that of unloaded nanoparticles, confirming the successful bond with silver ions. ζ-potential values for Ag-BMNPs and Ag-MNPs are close to or higher than zero in HEPES 50 mM at pH 7.4, while unloaded nanoparticles in the same buffer showed a higher density of negative charges on the surface: specifically, −32.00 ± 2.10 mV for BMNPs and −27.00 ± 3.00 mV for MNPs (*Table 7*). This change for nanoassemblies demonstrates the presence of silver ions on the surface, as negatively charged groups (carboxyl or hydroxyl groups) [249] previously present on the nanoparticle surfaces are now blocked by silver cations.

Sample	ζ-potential (mV)
MNPS	-27.00 ± 3.00
Ag-MNPS	-0.21 ± 3.00
BMNPS	-32.00 ± 2.10
Ag-BMNPs	+0.11 ± 0.02

Table 7. *ζ-potential outcomes for bare and silver ion-conjugated nanoparticles (MNPS and BMNPs). All the measurements have been performed in triplicate.*

NTA analysis of bare magnetic nanoparticles resuspended in 50 mM HEPES buffer at pH 7.4 revealed a heterogeneous size distribution, ranging from 41 nm to 93 nm, with a particle concentration of (8.87 ± 1.82) × 10^7 particles/mL (*Figure 10A*). These evidences are aligned with the dynamic light scattering (DLS) data presented by Donini et al. [249]. Upon coupling MNPs with silver, two distinct particle populations were identified at roughly 43 nm and 79 nm, with a concentration of (2.43 ± 0.11) × 10^8 particles/mL (*Figure 10B*). Conversely, bare biomimetic magnetic nanoparticles exhibited a more uniform distribution centred around 71 nm, with a concentration of (6.08 ± 0.75) × 10^7 particles/mL (*Figure 10C*), consistent with previous findings [245]. A heterogeneous profile appeared when BMNPs were functionalized with silver, defined by two major peaks at approximately 123 nm and 79 nm, and a particle concentration of (1.77 ± 0.44) × 10^8 particles/mL (*Figure 10D*).

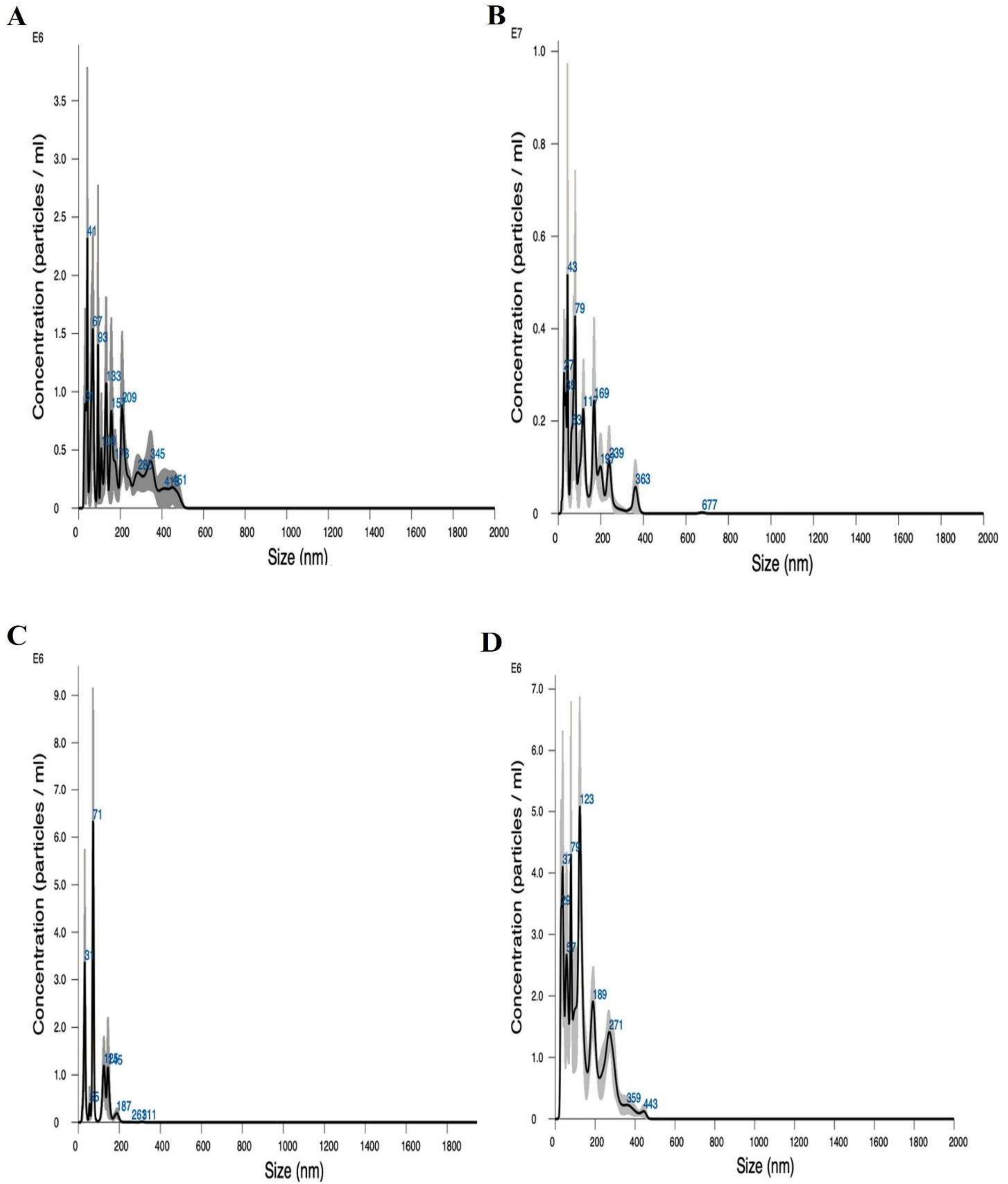


Figure 10. *NTA of (A) naked MNPs, (B) MNPs coupled with silver, (C) naked BMNPs, and (D) BMNPs coupled with silver.*

8. Antibacterial properties of the methyl-ester sulphonate detergent combined with silver-derivatized magnetic nanoparticles

The methyl-ester sulphonate detergent, the two types of silver magnetic nanoassemblies, and a mixture of the detergent and the nanoassemblies were tested against 30 *E. coli* and 30 *A. baumannii* strains. Therefore, we evaluated the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) to determine their antibacterial potential.

The most significative results were obtained combining the detergent with the nanoparticles. In particular, it was demonstrated that BMNPs-treated *E. coli* strains had a MIC_{50} of 0.063 (±0.0067) mg/mL and a MIC_{90} of 0.119 (±0.0067) mg/mL.

By contrast, *E. coli* strains treated with MNPs resulted in a MIC_{50} of 0.031 (±0.0049) mg/mL and a MIC_{90} of 0.063 (±0.0049) mg/mL. Interestingly, the MBC_{50} values were identical for both types of nanoparticles, precisely 0.063 (±0.0075) mg/mL, while the MBC_{90} values were 0.125 (±0.0075) mg/mL for both BMNPs and MNPs.

The tests conducted on *A. baumannii* demonstrated, overall, a superior inhibitory and bactericidal activity of the mixture of the detergent and the nanoassemblies: MIC_{50} resulted to be of 0.031 (±0.0018) and (±0.0022) mg/mL, respectively, for both BMNPs and MNPs; MIC_{90} was instead 0.031 (±0.0018) mg/mL for BMNPs, and 0.063 (±0.0022) mg/mL for MNPs.

Accordingly, MBC_{90} were exactly analogous: the results exhibited a MBC_{50} of 0.031 (±0.0023) and (±0.0029) mg/mL, respectively, for both BMNPs and MNPs, while MBC_{90} was 0.031 (±0.0023) mg/mL for BMNPs, and 0.063 (±0.0029) mg/mL for MNPs. These values suggested an analogous inhibiting and bactericidal activity of the detergent combined with the nanoassemblies, indicating major effectiveness against *A. baumannii* strains.

Overall, for both the bacterial species the bactericidal activity was verified by the absence of visible regrowth on agar plates, although no direct cell counting was performed after treatment; this is in line with standard microbiological definitions. These findings suggest that both MNPs and BMNPs exhibit comparable growth inhibition and bactericidal activity on *E. coli* and *A. baumannii* strains tested.

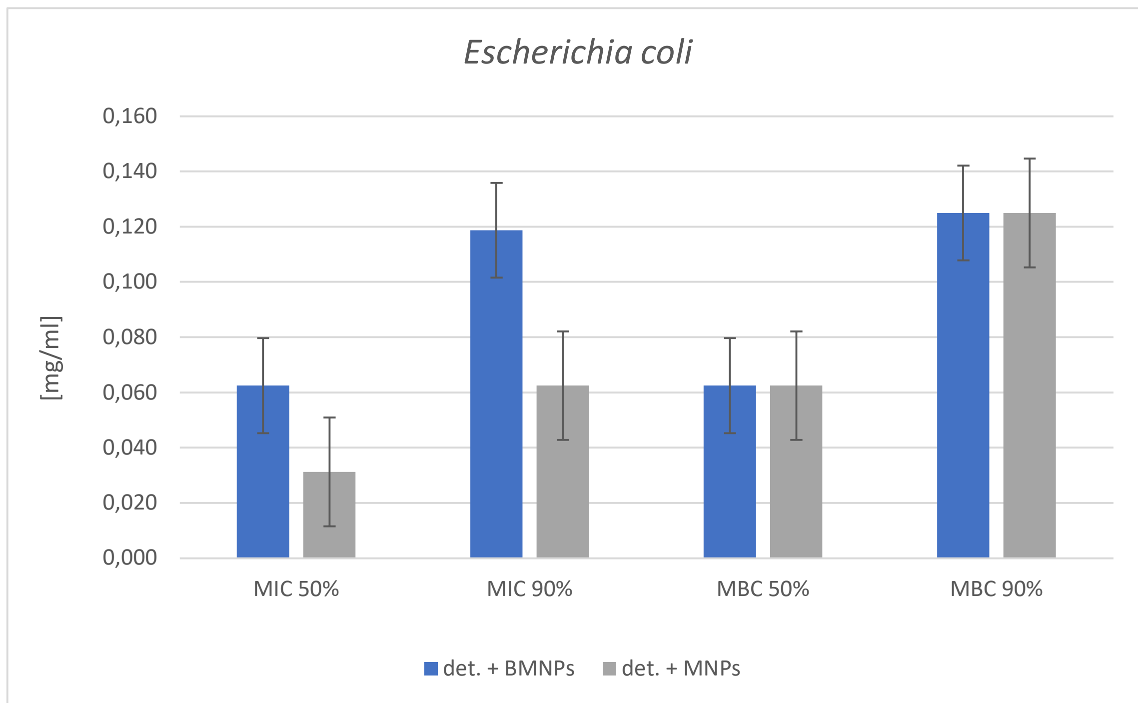


Figure 11. *Graph showing minimum inhibiting and minimum bactericidal concentration of the detergent complexed with the two types of nanoparticles against E. coli. Overall, MNPs showed a higher efficacy than BMNPs in inhibiting bacterial growth, as demonstrated by the lower level of* MIC_{50} *and* MIC_{90}.

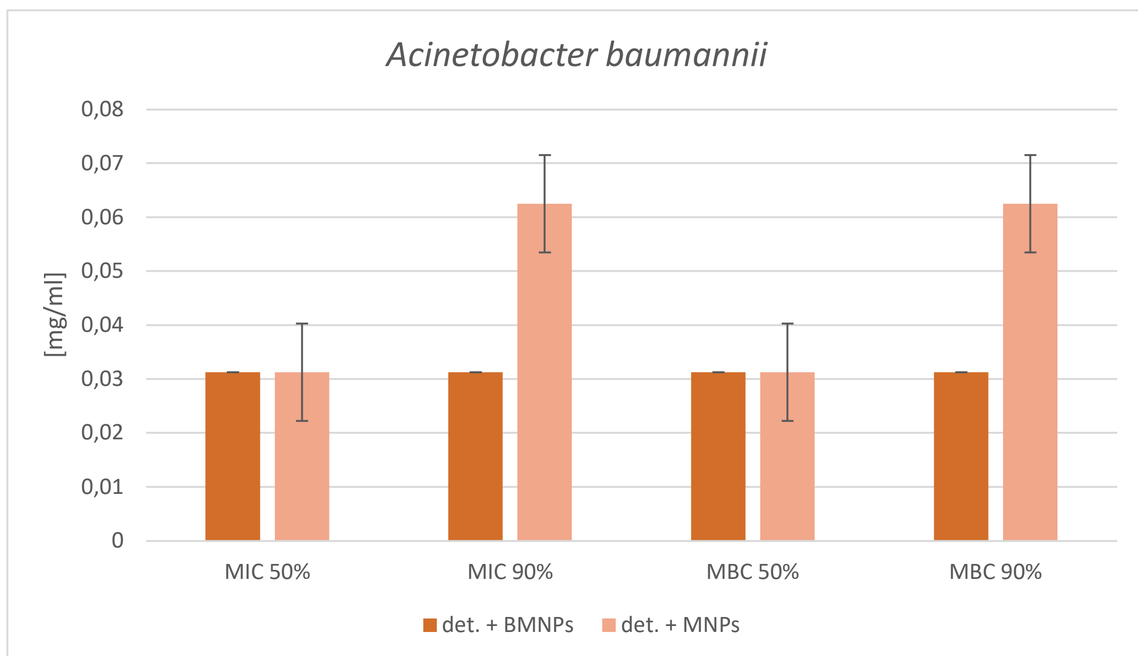


Figure 12. *Graph showing minimum inhibiting and minimum bactericidal concentration of the detergent complexed with the two types of nanoparticles against A. baumannii. Overall, BMNPs showed a higher efficacy than MNPs, requiring a lower concentration both to inhibit and to kill the bacteria, as shown by* MIC_{90} *and* MBC_{90}.

The detergent, alternatively combined with BMNPs or MNPs, was evaluated through MIC and MBC tests on the type-strains of six microorganisms' samples, obtaining the results reported in the tables below, respectively (*Table 8*, *Table 9*). An almost identical effect was revealed for the two types of nanoparticles within all the species tested; we also observed comparable results between inhibiting and bactericidal concentrations, with MBC slightly higher than MIC for some species.

It was noticed also a stronger effect among Gram-positive (*Enterococcus faecalis*, *Staphylococcus aureus*) than Gram-negative (*Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Enterobacter aerogenes*) bacteria.

These experiments should be performed on a higher number of strains for each specie to obtain proper MIC_{50}, MIC_{90}, MBC_{50} and MBC_{90} values. However, these results seem promising, as they suggest the efficacy of the detergent complexed with silver nanoparticles against a broad spectrum of microorganisms; notably, a pathogenic fungus (*Candida albicans*) was also included in this investigation, giving similar results when compared to bacteria, suggesting their effectiveness against an even wider spectrum of microorganisms.

MIC	D. + BMNPs	D. + MNPs
E. faecalis	0,031	0,031
P. aeruginosa	0,063	0,063
S. aureus	0,031	0,031
K. pneumoniae	0,125	0,125
E. aerogenes	0,063	0,063
C. albicans	0,063	0,031

Table 8. *Concentration of the two types of nanoparticles, expressed as mg/ml, mixed with the detergent (D.), correspondent to the MIC value for each type-strain sample.*

MBC	D. + BMNPs	D. + MNPs
E. faecalis	0,063	0,063
P. aeruginosa	0,063	0,063
S. aureus	0,031	0,031
K. pneumoniae	0,125	0,125
E. aerogenes	0,063	0,063
C. albicans	0,063	0,031

Table 9. *Concentration of the two types of nanoparticles, expressed as mg/ml, mixed with the detergent (D.), correspondent to the MBC value for each type-strain sample.*

9. Barrier performance results of the SAFE-HUG PRO wheelchair cover

The SAFE-HUG PRO Wheelchair Cover's barrier efficacy was evaluated using *E. coli* as the test organism, in a controlled in vitro test.

First, we looked for any potential microbial presence on the covers: the lack of optical density (OD) signals, cell counts and DNA levels showed the absence of any detectable microbial load. Thus, we examined the cover's performance under growth-promoting conditions within an incubator. We placed a sample of the cover in direct contact with a high bacterial load, specifically a suspension containing 5×10^7 cells, and then the non-exposed side was analysed after two different time points in incubator, namely 1 h and 24 h, using three complementary methods: OD600 (optical density), cells count, and DNA quantification. The results show a substantial reduction in bacterial transmission through the cover compared to the uncovered control, demonstrating the material's strong barrier properties (*Table 10*).

Table 10. *Barrier performance.*

Analysis Method	*With Cover (1h)*	*Control (1h)*	*With Cover (24h)*	*Control (24h)*
Optical density (OD600)	0.000	0.024 ± 0.0012	0.000	0.100 ± 0.009
Cells/mL	$(7 \pm 1.35) \times 10^4$	$(1.6 \pm 0.112) \times 10^8$	$(1 \pm 0.87) \times 10^5$	$(2.36 \pm 0.713) \times 10^8$
DNA (ng/μl)	Not detectable	0.2 ± 0.014	Not detectable	0.6 ± 0.054

Thus, the observed decrease in cell concentrations after 1 h exceeds three orders of magnitude ($>10^3$), while the decrease after 24 h corresponds with approximately three orders of magnitude.

DISCUSSION

Infection prevention and management in healthcare settings currently face important challenges: firstly, the conventional chemical disinfectants employed to clean medical devices and patients' rooms objects may result only partially effective against microbial contamination, and are associated with issues such as the contribution in environmental pollution; on the other hand, the antimicrobial resistance threat represents a growing concern worldwide. Together, these issues urgently require innovative and sustainable solutions [250], [251].

The present work proposes targeted solutions aimed at preventing as much as possible the spread of pathogenic bacteria potentially leading to infections development, focusing on crucial points to limit contamination in healthcare facilities: we assessed the effectiveness of a novel automated washer-disinfector, designed for non-critical items, such as steel bedpans; then, an eco-friendly detergent, combined with silver nanoparticles, was formulated as an alternative to common chemical disinfectants, and finally we tested the ability of wheelchair covers as protective barrier to prevent the transmission of microorganisms through these widely used facilities in healthcare settings. This integrated approach, aimed at intervening on different potential contexts of infection transmission, allows for a stronger risk reduction.

The SMEG BPW1260 has shown to be efficacious for bedpan thermal disinfection, in line with the European Standard EN ISO 15883. The results in fact demonstrated a significant ability in reducing bacterial contamination, making it a suitable choice for high hygiene standards settings, such as healthcare facilities and laboratories [252]. The effective drastic decrease in bacterial load was furtherly confirmed by the DNA extraction and quantification, which resulted in most cases significatively lower after disinfection or even not detectable. This supports the evidence of a true bactericidal effect, able to eliminate both the viable cells and their genetic material: the latter represents a desirable objective, considering the risk of gene transfer or residual pathogenicity [253]. Moreover, the incorporation of DNA quantification as a supplemental strategy to assess bactericidal efficacy is particularly advantageous and robust compared to traditional microbiological methods [253], [254]: in fact, these ones are not always able to reveal the presence of non-culturable microorganisms and are often time-consuming [255]; a typical example is represented by the traditional culture-based method of the colony-forming unit (CFU) count. Conversely, the strategy employed in this study took advantage of an integrated approach that combined direct cell counting for viability assessment, optical density measurement, and DNA quantification, enabling for a faster and more comprehensive microbial detection. This led to a reduction in the time required for microbiological validation and, at the same time, to improve the sensitivity and reliability of the

results, enhancing the accuracy of the contamination assessment and, in turn, of the bactericidal efficacy of disinfection procedures. This approach highlights also the need for standardized biological validation protocols for BWDs, necessary for a reliable performance evaluation across different clinical contexts. On the other hand, this study focused its attention also on sustainability, looking for strategies to improve both the operational performance in disinfection, and also the energy efficiency of the process. The A_0 parameter resulted very helpful in finding the optimal combination of time and temperature required to reach both an adequate disinfection level and a cost-effective efficiency according to the specific needs [256]. In particular, the A_0 600 program resulted to be the most suitable in our experimental settings, offering a significant bacterial load reduction and, at the same time, lower energy consumption and cycle time. The possibility of reducing the energy consumption and, in turn, the resources needed and the environmental impact is particularly important in this context, especially without compromising the efficiency of the disinfection process. Specifically, in our case the cycle duration was shortened from 35 to 28 minutes, while the energy consumption was decreased from 0.77 kWh to 0.42 kWh: these results may be relevant especially in high-throughput environments, where similar optimizations can lead to lower operational costs and increased workflow efficiency. The progressive adoption of energy-efficient cycles is in line with broad ecological and economic aims, which are connected also to institutional sustainability goals [257], [258]: in particular, supranational bodies such as the International Hospital Federation and the Pan-European Commission on Health and Sustainable Development recently recommended healthcare organizations to take greater responsibility in terms of environmental impact and carbon footprint due to excessive energy consumption, promoting instead sustainable good practices [259].

This work is limited to only two infectious bacteria, namely *Escherichia coli* and *Clostridium difficile,* that, although crucial in the context of HCAIs due to their widespread presence and frequency of association with infections, do not fully represent the broad spectrum of pathogens responsible of HCAIs. Future research should be focused on evaluating the effectiveness of the proposed approaches against a wider range of microorganisms, especially the multidrug-resistant ones, to further corroborate the applicability and robustness of the results.

Another of the main objectives of this study consisted in the formulation and validation of a novel nanofluidic detergent, with waste cooking oil serving as starting material and then complexed with silver-derivatized nanoparticles. One of the most noteworthy characteristics of this detergent is its sustainability: as it was prepared by repurposing waste materials, it represents a valuable alternative to conventional chemical detergents, and it is aligned with circular economy goals. This environmentally friendly detergent was successfully synthesized from waste cooking oil via a two-step process, that yielded sulphonated methyl-esters after transesterification and sulfonation

reactions. FTIR spectroscopy confirmed the presence of both ester and sulfonate groups; in particular, the transesterification reaction resulted in a 92 ± 5% conversion of waste oil into methyl ester, while a colour change from yellow to white evidenced the complete transformation of the methyl ester into methyl ester sulphonate. Notably, the predominant presence of C18 methyl esters is particularly desirable for our purposes, because it is indicative of enhanced detergency properties of methyl ester sulphonates [236].

The increasing emergence of multidrug and antibiotic-resistant pathogens has become such a concerning health issue that is making more and more urgent the need for alternative or complementary strategies to antibiotic therapy [128]. Among these alternatives, for the treatment of CDI, faecal microbiota transplantation (FMT) has emerged as an effective solution, which has gained interest mainly because of the limitations of other treatment options, and their inefficacy against recurrent CDI; in particular, the efficacy of FMT against recurrent and refractory CDI was demonstrated to be, respectively, of 85-90% and 55% [260], [261], [262], [263]. The mechanism through which this strategy results effective is probably the microbial antagonism: the competitive exclusion by other microbes that outcompete *C. difficile* lead it to the deprivation of nutrients, creating a hostile environment for its growth [264]. Although further investigations are needed to better clarify the mechanisms that undergo this process, FMT seems to be both safer and more effective, compared to many antimicrobials [261]. A significant clinical interest has been gained also by bacteriophage therapy, as an alternative to conventional antimicrobials. Bacteriophages work as natural antibiotics, both against Gram-negative and Gram-positive bacteria [265], [266]; they are viruses restricted to bacteria, that can be easily isolated from various sources and with lower costs when compared to antibiotics production [267]. The advantages of bacteriophage therapy are several, such as the specificity (that reduces the side-effects against human gut microbiota, usually associated with antibiotic therapy) [268], their ability to spread fast also in organs that antibiotics do not easily reach, the lack of resistance mechanisms developed by bacteria against bacteriophages [269], [270], [271]. Several studies investigated the effectiveness of phage therapy against *A. baumannii* [173], especially the antibiotic-resistant strains; among these ones, a study demonstrated the efficacy of phage therapy to treat carbapenem-resistant *A. baumannii* infection [272], [273].

Another of the most promising alternatives to antibiotic therapy is represented by metal nanoparticles. Currently, they are increasingly used to deliver medical products, including antibiotics, or directly as antibacterial agents. Metal and metal oxide nanoparticles have been demonstrated to exert their antimicrobial effect through different mechanisms: they can modify both the toxicity and the antibacterial activity of antibiotics, as well as favour the interaction between intra- and extracellular

components and ions; moreover, they are able to induce damages to the bacterial structures through reactive oxygen species, and also to inhibit DNA synthesis and enzyme activity [274], [275].

Silver ions (Ag^+) are known for their bactericidal properties. Considered this, among metal nanoparticles, silver nanoparticles (AgNPs) have emerged as effective antimicrobial agents, due to their broad-spectrum activity and unique physicochemical properties; their nanoscale size in fact favours their interaction with microbial surfaces [276].

In our study, ζ-potential measurements confirmed the successful functionalization of MNPs and BMNPs with silver ions, indicating a marked shift from strongly negative values (−27.00 ± 3.00 mV for MNPs and −32.00 ± 2.10 mV for BMNPs) to values near neutrality or slightly positive after silver binding. This revealed the presence of electrostatic interactions between the negatively charged nanoparticle surfaces and the positively charged silver ions, resulting in surface charge neutralization. These findings were further corroborated by nanoparticle tracking analysis, which showed increased heterogeneity and size distribution in the silver-functionalized samples (particularly in Ag-BMNPs), indicating the formation of more complex or larger nanoassemblies.

AgNPs were previously demonstrated to induce cell lysis and death through evidently compromising cell membranes of both *E. coli* and *C. difficile*: in this way, they allow for lower required dosages of conventional antibiotics, boosting antimicrobial efficacy [277], [278]. Some studies have shown that silver-functionalized magnetite nanoparticles exhibited a strong antibacterial activity by releasing silver ions and generating reactive oxygen species, causing cell death through cell membrane disruption, especially against *E. coli*. Conversely, recent studies on *C. difficile* are limited; nevertheless, AgNPs are known to be efficacious against Gram-positive anaerobes as well, suggesting that they may represent a promising approach also for targeting *C. difficile* [279], [280], [281].

Some studies have also highlighted the effectiveness of AgNPs against *A. baumannii*. In particular, these nanoparticles were found to enhance the antibacterial effect of antibiotics such as piperacillin and erythromycin against *A. baumannii* [282]; AgNPs efficacy, both as potential standalone antibiotic therapies and antibiotic adjuvants, was also demonstrated on *A. baumannii* together with other multidrug-resistant bacteria [283]. AgNPs synergy was observed when combined with colistin and imipenem against pan-drug resistant *A. baumannii* strains: specifically, this combination significantly lowered their MICs, with colistin-AgNP combinations showing the strongest effect [284]. Furthermore, another study investigated the effects of AgNPs in combination with antibiotics against persistent bacterial biofilms: together with amikacin, the nanoparticles were found to increase significantly the effect of the antibiotic alone, showing bactericidal effects within 48 h in several multidrug-resistant (MDR)/extensively drug-resistant (XDR) strains of different bacteria, among which *A. baumannii* [285].

The present work demonstrated the effectiveness of two types of silver-complexed nanoparticles, Ag-BMNPs and Ag-MNPs, acting as bactericidal agents when combined with the eco-friendly detergent derived from waste cooking oil. Therefore, the incorporation of these types of nanoassemblies into the sulfonated methyl ester-based detergent may represent a promising strategy for enhancing decontamination effectiveness in healthcare disinfection protocols.

MIC_{50} and MBC_{50}, similarly to MIC_{90} and MBC_{90}, demonstrated that both BMNPs and MNPs have comparable bacterial growth-inhibiting and bactericidal activity. This property is probably the result of the synergy between the membrane-disruptive properties of the MES detergent and the well-documented bactericidal effects of silver ions, which interfere with bacterial DNA replication and metabolic processes. Furthermore, the magnetic properties of the nanoparticles offer potential advantages for targeted application and, possibly, to post-use recovery, that represents an important property in terms of sustainability. As these promising results have been currently obtained only in vitro, further research is necessary to validate the nanoparticles-complexed detergent efficacy in real healthcare contexts, especially testing it on contaminated hospital surfaces.

Besides the improvement of antimicrobial efficacy, the integration of Ag-MNPs and Ag-BMNPs into a biodegradable, waste-derived detergent holds the beneficial adherence with circular economy purposes. The long-term stability of these nanoformulations and their effectiveness against a broader spectrum of pathogens, including multidrug-resistant organisms, should be object of future research.

Several eco-friendly compounds have recently been studied, to investigate their potential antimicrobial properties. Among these, ionic liquids (ILs) are proposed as environmentally safe substitutes for traditional solvents and surfactants; in particular, precursors obtained from natural waste, such as discarded coffee grounds, were used to create ionic liquid-based Gemini cationic surfactants (ILGCS), and showed a strong inhibiting effect on the growth of some Gram-positive bacteria (e.g., *L. monocytogenes* and *S. aureus*) and a moderate inhibition against Gram-negative strains such as *E. coli* and *Salmonella* sp. [286]. Electrochemically activated hypochlorous (HOCl) acid solutions have been also proposed as sustainable and low-impact cleaning and sanitizing agent, demonstrating an acceptable percentage of abatement of microbial charge and a significative difference when compared to water [287]. To achieve sustainability and effectiveness goals, new compounds have been developed to ensure virus inactivation, too: eco-friendly surfactants were evaluated for antiviral efficacy against herpes viruses, SuHV and HSV, and the retrovirus XMuLV, showing a significative inactivation efficacy [288]. Another study assessed the antiviral properties of the Probiotic Cleaning Hygiene System (PCHS), an eco-sustainable probiotic-based detergent able to stably abate pathogen contamination and antimicrobial resistance: this product demonstrated effective inactivation properties against several viruses tested, without a negative impact on antimicrobial

resistance issue and environmental pollution [289]. Moreover, as new promising solvents in the field of "green chemistry," deep eutectic solvents (DESs) have recently shown an important potential in different fields, among which their antimicrobial properties. Some of these compounds, such as tetrabutylammonium bromide:ethylene glycol, exhibited a significative antimicrobial activity and, notably, tetrabutylammonium bromide in glycerol can be considered an eco-friendly solvent due to its lower toxicity in both *in vivo* and *in vitro* environments [290].

Other solutions for alternative disinfection strategies, that are currently being studied, use approaches different from nanotechnologies and may be aimed or not at contributing to circular economy models. Such strategies include contact-less methods, like the use of gaseous ozone or essential oils in gaseous form. The latter are natural, biodegradable, and leave no harmful residues, representing an environmentally friendly option as well [291]; their antimicrobial activity may vary, depending on the oil composition and pathogen type [292]. On the other hand, gaseous ozone is known for its oxidizing properties, able to rapidly disinfect surfaces without chemical residues, but it requires specialized equipment and safety precautions due to its toxicity at high concentrations [293].

Another alternative method, particularly used for the high-level disinfection of contaminated endocavitary probes, is a hydrogen peroxide mist system: this method has shown complete inactivation of VRE and CRE-*Klebsiella pneumoniae* strains, a good efficacy against *Mycobacterium terrae* and *C. difficile* spores [294], and inactivation of human papilloma virus and other pathogens [295], [296]. However, proper cleaning of devices is recommended as necessary before the high-level disinfection process, to ensure its efficacy [210].

Finally, this work aimed at assessing the risk of HCAIs transmission associated with wheelchairs, which is frequently overlooked in routine infection control protocols [31], validating the effectiveness of a cover acting as a physical barrier between the wheelchair and the patient. We demonstrated that the SAFE-HUG PRO Wheelchair Cover effectively minimizes bacterial transfer, in an experimental setting as adherent as possible to the real conditions. Even after prolonged exposure to *E. coli*, the cover significantly prevented bacterial penetration to the non-contact surface. Conversely, unprotected wheelchair surfaces exhibited discrete bacterial loads and detectable levels of DNA, confirming their potential as reservoirs and vectors of microbial transmission. These findings support the need of these kinds of barriers as a simple and effective measure to improve existing hygiene practices: this is particularly recommended in high-risk healthcare environments, where indirect transmission routes, such as contaminated mobility aids, are often underestimated. Implementing these additional protective measures into standard protocols can make the difference in the context of pathogens transmission in healthcare facilities, improving patients' safety and matching with broader aims, connected to sustainability and HCAIs prevention.

CONCLUSION

In this work, novel approaches to face the challenges posed by HCAIs have been explored: no-touch thermal disinfection, eco-friendly nanoparticle-enhanced detergent, and wheelchair cover emerged as effective strategies for improving infection control in healthcare settings.

The SMEG BPW1260 washer-disinfector demonstrated strong bactericidal activity against *C. difficile* and *E. coli*, with the $A_0$600 cycle specifically providing enhanced energy efficiency without compromising effectiveness. Similarly, the novel biofluidic detergent showed effective antimicrobial properties, especially when combined with nanoparticles. Besides equipment disinfection, protective barriers as the cover we tested on wheelchairs play a key role in preventing microbial transmission: the SAFE-HUG PRO Wheelchair Cover demonstrated to efficiently prevent *E. coli* passage, acting as a physical barrier. These findings underline the importance of adopting physical containment strategies, such as antibacterial wheelchair covers, into broader infection prevention protocols.

Overall, these technologies provide complementary benefits that could result extremely helpful when integrated into standard procedures, carrying the potential to greatly lessen the burden of healthcare-associated infections (HCAIs), while also aiding in the shift toward more resilient and sustainable healthcare practices.

Together, all these findings may pave the way for future research aimed at optimizing their application in real-world contexts, to get clinically relevant insights into their infection prevention potential; additionally, important further objectives would be testing their efficacy across a broader spectrum of pathogens, and evaluating their long-term impact.

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