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Standardization of a Methodology for Disinfection Efficacy Against Microorganisms Isolated from a Pharmaceutical Industry Facility as a Contamination Control Strategy

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Abstract

Inadequate surface sanitization represents a significant risk to sterility assurance and regulatory compliance. Therefore an effective cleaning and disinfection programme is a critical component of contamination control strategies in pharmaceutical facilities manufacturing sterile medicinal products. This study aimed to standardize a carrier-based methodology for evaluating the efficacy of disinfectants against in-house environmental isolates recovered from a pharmaceutical industry facility. Nine representative strains (six bacteria and three fungi), selected based on historical environmental monitoring data (2012–2022), were characterized using matrix-assisted laser desorption/ionization - time-of-flight / mass spectrometry (MALDI-TOF MS) and molecular sequencing (16S rRNA or D2 LSU rDNA). Disinfectant efficacy was assessed on stainless-steel and low-density polyethylene surfaces using NF T 72-281:2014 with adaptations, testing alcohol 70%, sodium hypochlorite 0.5%, quaternary ammonium 0.05%, peracetic acid 0.5%, and accelerated hydrogen peroxide wipes. All agents demonstrated ≥5 $\log_{10}$ reductions against vegetative bacteria and fungi on both surfaces. However, variable sporicidal performance was observed, particularly for one *Bacillus cereus* group strain (B1342/15), which showed limited reduction on stainless-steel. These findings highlight inter-strain variability and the greater tolerance of surface-associated spores. The study reinforces the importance of carrier-based testing using in-house isolates to ensure realistic validation of disinfectants and to strengthen microbiological risk management within pharmaceutical contamination control strategies.

Keywords: disinfection; cleaning validation; biocide; antimicrobial efficacy; contamination control strategy; quality control; pharmaceutical industry; sterile medicinal products; GMP

1. Introduction

An effective cleaning and disinfecting programme is essential in controlled environments dedicated to the manufacture of pharmaceuticals products in order to minimize the risk of microbial contamination [1]. Sterile medicinal products may be exposed to contamination from multiple sources, including raw materials, process water, primary packaging components, the manufacturing environment, production equipment, and personnel involved in manufacturing activities. To facilitate efficient cleaning, maintenance, and compliant manufacturing operations current Good Manufacturing Practice (GMP) guidelines place strong emphasis on the appropriate design, construction, and layout of facilities, as well as on material and personnel flows [1–4].

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The cleaning and disinfection of surfaces represent a fundamental pillar of the contamination control strategy (CCS) in pharmaceutical industries manufacturing sterile medicinal products [1–4]. A robust cleaning and disinfection programme should therefore ensure compliance with predefined cleanliness criteria, effectively control microbial contamination. This is essential to ensure product manufacturing and, consequently, quality and safety, and compliance with GMP as required by regulatory authorities agencies [2–5]. Current regulatory frameworks, including European Medicines Agency (EMA) Annex 1 [2], World Health Organization [3], and Food and Drug Administration [4] GMP guidelines on aseptic processing emphasize that inadequate control of environmental and surface microbiota constitutes a significant risk to aseptic manufacturing and sterility assurance. In this context, cleaning and disinfection programmers must be scientifically justified, risk-based, and supported by documented evidence of effectiveness [6–8]. Consequently the selection of suitable chemical disinfectants and antiseptics, the demonstration of their bactericidal, fungicidal, and sporicidal efficacy; and the application of disinfectants in the sterile pharmaceutical manufacturing area must be evaluated [1,10].

The validation of cleaning and disinfection processes aims to demonstrate the consistent performance of sanitization procedures under actual manufacturing conditions. According to EMA Annex 1 [2] and United States Pharmacopeia (USP) Chapter ⟨1072⟩ Disinfectants and Antiseptics [1], validation studies should consider the disinfectant type, contact time, application method, and the nature of the surfaces treated. Surfaces commonly found in pharmaceutical facilities, such as stainless-steel (SS) and low-density polymeric materials, present distinct physicochemical properties that may influence disinfectant efficacy. Furthermore, both USP [1] and Parenteral Drug Association (PDA) Technical Report No. 70 [10] highlight the importance of incorporating environmental isolates into efficacy testing, as these in-house strains more accurately reflect the resident microbiota of a given facility and may exhibit increased tolerance or adaptive responses to biocidal agents when compared to standard reference strains [10].

Despite the regulatory emphasis on the use of in-house microorganisms [1,10], the implementation of such cleaning and disinfection validation studies remains challenging for many pharmaceutical quality control laboratories. In practice, numerous facilities do not maintain cryopreserved collections of environmental isolates or lack the specialized expertise, validated methods, and infrastructure required to perform these assays in accordance with regulatory expectations. Consequently, the efficacy testing is frequently outsourced to specialized service providers, leading to increased costs and reduced internal control over contamination management strategies [9]. These constraints reveal a persistent gap between regulatory guidance and routine industrial practice, underscoring the need for standardized, accessible, and reproducible methodologies that enable in-house validation of cleaning and disinfection processes while strengthening microbiological risk management and regulatory compliance in sterile pharmaceutical manufacturing environments.

The aim of this study was to describe a methodology for efficacy test of different chemical agents against selected in-house microorganisms isolated from a pharmaceutical industry facility.

2. Materials and Methods

2.1. Bacterial and Fungal Strains Selection and Culture Conditions

Bacterial and fungal strains were selected according to their frequency of isolation in a pharmaceutical facility producing immunobiologicals, located in Rio de Janeiro State, Brazil between 2012 and 2022. Based on this analysis, nine strains were selected from five different groups: Gram-positive non-spore forming bacteria (n=2), Gram-positive spore-forming bacteria (n=2), Gram-negative bacteria (n=2), yeasts (n=2) and filamentous fungus (n=1).

Bacterial and yeast stock cultures were prepared and maintained at <-70 ºC in Difco™ Skim Milk 30% (BD Biosciences, Le Pont de Claix, France), containing 30% glycerol (Merck KGaA, Darmstadt,

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Germany). The filamentous fungus was maintained at 5 ± 3 ºC in phosphate-buffered saline pH 7.2 (PBS; Sigma-Aldrich, Saint Louis, USA).

2.2. Strains Characterization

The strains were identified by matrix-assisted laser desorption/ionization - time-of-flight / mass spectrometry (MALDI-TOF/MS). Bacterial strains were seeded on Sheep Blood Agar (SBA) plates (BioCen do Brasil, São Paulo, Brazil) and incubated at 32.5 ± 2.5 °C for 48 h. Yeast and filamentous fungus strains were seeded in Potato dextrose agar (PDA) and incubated at 22.5 ± 2.5 °C for 6-7 days. A portion of a colony of each strain was applied to a slide in triplicate together with 0.5 µl of formic acid 70% and, after drying, 1 µl of alpha-cyano-4-hydroxycinnamic acid matrix solution (VITEK MS-CHCA, bioMérieux, Craponne, France). *Escherichia coli* ATCC 8739 was used as the control culture according to the manufacturer's instructions. The slides were analyzed using the VITEK® MS RUO equipment (bioMérieux, Craponne, France), and the results were analyzed by SARAMIS Premium software v. 4.0.0.14. Strains with ≥75% match to entries in the database were considered identified.

The bacterial strains were also identified by 16S rRNA gene Sanger sequencing analysis using MicroSEQ™ Full Gene 16S rDNA kit (Thermo Fisher Scientific, Waltham, USA) followed by analysis on the 3500 Series Genetic Analyzer (Applied Biosystems, Waltham, USA). DNA extraction was performed using PrepMan® Ultra Sample Preparation Reagent (Applied Biosystems, Waltham, USA), according to manufacturer's instruction. The sequences were processed using DNA Star LaserGene SeqMan software v. 7.0.0, and identification results were obtained from the website https://www.ezbiocloud.net/ (EzBioCloud Database Update 2025.04.21). All sequences were deposited at https://www.ncbi.nlm.nih.gov/. For Identification of bacteria by 16S RNA gene, the results showing an identification percentage of ≥98.7% were considered as species [11].

The fungi strains were seeded on PDA and incubated at 22.5 ± 2.5 ◦C for 3 days. DNA extraction was performed using PrepMan® Ultra Sample Preparation Reagent (Applied Biosystems, Waltham, USA), according to manufacturer's instruction. For amplification of D2 LSU rDNA domain, the MicroSEQ® D2 rDNA Fungal kit (ThermoFisher Scientific™, Waltham, USA) was used, according to the manufacturer's protocol followed by analysis on the 3500 Series Genetic Analyzer (Applied Biosystems, Waltham, USA). The sequences were processed using DNA Star LaserGene SeqMan v. 7.0.0 software, and the contig sequencing data was analyzed in the public databases GenBank (https://blast.ncbi.nlm.nih.gov/) and MycoBank (https://www.mycobank.org/). All sequences were deposited at https://www.ncbi.nlm.nih.gov/. Identification percentages ≥ 99.8%, ≥ 98.2%, and ≥ 96.2% for D2 sequences were considered for species, genus and family, respectively [12].

2.3. Chemical Agents Tested in Efficacy Test

Antimicrobial activity was performed using the methodology described on standard NF-T-72-281:2014 [13] simulating conditions of cleanliness used in the industrial sectors, with modifications.

The classification of the chemical agents tested was performed according to PDA [10] and were: 70% alcohol for 15 min (Merck, Darmstadt, Germany), 0.5% sodium hypochlorite for 15 min (Brasquímica, Belo Horizonte, Brazil), 0.05% quaternary ammonium for 20 min, peracetic acid 0.5% for 10 min (Divosan Forte VT6, Diversey®, Peróxidos do Brasil Ltd.a, Curitiba, Brazil) and Oxivir TB Wipes (OTW) - wipes containing 0.52% accelerated hydrogen peroxide (Diversey, Ontario, Canada) for 5 min; according to Table 1.

Table 1. Antimicrobial chemical agents tested for efficacy test.

Classification[1]	Chemical agents	Concentration (%)	Contact time (min)	Test
Sanitizer	Alcohol	70	15	Bactericidal and yeasticidal activity
Disinfectant	Sodium hypochlorite	0.5	15	Bactericidal and yeasticidal activity
Disinfectant	Quaternary ammonium	0.05	20	Bactericidal and yeasticidal activity
Sporicide	Peracetic acid	0.5	10	Sporicidal and fungicidal activity

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Sporicide	Oxivir TB Wipes containing accelerated hydrogen peroxide[2]	0.52	5	Bactericidal, yeasticidal, fungicidal and sporicidal activity

[1]Classification according to Parenteral Drug Association Technical Report No. 70 [10].

2.3.1. Carriers' Preparation

Stainless steel (SS) 2 cm diameter discs were used as carriers to simulate SS surface. For simulation of plastic surface, low-density-polyethylene (LDP) bags (Pall, California, USA) were cut in squares of 3 ± 0.5 cm^2.

In a biological safety cabin (BSC), LDP carriers were immersed in a flask containing 70%$_{(v/v)}$ isopropanol solution (Merck, Darmstadt, Germany) for 15 min. After, the carries were placed in Petri dishes (Interlab, São Paulo, Brazil) until completely drying by evaporation. The SS carriers were sterilized by autoclaving for 121 ºC/ 30 min. To verify the efficacy of the sterilization procedure, two carriers of each type (LDP and SS) were added to tubes containing 20 mL of brain heart infusion broth (BHI; Merck KGaA, Darmstadt, Germany) and incubated at 32.5 ± 2.5 ºC for 14 days.

2.3.2. Preparation and Enumeration of Suspension Tests

Bacterial strains used for bactericidal activity evaluation were cultured in Tryptic Soy Agar (TSA, Biocen, São Paulo, Brazil) and incubated at 37.0 ± 1.0 ºC for 18-24 h followed by a second subculture under the same conditions. A portion of the overnight culture was suspended in 10 mL in PBS and homogenized. The suspensions were adjusted to final concentration of 5 x 10^7 to 5 x 10^9 CFU/mL (Gram-negative bacteria) and 5 x 10^7 to 2 x 10^9 CFU/mL (Gram-positive non-spore forming bacteria) using a VITEK® DENSICHEK® (bioMérieux, Craponne, France). To confirm the inoculum density, the suspension was diluted in PBS and 0.1 mL spread on TSA followed by incubation at 37.0 ± 1.0 ºC for 18-24 h.

For bacteria spore suspension preparation, the strain was cultured in TSA and incubated at 30.0 ± 1.0 ºC for 7-12 days. A second cultivation was prepared and incubated under the same conditions A Wirtz-Conklin coloration was performed to confirm that sporulation had started. A portion of the bacterial grown was suspend in 10 mL of sterilized Milli-Q water and homogenized. The suspension was submitted to serial dilution in PBS and 0.1 mL were spread in TSA, incubated at 30.0 ± 1 ºC for 18-24 h for inoculum confirmation. The spore stock suspension was maintained at 5 ± 3 ºC until the day of the test. Then, the suspension was diluted to a final concentration of 2 x 10^5 to 5 x 10^5 CFU/ mL and 0.5%(v/v) of Skim Milk in PBS. The suspension was serial diluted in Milli-Q water and 0.1 mL was spread in TSA, incubated at 30.0 ± 1 ºC for 18-24 h for inoculum confirmation.

For yeast suspension preparation, the strain was cultured in PDA and incubated at 22.5 ± 2.5 ◦C for 42-48 h. A second cultivation was prepared in the same conditions. A portion of the yeast grown was suspended in 10 mL in PBS and homogenized. The suspensions were adjusted to final concentration of 2 x 10^7 to 1 x 10^8 CFU/mL using a VITEK® DENSICHEK®. The suspension was submitted to serial dilution in PBS and 0.1 mL was spread in PDA, incubated at 22.5 ± 2.5 ºC for 42-48 h for inoculum confirmation.

For fungal conidia suspension preparation, the strain was cultured in PDA and incubated at 22.5 ± 2.5 ◦C for 7-9 days. A second cultivation was prepared in the same conditions. A portion of the fungi grown was suspended in a tube with 10 mL of PBS containing 0.05%$_{(v/v)}$ polysorbate 80 and sterile glass beads. The suspension was vigorous homogenized and filtered through a glass funnel containing hydrophobic cotton. The numberof conidia was determined using a Neubauer chamber and the stock suspension was stored at 5 ± 3 ºC (not more than two days). In the day of the test, the suspension was diluted to a final concentration of 5 x 10^6 to 1 x 10^7 CFU/ mL in 0.5%$_{(v/v)}$ of Skim Milk in PBS. The suspension was submitted to serial dilution in PBS and 0.1 mL was spread in PDA, incubated at 22.5 ± 2.5 ºC for 7-9 days for inoculum confirmation.

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2.3.3. Evaluation of Residue Effect and Efficacy Test

Initially, a preliminary test was conducted to assess the presence of inhibitory activity due to possible residues of chemical agents in the test.

Four carriers of each type (SS and LDP) were placed in one Petri dish and spiked with 50 µL of PBS. After 1 h, the spiking inocula were completely dried and the two carriers of each type were disinfected according to the procedures described in Table 1. Each carrier was added to tubes containing 10 mL of PBS and homogenized for 30 s. For evaluation of residue effect, the inoculum (0.1 mL) of RE carriers for each strain was mixed with the residue (0.1 mL) of each correspondent chemical agent tested and spread in agar plating. For all strains tested, absence of residual effect was considered when the viable count found in the presence of each specific residue was 50-200% of the IC. A schematic diagram of residue preparation is showed in Figure 1A.

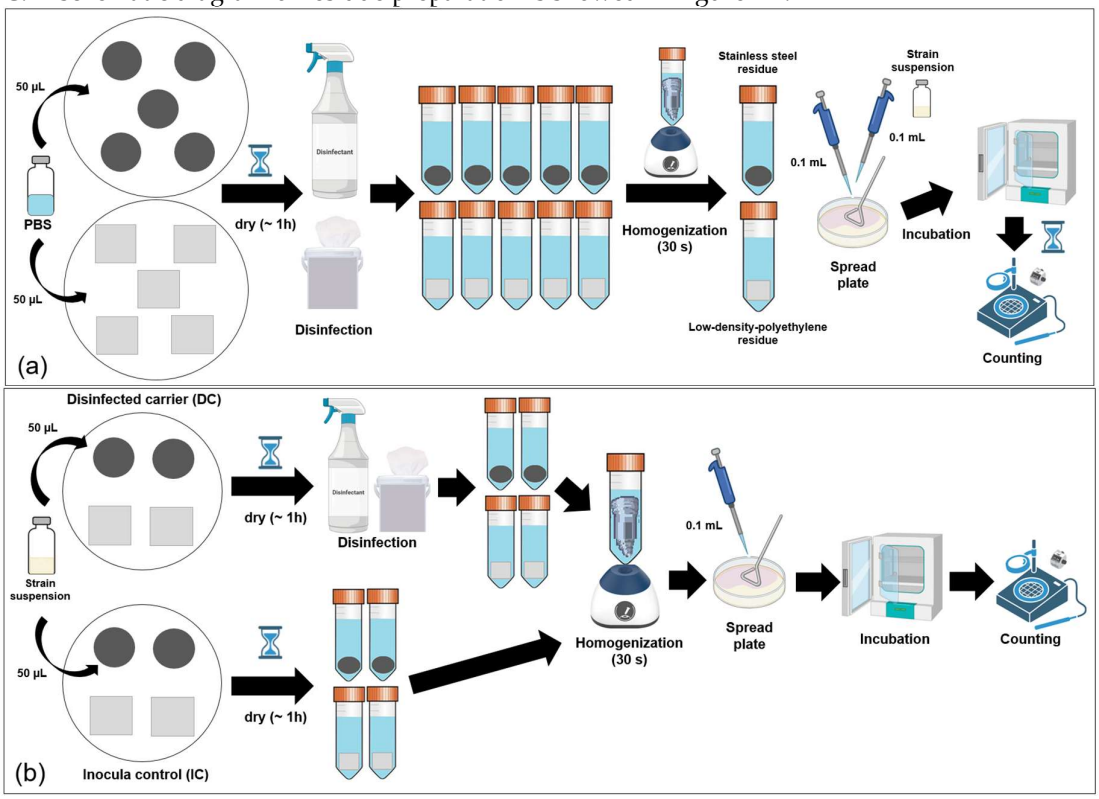

Figure 1. Schematic diagram for preparation of stainless-steel (2.0 cm diameter) and low-density-polyethylene (square pieces of 3.0 cm^2) residue for evaluation of residual effect (a), and of sample spiking in the stainless-steel discs and low-density-polyethylene for disinfectant evaluation (b). PBS: phosphate-buffered saline pH 7.2.

For each strain, eight carriers (four of each type) were spiked with 50 µL of each suspension, spread with a bacteriological loop (Inlab, São Paulo, Brazil) and maintained in the plates opened inside the BSC until completely dry (~ 1h).

For each surface (SS and LDP), two carriers were used as inoculum controls (IC) and two were the disinfected ones (DC) (Figure 1.B). The DC were disinfected according to the procedures described in Table 1. After, each IC and DC carriers was transferred to a tube containing 10 mL of PBS and homogenized for 30 s. The number of colony forming unit (cfu)/carrier was determined by agar plating (TSA for bacteria and PDA for fungi) after serial dilution. Plates were incubated at 37.0 ± 1.0 ºC for 18-24 h for bacteria suspensions, 30.0 ± 1 ºC for 18-24 h for bacteria spores suspensions, 22.5 ± 2.5 ºC for 42-48 h for yeast suspensions, and 22.5 ± 2.5 ºC for 7-9 days for fungal conidia suspensions.

When no colonies were found in any dilution, the assay detection limit was calculated as one cfu in the total volume of lowest dilution inoculated.

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2.3.3. Calculation and Interpretation of the Results

The average of the two carries were calculated. The $\log_{10}$ CFU percent of reduction was calculated by comparing the number of CFU of the non-disinfected (IC) with the disinfected samples (DC), using the following equation:

% reduction ($\log_{10}$ CFU/carrier) = 100 – [(average of disinfected carriers x 100)/(average of inoculum controls carriers)] …… (1)

The results obtained were compared to the criteria described in NF-T-72-281:2014 [13], in PDA Technical Report No. 70 [10] and in the USP [1], as described in Table 2.

Table 2. Description of applications and criteria for evaluating the chemical agent efficacy test on stainless-steel and low-density-polyethylene surfaces.

Reference	Application	Microorganisms	Recommended initial inoculum (log)	Recommended reduction (log)
NF T 72-281:2014 [13]	Evaluation of airborne surface disinfection in the following sectors: human health;) veterinary; and agriculture, food, industry by physical and/or chemical processes	Vegetative bacteria	≥6	≥ 5
		Bacterial spores	≥4	≥ 3
		Yeasts and fungi	≥5	≥ 4
Parenteral Drug Association Technical Report No. 70 [10]	Evaluation of surface disinfection efficacy in production areas of pharmaceutical industries	Non-spore-forming Bacteria and fungi spores	3 to 5	> 1
United States Pharmacopeia [1]		Vegetative bacteria	Not informed	≥ 3
		Bacterial spores		≥ 2

3. Results

3.1. Bacterial and Fungi Strains Selection and Characterization

Between 2012 and 2022, 114 bacterial strains were isolated from cleaning validation tests. The most prevalent genera were *Micrococcus* (n=12; 10.5%), *Sphingomonas* (n=12; 10.5%), *Pseudomonas* (n=11; 9.6%), and *Acinetobacter* (n=11; 9.6%). Regarding spore-producing bacteria, at least one strain from the following genera was identified: *Paenibacillus*, *Alicyclobacillus*, *Bacillus*, *Brevibacillus*, and *Lysinibacillus*. Thirty-nine yeast strains were identified, and the most prevalent genera were *Candida* (n=5; 12.8%) and *Rhodotorula* (n=3; 7.7%). Thirty-three filamentous fungal strains were identified, and the most prevalent genera were *Penicillium* (n=15; 44.5%) and *Cladosporium* (n=5; 15.1%). Based on these results, nine strains were selected from this collection and characterized as described in Table 3.

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Table 3. Characterization of bacterial (n=6), yeast (n=2) and filamentous fungi (n=1) strains selected for the efficacy tests.

Microorganisms and characteristics	**Id.**	**VITEK®2 (%)**	**VITEK®MS (%)**	**Molecular characterization**		
				16S rRNA (bacteria) or D2 (fungi)	Size (bp[1])	NCBI number[2]
Bacterial strains						
Gram-negative bacilli	B0380/16	*Pseudomonas aeruginosa* (99.0)	*Pseudomonas aeruginosa* (99.0)	*Pseudomonas aeruginosa* (99.93)	1,433	OR656695
Gram-negative bacilli	B0747/20	*Acinetobacter baumannii* complex (98.0)	*Acinetobacter haemolyticus* (99.9)	*Acinetobacter haemolyticus* (99.86)/ *A. beijerinckii* (98.82)	1,470	OR656731
Gram-positive cocci	B1464/15	*Micrococcus luteus* (99.9)	*Micrococcus luteus* (99.9)	*Micrococcus luteus* (99.52)/ *M. porci* (98.96)/ *M. endophyticus* (98.94)	1,483	OR656739
Gram-positive cocci	B1472/15	*Kocuria rosea* (99.0)	NI[3]	*Kocuria turfanensis* (99.52)/ *K. oceani* (98.90)	1,484	OR656740
Gram-positive bacilli	B1342/15	*Bacillus cereus/thurigiensis/ mycoides* (88.0)	*Bacillus cereus* group (99.9)	*Bacillus cereus* (99.93)/ *B. paranthracis* (99.86)/ *B. albus* (99.86)/ *B. luti* (99.86)/ *B. nitratireducens* (99.86)/ *B. sanguinis* (99.86)/ *B. dicomae* (99.86)/ *B. basilensis* (99.86)/ *B. wiedmannii* (99.79)/ *B. paramycoides* (99.79)/ *B. tropicus* (99.79)/ *B. anthracis* (99.79)/ *B. proteolyticus* (99.73)/ *B. fungorum* (99.73)/ *B. mobilis* (99.66)/	1,478	OR656749

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				B. pacificus (99.66)/ *B. paramobilis* (99.65)/ *B. toyonensis* (99.59)/ *B. arachidis* (99.57)/ *B. pseudomycoides* (99.52)/ *B. hominis* (99.51)/ *B. mycoides* (99.38)/ *B. gaemokensis* (98.90)		
Gram-positive bacilli	B0284/17	*Bacillus pumilus* (85.0)	*Bacillus pumilus* (99.9)	*Bacillus safensis* subsp. *safensis* (99.86)/ *B. safensis* subsp. *osmophilus* (99.86)/ *B. australimaris* (99.71)/ *B. pumilus* (99.64)/ *B. zhangzhouensis* (99.64)/ B. *altitudinis* (99.43)/ *B. xiamenensis* (99.36)	1,403	OR656689
Fungi strains						
Yeast	L-11	*Candida parapsilosis* (95.0)	*Candida parapsilosis* (99.9)	*Candida parapsilosis* (99.69)/ *Nakaseomyces glabratus* (*Candida glabrata*) (99.69)	320	OR731399
Yeast	L-51	*Cryptococcus laurentii*/ *Rhodotorula mucilaginosa*/*glutinis* (95.0)	*Rhodotorula mucilaginosa* (99.9)	*Rhodotorula mucilaginosa* (99.38)	320	OR731402
Filamentous fungi	F-21/18	NA[4]	NI	*Penicillium lanosocoeruleum* (100)/ *P. chrysogenum* (100)/ *P. goetzii* (100)/ *P. rubens* (100)/ *P. nalgiovense* (100)/ *P. tardochrysogenum*	324	OR734960

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			(100) / *P. westlingii* (100)/ *P. aurantiogriseum* (100)	

[1]- *base pairs;* [2]- *National Center for Biotechnology Information;* [3]- *Not identified (≤75%);* [4]- *Not applied.3.2. Efficacy test.*

All strains were within the acceptance range (50-200%), except for *M. luteus* (B1464/15) with the use of 0.5% hypochlorite on SS (recuperation of 11.7%), *C. parapsilosis* (L-11) with the use of Oxivir TB Wipes on the PBD surface (recuperation of 600%). However, since these results were only identified on these surfaces, for these disinfectants and only with these strains, and not as a systemic effect per strain or type of surface, it was decided to proceed with the efficacy test on those surfaces.

The results obtained in the efficacy tests are presented in Table 4. For non-spore-forming bacteria and yeasts, a reduction of ≥6.04 and ≥5.15 $\log_{10}$ was observed in all chemical agents and surfaces tested, respectively, meeting the recommendations in all standards (Table 2). For bacterial spores, the reduction of the *Bacillus* spp. strain B1342/15 ranged from 0.13 to 2.11 $\log_{10}$. Consequently it only complied with the recommendation of PDA Technical Report No. 70 [10] on the PBD surface using Oxivir TB Wipes. The *Bacillus* spp. strain B0284/17 showed a reduction of ≥ 1.65 $\log_{10}$, meeting the recommendation of PDA Technical Report No. 70 [10]. Using the recommendation of the USP [1], Oxivir TB Wipes on SS surfaces did not meet the recommended reduction (≥ 2 $\log_{10}$). Compared to the recommendations in NF T 72-281:2014 [13], the only disinfectant that showed sporicidal activity was 0.5% peracetic acid on PBD. For the evaluation of the fungicidal activity of the *Penicillium* spp. strain F21/18, there was a reduction in SS of 4.04 and 4.44 $\log_{10}$ with the sporicides peracetic acid and Oxivir TB Wipes, respectively, meeting the standards of NF T 72-281:2014 [13] and PDA Technical Report No. 70 [10]. In PBD surface, a reduction of 3.41 and 3.56 $\log_{10}$ with peracetic acid and Oxivir TB Wipes, respectively, was observed, meeting only the PDA Technical Report No. 70 [10] standard for fungicidal evaluation.

Table 4. Efficacy of chemical agents in stainless-steel and low-density-polyethylene surfaces.

Strain	Product (Contact time)	Surface	$\log_{10}$ CFU[1] (IC[2] carrier)	Reduction $\log_{10}$ (CFU/carrier)
Pseudomonas aeruginosa (B0380/16)	Alcohol 70% (15 min)	SS[3]	7.77	≥ 6.77
		LDP[4]	7.64	6.94
	Sodium hypochlorite 0.5% (15 min)	SS	7.77	7.38
		LDP	7.64	≥ 6.64
	Quaternary ammonium 0.05% (15 min)	SS	7.77	≥ 6.77
		LDP	7.64	7.24
	Oxivir TB Wipes 0.52% (5 min)	SS	7.77	≥ 6.77
		LDP	7.64	6.94
Acinetobacter haemolyticus (B0747/20)	Alcohol 70% (15 min)	SS	7.82	≥ 6.82
		LDP	7.76	7.36
	Sodium hypochlorite 0.5% (15 min)	SS	7.82	≥ 6.82
		LDP	7.76	≥ 6.76
	Quaternary ammonium 0.05% (15 min)	SS	7.82	≥ 6.82
		LDP	7.76	≥ 6.76
	Oxivir TB Wipes 0.52% (5 min)	SS	7.82	7.42
		LDP	7.76	≥ 6.76
Micrococcus luteus (B1464/15)	Alcohol 70% (15 min)	SS	8.92	≥ 7.92
		LDP	7.04	≥ 6.04
	Sodium hypochlorite 0.5% (15 min)	SS	8.92	≥ 7.92
		LDP	7.04	≥ 6.04
	Quaternary ammonium 0.05% (15 min)	SS	8.92	≥ 7.92
		LDP	7.04	≥ 6.04
	Oxivir TB Wipes 0.52% (5 min)	SS	8.92	≥ 7.92
		LDP	7.04	≥ 6.04

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Kocuria spp. (B1472/15)	Alcohol 70% (15 min)	SS	7.34	≥ 6.34
		LDP	7.21	≥ 6.35
	Sodium hypochlorite 0.5% (15 min)	SS	7.34	≥ 6.34
		LDP	7.21	≥ 6.35
	Quaternary ammonium 0.05% (15 min)	SS	7.34	≥ 6.34
		LDP	7.21	≥ 6.35
	Oxivir TB Wipes 0.52% (5 min)	SS	7.34	≥ 6.34
		LDP	7.21	≥ 6.35
Bacillus spp. (B1342/15)	Peracetic acid 0.5% (10 min)	SS	4.55	0.13
		LDP	4.91	0.61
	Oxivir TB Wipes 0.52% (5 min)	SS	4.55	0.55
		LDP	4.91	2.11
Bacillus spp. (B0284/17)	Peracetic acid 0.5% (10 min)	SS	4.50	2.40
		LDP	4.54	3.54
	Oxivir TB Wipes 0.52% (5 min)	SS	4.50	1.65
		LDP	4.54	2.54
Candida parapsilosis (L-11)	Alcohol 70% (15 min)	SS	6.58	≥ 5.58
		LDP	6.38	≥ 5.38
	Sodium hypochlorite 0.5% (15 min)	SS	6.58	≥ 5.58
		LDP	6.38	≥ 5.38
	Quaternary ammonium 0.05% (15 min)	SS	6.58	≥ 5.58
		LDP	6.38	≥ 5.38
	Oxivir TB Wipes 0.52% (5 min)	SS	6.58	≥ 5.58
		LDP	6.38	≥ 5.38
Rhodotorula mucilaginosa (L-51)	Alcohol 70% (15 min)	SS	6.20	≥ 5.20
		LDP	6.15	≥ 5.15
	Sodium hypochlorite 0.5% (15 min)	SS	6.20	≥ 5.20
		LDP	6.15	≥ 5.15
	Quaternary ammonium 0.05% (15 min)	SS	6.20	≥ 5.20
		LDP	6.15	≥ 5.15
	Oxivir TB Wipes 0.52% (5 min)	SS	6.20	≥ 5.20
		LDP	6.15	≥ 5.15
Penicillium spp. (F21/18)	Peracetic acid 0.5% (10 min)	SS	5.44	4.04
		LDP	4.41	3.56
	Oxivir TB Wipes 0.52% (5 min)	SS	5.44	4.44
		LDP	4.41	3.41

[1]- Colony forming unit; [2]- inoculum control; [3]- stainless-steel; [4]- low-density-polyethylene.

4. Discussion

The purpose of an industrial cleaning and disinfection program is not only to control microbial contamination, but also to serve as corrective action [10]. Many aspects need to be considered when selecting a chemical agent for using in a pharmaceutical manufacturing area. These include the number and types of microorganisms to be controlled; the spectrum of activity of commercially available chemical agents; the concentration, application method, and contact time of the disinfectant. The nature of the surface material being disinfected and its compatibility with the disinfectant is also important due to the corrosiveness of the chemical agents to equipment with repeated application. Planned disinfectant rotation and the steps that need to be taken to avoid the contamination of pharmaceutical products by a chemical agent must also be considered [1]. In this study, five chemical agents with different classifications were tested, using the concentrations and contact time preconized in the cleaning and disinfecting programme of the pharmaceutical facility (Table 1).

The emergence of antimicrobial resistance to antibiotics is a well-established and extensively documented phenomenon [14]. In contrast, the development of resistance to disinfectants is generally considered less probable at clinically or industrially relevant levels, given that disinfectants act as broad-spectrum biocidal agents with greater lethality than antibiotics. Moreover, these agents are typically applied at high concentrations to relatively low numbers of microorganisms, mainly in

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classified (grade A, B or C areas) that are often in a metabolically inactive state, thereby reducing the selective pressure that drives antimicrobial resistance development [1]. Nevertheless, microorganisms most frequently recovered through environmental monitoring programmes should be periodically evaluated through susceptibility testing against the disinfectants employed in the CCS. This practice is necessary due to the interspecies variability in tolerance to different biocidal agents. It also supports ongoing verification of disinfectant efficacy against the resident microbiota in each specific manufacturing environment and should include various surface types [1]. In the present study, six bacterial and three fungal strains isolated from cleaning validation tests at a pharmaceutical facility were selected as representing the most persistent strains in this environmental. These strains were characterized using a polyphasic approach, including molecular characterization (Table 3), in order to assure the strains identity. However, some strains as B1342/15 (*Bacillus* spp.) and F-21/18 (*Penicillium* spp.) could not be identified until species level. The difficulty in identified *Bacillus* and related genus strains isolated from pharmaceutical environmental were already reported in other studies [15–17]. This is due to high similarity in phenotypical characteristic and 16S rRNA sequence within the *Bacillus cereus* group. [18]. Identification of filamentous fungi is also laborious and not performed in many laboratories [19]. Phenotypic characterization methods, based mainly on macroscopic features of the culture and microscopic features of the reproductive structures, are subjective and need mycologist with experience for analysis. The alternative automated commercial systems as MALDI-TOF MS and internal transcribed spacer regions and/or D2 LSU rDNA domain sequencing can be used, but they generally also do not achieve a species identification result due to database limitations [20].

The efficacy test must have realistic acceptance criteria [1]. According to PDA Technical Report No. 70 [10], as normal clean room bioburden level is very low, the inoculum levels for testing would ideally depict levels seen in the controlled area. However, as this would not be practical in a test environment, a higher inoculum level should be used though it should not exceed 10^5 cfu/ml. In this study, in-house micro-organisms with high inocula (4.55 to 8.92 $\log_{10}$ CFU) were tested, but these cannot represent the real scenario in clean rooms. However, even using high levels, all disinfectants, in respectively contact time, tested showed bactericidal and yeasticidal activity (reduction ≥5.15 $\log_{10}$ CFU), and sporicidal activity (reduction ≥3.41 $\log_{10}$ CFU) against fungi spores (Table 4). The suggested minimum log reduction recommended by PDA [10] is > 1 log (Table 2). This was achieved against *Bacillus* spp. strain B0284/17 (reduction ≥1.65 $\log_{10}$ CFU) but not for *Bacillus* spp. B1342/15 using peracetic acid 0.5% and Oxivir TB Wipes 0.52% on SS surface (Table 4).

A sporicide is a compound that destroys all vegetative micro-organisms and bacterial and fungal spores [9,10]. Bacterial spore structures are important for protective role in biocide resistance [21,22]. In the present study, the sporicidal efficacy of peracetic acid 0.5% and Oxivir TB Wipes were tested (Table 1) against bacterial and fungi spores. Peracetic acid 0.5%/10 min was not sufficient for elimination of *Bacillus* B1342/15 spores (reduction of 0.13 to 2.11 $\log_{10}$ CFU), but was more effective against *Bacillus* B0284/17 (reduction of 1.65 to 3.54 $\log_{10}$ CFU). These results may be due to interspecies variation to the disinfectants [21]. Ceccanti et al. [6] evaluated the sporicidal effect against *B. subtilis* ATCC 6633, *B. cereus* ABIO 845 and *B. sphaericus* ABIO 229 (environmental in-house isolates). The authors reported that a 70% suitable dilution of the ready-to-use peracetic acid solution (commercially available 0.08% peracetic acid and 1.0% hydrogen peroxide solution) with a contact time of 10 min was effective (>2 log spore reduction) in both clean and dirty conditions on Teflon, linoleum, polycarbonate and SS surfaces. The findings of the present study are consistent with those reported by André et al. [23], who demonstrated that sporicidal efficacy may significantly differ between planktonic and surface-adhered spores. Similarly, the results from this study showed limited log reductions of Oxivir TB Wipes 0.52% for *Bacillus* spp. spores, despite adequate performance against vegetative cells and yeasts (Table 4). This fact showed that surface-associated spores may demonstrate enhanced tolerance due to limited disinfectant penetration, reduced metabolic activity, and potential protective surface interactions [21].

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These data reinforce that cleaning and disinfection validation protocols based solely on suspension tests (not using carriers simulating the surfaces) may overestimate real-world efficacy. Furthermore, the inter-strain variability observed among *Bacillus* strains highlights the importance of incorporating in-house environmental strains into disinfectant efficacy studies, as recommended by USP [1] and PDA Technical Report 70 [10].

5. Conclusions

In conclusion, all disinfectants tested showed bactericidal and yeasticidal activity against in-house strains (n=6) on SS and LDP surfaces. Disinfection with peracetic acid 0.5% for 10 min was not sufficient to eliminate bacterial spores of one *Bacillus* strain in both surfaces. Oxivir TB Wipes was not effective to eliminate bacterial spores on SS surface. For these cases, other studies are necessary for determination of the optimum concentration and contact time. These data are important to complement individual risk assessments of biological production processes, and contribute to CCS in the industry facility.

The findings of the present study emphasizes that chemical agents efficacy must be demonstrated under conditions representative of actual manufacturing environments. In alignment with these recommendations, the current study employed carrier-based testing on SS and LDP surfaces, thereby simulating real environmental conditions. The log reductions observed for *Bacillus* spp. B1342/15 strain highlights that spore-forming microorganisms can exhibit increased tolerance when attached to surfaces.

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Data Availability Statement: The sequencing data for the 16S rRNA and ITS genes, which supports the findings of this study, have been deposited in the National Center for Biotechnology Information (NCBI) repository. Accession numbers are listed in Table 3. All other data are included within this article.

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Conflicts of Interest: Stephen James Forsythe is employed by Foodmicrobe.com Ltd., the remaining authors declare that they have no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

CCS	Contamination control strategy
EMA	European Medicines Agency
EU	European Union
GMP	Good Manufacturing Practice
LDP	Low-density-polyethylene
PDA	Parenteral Drug Association
SS	Stainless-steel
USP	United States Pharmacopeia

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