Hard Surface Biocontrol in Hospitals Using Microbial-Based Cleaning Products

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Abstract

Background: Healthcare-Associated Infections (HAIs) are one of the most frequent complications occurring in healthcare facilities. Contaminated environmental surfaces provide an important potential source for transmission of many healthcare-associated pathogens, thus indicating the need for new and sustainable strategies.

Aim: This study aims to evaluate the effect of a novel cleaning procedure based on the mechanism of biocontrol, on the presence and survival of several microorganisms responsible for HAIs (i.e. coliforms, Staphylococcus aureus, Clostridium difficile, and Candida albicans) on hard surfaces in a hospital setting.

Methods: The effect of microbial cleaning, containing spores of food grade Bacillus subtilis, Bacillus pumilus and Bacillus megaterium, in comparison with conventional cleaning protocols, was evaluated for 24 weeks in three independent hospitals (one in Belgium and two in Italy) and approximately 20000 microbial surface samples were collected.

Results: Microbial cleaning, as part of the daily cleaning protocol, resulted in a reduction of HAI-related pathogens by 50 to 89%. This effect was achieved after 3–4 weeks and the reduction in the pathogen load was stable over time. Moreover, by using microbial or conventional cleaning alternatively, we found that this effect was directly related to the new procedure, as indicated by the raise in CFU/m² when microbial cleaning was replaced by the conventional procedure. Although many questions remain regarding the actual mechanisms involved, this study demonstrates that microbial cleaning is a more effective and sustainable alternative to chemical cleaning and non-specific disinfection in healthcare facilities.

Conclusions: This study indicates microbial cleaning as an effective strategy in continuously lowering the number of HAI-related microorganisms on surfaces. The first indications on the actual level of HAIs in the trial hospitals monitored on a continuous basis are very promising, and may pave the way for a novel and cost-effective strategy to counteract (bio)control healthcare-associated pathogens.


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Introduction

Healthcare-Associated Infections (HAIs) are one of the most frequent complications occurring in healthcare facilities and represent a problematic concern regarding the safety and quality of healthcare worldwide [1], as also stated in a recent report by the World Health Organization estimating hospital-wide prevalence in high-income countries at 8% [2]. The European Center for Disease Control point prevalence study confirmed that healthcare-associated infections are a major public health problem in Europe with a prevalence of 5.7% (4.5–7.4%) which means 81.089 (64.624–105.895) patients with one HAI for each day in European acute care hospitals [3]. In particular, this European survey reported a similar estimation of nosocomial infections for Italy and Belgium, where the percentage of patients with HAIs has been calculated as 6.3% (5.4–7.4%) and 7.1% (6.1–8.3%), respectively [1]. Based on this study, the estimated total annual number of patients with an HAI in European acute care hospitals in 2011–2012 was 3.2 million, albeit with a wide confidence interval from
1.9 to 5.2 million patients. Similar incidences were measured in the United States [4]. Besides human suffering, also impressive economic costs are related to HAI management. Indeed, as reported by the Centers for Disease Control and Prevention (CDC), it has been estimated that the overall annual direct medical costs for healthcare-associated infections in hospitals ranges from 33.7 to 45 billion dollars in the United States [5]. In addition, the management, prevention and monitoring of HAI nowadays still represents a challenge for healthcare facilities [6,7].

The microorganisms most frequently isolated from HAI are, in decreasing order, *Escherichia coli* (15.9%), *Staphylococcus aureus* (12.3%), *Enterococcus spp.* (9.6%), *Pseudomonas aeruginosa* (8.9%), *Klebsiella spp.* (8.7%), coagulase-negative staphylococci (7.5%), *Candida spp.* (6.1%), *Clostridium difficile* (5.4%), *Enterobacter spp.* (4.2%), *Proteus spp.* (3.8%) and *Acinetobacter spp.* (3.6%) [3].

A very controversial and debated question is the qualitative and quantitative role of the environment in the patient contamination process, particularly the role of confinement and furnishing surfaces. It is well known that surfaces act as reservoirs for microorganisms and could contribute to the transmission of hospital pathogens, increasing the risk of cross-contamination through indirect contact with the patient [8–10]. To reduce such risks, sanitation procedures are applied to every surface that directly or indirectly may come in contact with people. Despite experimental evidence suggesting that a reasonable use of disinfectants is recommended, their routine use is still controversial [11,12]. Nevertheless, a proper surface disinfection is recommended by all international guidelines as an important procedure for preventing infections [13–17], and considerable evidence exist concerning the benefits of hospital cleanliness towards reducing HAI [18]. Indeed, failure to ensure proper cleaning and sterilization or disinfection may lead to patient-to-patient transmission of pathogens [19].

However, the widespread use of chemical disinfectants presents risks towards the environment and the safety of personnel. It is clear that microorganisms can adapt to a variety of environmental physical and chemical conditions, and it is therefore not surprising that resistance to extensively used antiseptics and disinfectants has been reported [20,21]. For these reasons, the importance of cleaning procedures that are aimed to control the load of pathogenic bacteria indicates that a new and sustainable strategy is necessary.

A very promising approach, as suggested by Falagas & Makris in 2009, is the use of non-pathogenic microorganisms, namely probiotics and defined as living microorganisms able to confer a health benefit on the host, to colonize hard surfaces in order to counteract the proliferation of other bacterial species [22], according to the competitive exclusion principle (Gause’s law) [23–25]. This concept has been designated as biocontrol when the application is antagonistic towards a certain pathogen [26], and has already successfully been applied to the abatement of Legionella in water systems [27].

Several investigators have pointed to evidence that probiotic type microorganisms and their biosurfactants may antagonize the growth of nosocomial pathogens on inanimate surfaces [28–32]. However, the actual application of probiotic type microorganisms on hard surfaces as a cleaning procedure has never been tested. Therefore, this study evaluates the effects of microbial cleaning of hard surfaces in hospitals on the presence and/or survival of HAI-related microorganisms on such treated surfaces. In order to facilitate the statistic meaningfulness of the results, the study was performed in three independent hospitals (one in Belgium and two in Italy). Comparison was made with chemical cleaning and disinfection protocols. This study aims to provide sufficient data to conclude whether the technique of biocontrol of hospital surfaces can act as a sustainable alternative to chemical disinfectants.

**Methods**

**Preliminary tests**

Prior to the actual field trials in the hospitals, a number of preliminary tests were done at Ghent University, Ferrara University and AZ Lokeren hospital in order to determine the most suitable formulation of the microbial cleaning products to be used. Mainly the identity and concentration of the microorganisms used in the cleaning products were chosen in view of the average microbiological load on hard surfaces, pH, temperature and humidity. The microbial cleaning products used in the field trials comprised spores of *Bacillus subtilis*, *Bacillus subtilis* and *Bacillus subtilis* and *Bacillus subtilis* with a fixed quantity of $5 \times 10^7$ CFU per ml of product concentrate. All products were manufactured by Chrals (Lommel, Belgium) and supplied to AZ Lokeren by Chrals and the two Italian hospitals by Copma srl. (Ferrara, Italy).

In order to prevent bias in the eventual hospital trials due to the detergents in the products used, several field trials in the AZ Lokeren hospital were performed to compare the effect between the microbial and non-microbial version of the products to be used in the hospital trials (data not shown).

**Ethics Statement**

The study protocol was reviewed and approved by the local Ethics Committees. The trials in the two Hospitals residing in Ferrara (Sant’Anna and San Giorgio) were approved by the Ethics Committee, named Comitato Unico della Provincia di Ferrara (Unique Committee of the Ferrara Province), of the Azienda Ospedaliere-Universitaria of Ferrara (Ferrara, Italy). For the AZ Lokeren setting, the study was evaluated and approved by the AZ Lokeren Ethic Comité (Lokeren, Belgium). The two Ethics Committees stated that a formal authorization was not necessary because the probiotic products would not be directly administered to patients but exploited for cleaning of hospital surfaces only. For this reason, the Committees waived the need for written informed consent from participants because of the observational nature of the study.

**Hospital trial setup**

Three independent hospital trials, separated in time and location were performed. In each trial setting, comparison was made between cleaning with microbial cleaning products and the conventional hygiene protocols (using chemical cleaning products and disinfectants). Control cleaning products in AZ Lokeren consisted of chemical detergents (Ecolab, Groot-Bijgaarden, Belgium) and in both Italian hospitals chlorine based detergents (Actichlor for all washable surfaces, Diversey S.p.A., Italy) were applied. The microbial cleaning products in all three hospitals comprised a floor cleaner, interior cleaner and bathroom cleaner (Chrisal, Lommel, Belgium). Comparison between control and microbial cleaning was made both over time and on units with identical infrastructure within the hospital (e.g. two floors of geriatrics in AZ Lokeren). Except for the products, all other parameters related to the cleaning procedures (e.g. frequency, equipment) were the same between control and microbial cleaning. Cleaning in AZ Lokeren was performed according to the existing hygiene protocol of the hospital, and cleaning in the two Italian hospitals was performed according to the Probiothec Cleaning Hygiene System (PGCHS) by Copma srl. Cleaning staff
was not aware whether or not they were operating with microbial cleaning products.

Description of hospital wards

San Giorgio Rehabilitation Hospital. The San Giorgio hospital (total surface 12,300 m²) is a centre for the rehabilitation of acquired severe brain-damaged disorders. The wards involved in this study are distributed on a total of three floors, with each floor (4,100 m²) consisting of two identical and specular wards, namely the Severe Brain-damaged Unit and the Rehabilitation Medicine Unit. These two wards are separated by a central corridor and each consists of 22 recovery rooms (28 m²) with two beds and a toilet (4.2 m²). In these settings, treated surfaces consisted of room, corridor, and floors and toilets. In addition, cleaning procedures and samplings were also performed in the six gymniasms used for patient rehabilitation.

Sant’Anna University Hospital. The wards involved in the study within the Sant’Anna University Hospital are referred to as an in-patients general medicine ward, consisting of two identical areas (named S- and T-Area, each with a surface of 550 m²), and an out-patient ward containing the Ophthalmology/Cardiology department (total surface 12,300 m²). In these settings, treated surfaces consisted of room, corridor, and floors and toilets.

AZ Lokeren Hospital. In the Lokeren hospital setting, two structurally identical (500 m² per unit) geriatrics units were involved in the study. The wards host elderly people suffering from a wide range of illness and pathologic states, including surgery, daycare, and some cases in which the treatment of infections is needed.

Microbiological tests

The effect of the microbial and conventional cleaning products on HAI related microorganisms on surfaces was assessed by means of surface sampling and culture-based microbiology tests. The following HAI-related microorganisms were monitored: Staphylococcus aureus and Escherichia coli in all three hospitals; Clostridium spp. in AZ Lokeren hospital; Candida albicans in Sant’Anna and San Giorgio hospital. During six weeks in Sant’Anna University hospital, 24 weeks in AZ Lokeren hospital and 66 weeks in San Giorgio Hospital, a total of 20000 microbiological samples were collected (between six and eight hours after cleaning) from a broad variety of surfaces such as floors, doors, showers, window sils, toilets, sinks, made of stone, wood, plastic, glass or metal.

All surface samples were performed in triplicate by contact RODAC (Replicate Organism Detection and Counting) plates, used for microbiological monitoring surface equivalent to 24 cm² of the surface. The following growth media were used: McConkey Agar (BBL MacConkey Agar, BD) as selective and differential medium for the detection and enumeration of Enterobacteriaceae (especially the group of coliform bacteria); Baird Parker Agar (Merk Millipore Baird-Parker Agar) as moderately selective and differential medium for the detection and enumeration of coagulase-positive staphylococci, and used for detecting Staphylococcus aureus; Clostridium difficile agar (BBL Clostridium difficile Agar, BD) for the selective detection of Clostridium difficile; and Sabouraud Dextrose Contact Agar added with chloramphenicol (Merk Millipore) as selective medium for detection and enumeration of Candida albicans. Incubation was done aerobically at 37°C (48 h–72 h) for McConkey, Baird Parker, Cetrimide Agar and Sabouraud Dextrose Contact Agar, and anaerobically using anaerobic jars (GasPak, BD) at 37°C (72 h) for Clostridium difficile agar. Colony Forming Units (CFU) on all agar plates were manually counted after their respective incubation period. Occasionally isolates from the above agar plates were identified to check the selectivity of the used media. Identification of isolates was assessed by API 20 E (bioMérieux, Inc, Durham, NC, USA), BBL Enterotube II (BD Diagnostic Systems) for Escherichia coli, API Staph (bioMérieux, Inc) for Staphylococcus aureus, and API 20 C Aux (bioMérieux, Inc) for Candida albicans.

Antibiograms

The protocol used was based on the Kirby-Bauer disk diffusion antimicrobial susceptibility test [33–35], following the criteria outlined by the Clinical and Laboratory Standard Institute (CLSI) [36,37]. Briefly, a 90 mm Mueller-Hinton agar plate was inoculated with a suspension of Bacillus subtilis (ATCC 6633), or Bacillus strains isolates, corresponding to a 0.5 McFarland standard. After 15 minutes, a paper disk (Oxoid Ltd., Thermo Scientific, Basingstoke, Hampshire, United Kingdom) impregnated with a known concentration of an antimicrobial compound was added, and plates were incubated at 37°C for 24–48 hours. The zones of inhibition (expressed in mm) were measured, and the interpretation of results was based on CLSI reference criteria (Table S1) [38].

Molecular analyses

The presence of antimicrobial resistance genes in the Bacillus collected isolates was evaluated by a real time quantitative PCR (qPCR) method. Microbial genomic DNA was extracted from each isolate by the QIAamp UCP Pathogen Mini Kit (Qiagen, Hilden, Germany), adjusting the instruction of the manufacturer for optimal lysis of Gram-positive bacilli and spores. Two micrograms of extracted DNA were analyzed by the Microbial DNA qPCR Array for Antibiotic Resistance Genes (Qiagen, Hilden, Germany), using an Applied Biosystem 7900 instrument and following the manufacturer’s indications. The analysis was performed on the strains originally present in the cleaning products and on 20 different isolates from the different hospital settings involved in the study. Microbial DNA-free water (Qiagen) and Microbial DNA Positive Control (Qiagen) were used as negative and positive controls, respectively. DNA from E. coli JM101 strain was also used as a negative control. Preliminary tests showed that the sensitivity of the assay was about 10 copies for each target gene.

Statistics

The statistical analysis was based on the generalized linear model for repeated measures. This method combines the repeated measures of a marginal model with the generalized linear model for residuals following the Poisson distribution and the logarithm as link function. Comparisons between follow up and basal times were performed by using Wald Statistics with the Sidak correction for multiple comparisons. All statistical analyses were performed using the SPSS v 19.0 software (IBM Corp., Armonk, NY; USA).

Results

Prior to hospital trials, a broad range of in vitro and small scale field trials were performed in order to determine both the best possible test protocols and product formulations for the actual study, as well as to provide relevant information to the ethics committees. The focus of the study was on microbiological counts and no evaluations of actual dirt removal were assessed. The use of Bacillus strains in the cleaning products showed a much higher...
Microbiological influence on hard surfaces compared to other genera (such as Lactobacillus and Pseudomonas), mainly due to their sporulation capacity (data not shown). Based on the average total count values of several types of hard surfaces in hospitals, the optimal concentration of Bacillus spores to be employed in the product formulations was determined. Before starting the hospital trials, we decided that the amount of product bacteria to be left on surfaces upon cleaning should be of the same magnitude as the average total count observed before the microbial cleaning application. Finally, a number of laboratory scale tests demonstrated that the same product formulations, with or without the addition of the Bacillus spores, produced significantly different effects on pathogen load on the treated surfaces.

The study was conducted in three different hospitals, and approximately 20000 microbial surface samples were collected. The microbial analyses focused on Staphylococcus aureus, coliform bacteria, Clostridium difficile, and Candida albicans as representatives or indicators for proper hygiene and HAI-related microorganisms. In order to allow proper comparison among the different hospital settings, results are presented as relative values to the control, which is referred to the value of microbial surface contamination (CFU count) at the beginning of the trials (week 0), as shown in Tables S2, S3, S4, S5.

Notably, the effect of microbial cleaning on the different HAI-related pathogens displayed similar trends among the three hospitals under investigation. In the Sant’Anna University hospital, the microbial sampling lasted six weeks, whereas the other two hospital were monitored until week 24 (AZ Lokeren) and week 66 (San Giorgio Hospital). However, because no significant differences in counts were observed between week 24 and 66 at the other two hospital, the microbial sampling lasted six weeks, whereas the microbial cleaning was replaced by the conventional cleaning products. Indeed, the observed reduction was significant for all the monitored hospital settings (Table S2).

Coliforms and E. coli

Figure 1 shows the mean relative values for coliform bacteria load as a general indicator for hygiene. The reference values measured at week 0 for coliforms, with E.coli representing about one half of the count, are reported in Table S2. However, individual surface counts could fluctuate strongly, especially in specific areas such as bathrooms or geriatric departments. Noticeably, the beginning of microbial cleaning resulted in a fast reduction of coliform and E. coli counts, achieving a maximum effect approximately after two weeks of daily cleaning. The average reduction over time was 74±21% for the coliforms and 89±18% for E. coli. The observed reduction was statistically significant for all the monitored hospital settings (Table S2).

S. aureus

The average levels of S. aureus on tested surfaces and the corresponding percentage reduction over time are reported in Table S3 and Figure 2, respectively. For the sake of clarity, no distinction was made between antibiotic-resistant or antibiotic-sensitive S. aureus. After 10 days of microbial cleaning within the San Giorgio Hospital setting, the S. aureus counts on surfaces dropped, on average, by 58±12%, and 78±15% after six weeks, with a reduction that was highly significant in all the hospital settings (Table S3). In both conventional and microbial cleaning, S. aureus counts on treated surfaces showed a good stability with no large exceptions throughout the study (data not shown), suggesting that this organism is not very susceptible to different environmental conditions within a hospital and might therefore survive most common conditions.

C. difficile

A less abundant but still common HAI-related organism is Clostridium difficile, with average counts of about 500 CFU/m² (Table S4), which is near the detection limit of the test protocols used. These microorganisms were monitored on a regular basis only in the AZ Lokeren setting. C. difficile showed much larger specific counts on different surfaces and time points, which, in combination with the overall lower mean counts, resulted in larger standard deviations, thus making the effect of microbial cleaning barely significant when compared to control. The overall average reduction in C. difficile load was achieved very quickly after three days of microbial cleaning, with a reduction corresponding to 55±47% (Figure 3). Ongoing measurements in the next 24 weeks indicated that C. difficile levels on microbial cleaned surfaces dropped below the detection limit of the analysis method. The observed reduction was significant for weeks 4 (p = 0.048) and 12 (p = 0.007), and particularly for weeks 18 (p<0.0005) and 24 (p = 0.004), indicating the long-term effect on C. difficile load reduction.

C. albicans

Candida albicans load was measured on a regular basis in the two Italian hospital settings. Figure 4 shows the mean relative values of load reduction. The average control values of C. albicans are reported in Table S5. Microbial cleaning resulted in a fast load reduction, corresponding to a value of 82±19%, after one week and, noticeably, the load of C. albicans was stably maintained at low levels over the next weeks. As of week two, C. albicans counts residing on the microbial cleaned surfaces was hardly above the detection limit of the test protocols. Indeed, the observed reduction was highly significant in both the hospital settings (Table S5).

Time trial with conventional and microbial cleaning

A separate experiment was conducted within the geriatrics department in the AZ Lokeren setting, where the conventional and microbial cleaning procedures were alternatively applied and the bacterial load was monitored for 10 weeks. Microbial cleaning (from week 0 to week 2) was applied after two weeks of conventional cleaning (from week 2 to week 4), which was then applied for a second period spanning the last 8 weeks (from week 2 to week 10). Consistently with previous observations, the microbial cleaning strongly reduced both coliforms and S. aureus load, whose counts dropped from 9250±1750 CFU/m² to 3200±1200 CFU/m² and from 4200±1200 CFU/m² to 950±150 CFU/m² after 2 weeks, respectively. Conversely, when the microbial cleaning was replaced by the conventional procedure, both loads raised to colony counts comparable to those observed before the application of the microbial cleaning (between weeks 2 and 0) (Figure 5). The application of the
Microbial cleaning led to a significant decrease in the pathogenic load of coliforms \( (p < 0.0001, \text{from week 0.5 to week 3}) \) and *S. aureus* \( (p = 0.003, \text{from week 1 to week 3}) \) in comparison with that measured at week 0, whereas no significant differences were observed after replacement of the microbial cleaning with the conventional procedure.

Overall, our data on microbial cleaning indicated that the strong reduction of the pathogen load was stably maintained over time, and the observed effect was directly related to its application, as indicated by comparison with conventional cleaning.

**Susceptibility/resistance of Bacillus isolates to antibiotics**

In order to monitor the appearance of antimicrobial resistances in *Bacillus* strains, we performed both conventional antibiograms and molecular analyses on *Bacillus* isolates collected on the treated surfaces. Preliminary results from antibiograms showed that the Bacilli tested, deriving from both the PCHS products and from isolates, displayed the known natural resistance to penicillin. The susceptibility profile for the other antibiotics tested (cefoperazone, cefalotin and gentamicin) was comparable to that of the ATCC reference strain, with the only exception of clindamycin, for which intermediate values were observed (Table 1).

To increase the number of antimicrobial resistance factors analyzed, 20 *Bacillus* isolates, which were collected from 6 to 12 months after the beginning of the hospital trial, were also analyzed by a qPCR assay capable of detecting 87 different genes associated to antibiotic resistance. Prior to testing *Bacillus* isolates, the method was set up on known *Bacillus* spp. DNA samples, which indicated this technique as a reliable tool for subsequent analysis.

The analysis of the probiotic-based cleaning products revealed the existence of constitutive resistance genes in the *Bacillus* species contained in the original product formulation, confirming previous literature reports (Figure 6).

Noticeably, new or acquired resistance genes were completely absent in all the *Bacillus* isolates tested, indicating that these bacteria did not undergo mutagenicity or gene transfer events, even after 12 months after application, thus confirming the results observed in the antibiogram assays.

**Discussion**

Given the latest official reports on the prevalence of HAIs [3] and the evolution of microbial resistance to antibiotics [39] and disinfectants [20], it is clear that new and sustainable strategies to address contaminated surfaces in hospital settings are of great interest. Besides measures that deal with person-to-person transmission of pathogens, contaminated environmental surfaces provide an important potential source for transmission of many healthcare-associated pathogens due to their persistence on such inanimate surfaces [9,10,40]. This highlights the importance of cleaning procedures aimed to control the load of pathogenic bacteria that reside on hospital surfaces and, as a consequence, of HAIs, as indicated by considerable evidence about the benefits of hospital cleanliness towards reducing HAIs [18].

This study exploited the hypothesis proposed by Falagas & Makris [22] to use non-pathogenic microorganisms, namely probiotics, as part of daily cleaning products to lower the incidence of HAI-related microorganisms in healthcare facilities. Evidences on the efficacy of probiotics for the prevention and treatment of infections have been observed both in vitro and in vivo [41–43].

A variety of HAI-related microorganisms exist and for this study the selection was mostly determined by the relevance and relative abundance of the microorganisms in the hospitals willing to participate to the present study. Coliforms were selected as an indicator for overall hygiene and cleaning assessment. Although some HAIs are associated with outbreaks and others show rather constant prevalence, the related microorganisms can be found on hard surfaces at any time as presented in this study. This implies that surfaces act as a reservoir that might initiate an outbreak.
when people get contaminated and start spreading the pathogens amongst each other.

The aim of this study was to collect a substantial amount of data from different independent healthcare facilities and the 20000 microbial surface samples contribute to the significance of the results. However, the amount of analyses makes it impossible to present all specific data and observations in this study. Therefore, given the rather compatible mean microbial counts between the several hospitals, the witnessed trends in surface microbiology upon microbial cleaning was presented.

In general, it was observed that microbial cleaning of hard surfaces resulted in a clear change of the microbiology within two weeks. With the exception of *Clostridium difficile*, a very significant decrease in HAI-related microorganisms, compared to conventional cleaning and disinfection, was observed. As of two weeks of microbial cleaning, the reduced HAI-related microorganisms count remained constant and mostly just above detection limit of the test protocol. The standard deviation of some values was high (likely due to large differences in the type of tested surfaces), but still the difference was clearly significant to demonstrate the effects exerted by the microbial cleaning compared to the control. Our results indicate that microbial cleaning clearly lowered the presence of HAI-associated microorganisms on hard surfaces compared to conventional cleaning procedures, without significantly lowering the total microbial counts. In addition, our data show that when microbial cleaning was switched to the conventional procedure, the HAI-related microorganism counts raised to the original [higher] levels observed before the beginning of microbial cleaning. This observation indicates that microbial cleaning has a time-limited

![Figure 4. Effect of microbial cleaning on *Candida albicans* surface counts.](https://doi.org/10.1371/journal.pone.0108598.g004)

Figure 4. Effect of microbial cleaning on *Candida albicans* surface counts. Surface counts are reported as relative percentage of reduction compared to the control, which was cleaned with conventional (disinfecting) cleaning products. The analysis indicated that results observed were statistically significant (Table S5).

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effect on the counts of HAI-related microorganisms and that microbial cleaning needs to be continuously applied on regular basis in order to stably maintain the reduced levels of these microorganisms. Microbial cleaning therefore resulted in the partial non-permanent replacement of HAI-related microorganisms by the *Bacillus* strains used in the microbial cleaning products.

Although this study did not aim to investigate the actual mechanisms involved in the observed changes in surface microbiota, some assumptions can be made based on existing literature. The most relevant mechanism underlying the observed effect is likely due to competitive exclusion: *Bacillus* strains applied on the surfaces within the cleaning solution may compete, in terms of nutrients and space, with the microbiota already present on the surfaces [44]. Woo & Ahn have suggested that competitive exclusion can also destabilize certain biofilms [44], which was observed in the present study as removal of grout on certain hard surfaces after several weeks of microbial cleaning. Such biofilm removal on hard surfaces by means of microbial cleaning deserves further research. Another mechanism that is likely to contribute to the changes in microbiota upon microbial cleaning, and also destabilize biofilm formation, is quorum sensing and quorum quenching [45]. The constant artificial introduction of dominant counts of *Bacillus spp.* through the cleaning products could destabilize the microbial population dynamics on surfaces and in biofilm (e.g. grout). Given the observed general effect of microbial

### Table 1. Antibiograms on *Bacillus spp.* from the cleaning products and isolates.

<table>
<thead>
<tr>
<th>Zone Diameter Interpretive Criteria (mm)</th>
<th>Beta-lactams</th>
<th>Aminoglycosides</th>
<th>Lincosamides</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Penicillin</td>
<td>Cefoperazone</td>
<td>Cefalotin</td>
</tr>
<tr>
<td></td>
<td>(10 U)</td>
<td>(30 µg)</td>
<td>(30 µg)</td>
</tr>
<tr>
<td>ATCC 6633</td>
<td>7 R&lt;sup&gt;a&lt;/sup&gt;</td>
<td>20 I&lt;sup&gt;b&lt;/sup&gt;</td>
<td>30 S</td>
</tr>
<tr>
<td>PCHS product</td>
<td>14 R</td>
<td>19 I</td>
<td>24 S</td>
</tr>
<tr>
<td><em>Bacillus spp.</em> isolate 1</td>
<td>12 R</td>
<td>19 I</td>
<td>20 S</td>
</tr>
<tr>
<td><em>Bacillus spp.</em> isolate 2</td>
<td>15 R</td>
<td>20 I</td>
<td>30 S</td>
</tr>
<tr>
<td><em>Bacillus spp.</em> isolate 3</td>
<td>10 R</td>
<td>30 S&lt;sup&gt;c&lt;/sup&gt;</td>
<td>35 S</td>
</tr>
</tbody>
</table>

<sup>a</sup>Resistant.  
<sup>b</sup>Intermediate.  
<sup>c</sup>Susceptible.

*Note:* for reference criteria see Table S1.

Antibiograms were conducted on *Bacilli* from the PCHS products and isolates, with ATCC 6633 strain as reference. Results are reported as diameter (expressed in mm) of the zones of inhibition and the corresponding interpretive criteria, in accordance to the CLSI reference criteria [38] (Table S1).

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cleaning on several HAI-related microorganisms, it is likely that non-selective effects such as competitive exclusion and quorum sensing are the most important mechanisms involved. Besides these general mechanisms, other more specific interactions could contribute to the reduction effect on one or more HAI-related microorganisms. For instance, the production of either bacteriocins [46] or some enzymes known to be produced by Bacillus spp. might affect other organisms to a higher extent. Regarding Clostridium, the authors suggest that the cortex-lytic enzymes involved in the germination of the Bacillus spores [47] in the microbial cleaning products might also trigger the germination of Clostridium spores on surfaces. The germinated Clostridium cells

Figure 6. Antimicrobial resistance genes profile by qPCR assay. The DNA of the Bacillus spp. from the cleaning products (Original) and from 20 Bacillus spp. colonies isolated from the treated surfaces up to 12 months after the beginning of the cleaning protocol (Isolates), was analyzed by qPCR Microarray to detect the presence of antibiotic resistance genes (R genes). DNA from E. coli JM101 strain was used as negative control (NTC). Results are reported as folds of gene copy number comparing the detected values with those obtained in the negative control DNA, both normalized for bacterial cell number. Those genes coding for antibiotics that are not included in the figure (i.e. vancomycin, tetracyclins) were negative both in Original and Isolates collections. Results are reported as mean ± standard deviation.

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might subsequently be killed by oxygen or could suffer from competitive exclusion. Regardless the actual mechanisms involved, this study demonstrates that microbial cleaning in healthcare facilities manages to significantly lower the surface counts of a variety of HAI-associated microorganisms.

The most important question concerns the safety of applying microbial cleaning as biocontrol system in healthcare facilities. Obviously the identification and safety assessment of the bacterial strains used in the products is of utmost importance, as well as the production processes involved throughout the whole production chain. The strains used in this study (Bacillus subtilis, Bacillus pumilus and Bacillus megaterium), were food grade organisms, for which substantial safety and toxicity data existed by the manufacturer, and are also known to be not harmful to humans [48–51]. In addition, all production and quality control processes were ISO9001:2008 certified. Furthermore, the use of probiotics for several biotechnological and biopharmaceutical applications, as indicated by the very recent employment of B. subtilis as a biocontrol agent in aquaculture [52], agriculture [53–55], as adjuvant [56,57] or, in the spore form, as delivery system for biocontrol agent in aquaculture [52], agriculture [53–55], as an antibiotic resistance. We recently implemented our studies by exploiting antibiogram tests, which have been indicated by the very recent employment of B. subtilis as a biocontrol agent in aquaculture [52], agriculture [53–55], as adjuvant [56,57] or, in the spore form, as delivery system for biocontrol systems [66]. Although these assays were performed on only 20 Bacillus spp. isolates, these preliminary results show that they did not display acquisition of new resistance genes, even in a period of 12 months, thus strengthening the hypothesis that the use of Bacillus spores in cleaning products might be considered as safe. On the other hand, results also show that this method can be reliable for the evaluation of antimicrobial resistance factors in Bacillus, and designate this assay as an important implementation for future studies also focused on safety concerns.

Conclusions

Given the recent and fast evolution of multi-resistant pathogens in healthcare facilities there is a need for sustainable and effective alternatives to the cleaning and disinfection chemicals used today. This study demonstrates that a microbial (probiotic-based) cleaning is more effective in the long-term lowering of the number of HAI-related microorganisms on surfaces, when compared to conventional cleaning products, even those containing disinfectant molecules such as chlorine. The first indications on the percentages of HAIs in the trial hospitals monitored on a continuous basis throughout the study are very promising and may pave the way for a novel and cost-effective strategy to counteract or (bio)control healthcare-associated pathogens.

When it comes down to risk management, one has to decide whether a patient should stay in an environment dominated by food grade microorganisms or in an environment harboring an elevated level of increasingly resistant pathogens.

Supporting Information

Table S1 Reference criteria for interpretation of the zones of inhibition in the disk diffusion antimicrobial susceptibility test. The table shows the disk diffusion interpretive criteria for the correlation of the diameter (expressed in mm) of the zones of inhibition with the corresponding interpretation, referred as Susceptible (S), Intermediate (I) and Resistant (R), according to the CLSI reference criteria [38].

(XLSX)

Table S2 Statistical analysis on the reduction of coliforms load. Results are expressed as CFU/m².

(XLSX)

Table S3 Statistical analysis on the reduction of Staphylococcus aureus load. Results are expressed as CFU/m².

(XLSX)

Table S4 Statistical analysis on the reduction of Clostridium difficile load. Results are expressed as CFU/m².

(XLSX)

Table S5 Statistical analysis on the reduction of Candida albicans load. Results are expressed as CFU/m².

(XLSX)
Author Contributions
Conceived and designed the experiments: RT PA PGB EC SM. Performed the experiments: AV RT AF EC. Analyzed the data: SM RT AF EC DP

References