BJPS

Brazilian Journal of Pharmaceutical Sciences

http://dx.doi.org/10.1590/s2175-97902024e23664

Article

Culturable microbiological profile of a non-sterile drugs pharmaceutical production environment

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For places where non-sterile drug production occurs, regulatory bodies recommend monitoring of the environmental bioburden. This procedure provides information regarding possible microbiological risks to which the products may be exposed, so that subsequent action measures may be implemented. The aim of the present work was to quantify and characterize the microorganisms present in Grade D (ISO 8) cleanrooms of a Brazilian pharmaceutical industry, identifying any possible seasonal climatic influences on these environments. Sampling was performed by surface and air monitoring, over 12 months during the year 2019, in rooms that were in operation. For both sampling methods, no statistically significant differences in bacteria and fungi counts were found between months or seasonal periods. Microorganisms that presented higher incidence included *Staphylococcus epidermidis* (15%) and *Micrococcus* spp. (13%), common to the human microbiota, and the fungi *Cladosporium* sp. (23%) and *Penicillium* sp. (21%), typical of the external environment. The results showed that microbial contamination in the Grade D cleanrooms was within the permissible maximum levels and remained similar throughout the year. Microbiological quality control in the clean areas of the pharmaceutical industry investigated was considered effective, with regular maintenance being necessary to keep bioburden levels controlled.

Keywords: Microbiota, environmental monitoring, cleanroom, airborne microbes, indoor air, microbiological air quality.

INTRODUCTION

Monitoring and control of the bioburden inside the pharmaceutical industrial environment is essential to ensure the microbiological safety of the products at the end of the production chain. For this reason, regulatory agencies stipulate maximum acceptable values for microbial contamination of the air and surfaces in these environments, according to different cleanroom classifications. In Brazil, these limits are established by Normative Instruction No. 35/2019 of the Brazilian Health Regulatory Agency (Anvisa), (2019), which resembles ISO 14644-1 (ISO, 2015), except for a distinction regarding the use of the environments, since according to the Brazilian regulations they can be classified as "at rest" or "in operation".

The analysis of environmental microbiological monitoring data makes it possible to identify trends in the growth of microbial populations, examine their causes and origins to enable them to be contained, determine the presence of potential pathogens, and select appropriate sanitizers for use in cleanrooms (Ashour, Mansy, Eissa, 2011; Mahnert *et al.*, 2015; Sandle, 2011).

Despite these efforts, according to the Food and Drug Administration, 211 types of drugs were recalled from the market between 2018 and 2019 in the United States. Of these, 13% occurred due to microbiological contamination (FDA, 2020). This raises concern, since the pharmaceutical industrial environment exerts selective pressure on the microorganisms present there, especially in antibiotic-producing facilities, which can selectively favor those organisms that are more tolerant to abiotic

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stresses such as low humidity, low water activity, antibiotics, and sanitizers (Hamdy *et al.*, 2018).

In addition, external factors such as equipment, seasonality, and the transit of people and supplies between environments can potentially influence the transfer of microorganisms found in cleanrooms (Kawai *et al.*, 2019; Pacchioni *et al.*, 2018; Park *et al.*, 2014). This reinforces the need for continuous monitoring, taking into consideration the possible variations in the microbiological profiles of cleanrooms between industries in different locations or producing different products. However, environmental monitoring studies in pharmaceutical production areas are scarce, especially regarding the evaluation of bioburdens in Brazilian pharmaceutical industry cleanrooms. Therefore, the objective here was to assess the culturable microbiota profiles in production environments (Grade D / ISO 8) of a pharmaceutical industry located in the interior of São Paulo state, Brazil, identifying seasonal trends and determining the primary microbial contaminants present in the areas evaluated.

MATERIAL AND METHODS

Pharmaceutical manufacturing facility

From January to December 2019, monthly air and surface sampling was performed in areas where there was production of oral liquid medicines and tablets (Grade D cleanrooms), in a pharmaceutical industrial facility located in the metropolitan region of Campinas, São Paulo state, Brazil (a subtropical region). The number and locations of sampling sites were defined considering the areas of the rooms and the most critical contamination points. As a result, 34 air and 66 surface sampling locations were established, distributed among 33 rooms in 5 production buildings. The monitoring was only carried out when the rooms were in operation and production was in progress.

Surface and air sampling procedures

For surface monitoring, samples were collected using RODAC® (Laborclin, Brazil) contact plates containing the following formulation: TSA (Trypticase soy agar, Difco, USA), soy lecithin (Thermo Fisher Scientific, USA), and Tween 80 (INLAB, Brazil). The test consisted of placing the culture medium on the desired flat surface and gently rotating the plate three times, ensuring that the entire agar was exposed to the sampled surface.

Air monitoring was performed according to the active method, in production areas where the product or raw material had some degree of exposure to the environment. The equipment used was an air sampler for viable particles (AIR IDEAL® 3P®, bioMérieux, Crapone, France), adjusted to sample 1000 L of air per collection (at a flow rate of 100 L/min for 10 min), with the sample being deposited in a Petri dish containing TSA medium. The plates were incubated at 20-25 ºC for 3-5 days, after which they were transferred to an incubator where they were kept at 30-35 ºC for a further 2-3 days, according to procedures established by the company.

Microbial quantification and identification

At the end of the incubation period, the Petri dishes were evaluated and for those that showed microbial growth, the numbers of colony-forming units (CFU) were counted and tabulated. For air sampling, the values were expressed in CFU/m³, while units of CFU/25 cm² were used for the surface sampling data. After counting, the CFUs were distinguished morphologically, before being subjected to the identification process. Only the yeast or bacteria colonies that presented morphological differences were selected for replating on TSA agar and were later submitted to Gram staining, followed by identification using biochemical kits, according to the manufacturer's specifications (API®, bioMérieux, Crapone, France). The selection of isolates was performed similarly for the filamentous fungi colonies, with the samples being sent to a specialized company for microbial identification. The identified microorganisms were ranked annually and monthly, according to their incidence.

Collected data grouping

The sampling results were grouped into different periods to enable understanding of the influence of

external climatic conditions on the microbiota found in the production areas. The first grouping was according to the collection months, resulting in twelve distinct groups (January to December). The second organization of the data was according to temperature and historical rainfall volume for the Campinas region (CEPAGRI, 2019), resulting in two groups: dry period (April to September) and rainy period (October to March).

To obtain an overall profile of the microbiota in the industrial plant, the microorganisms were grouped by type (bacteria and fungi, where the latter included filamentous fungi and yeasts) and the ten most frequent microorganisms in each group were identified. For analysis of the monthly frequency pattern in relation to the overall profile for the industrial plant, the data were separated by month and organized in a similar way as described above, identifying the five most frequent microorganisms in each month.

Statistical analysis

The data were grouped according to month, rain conditions, and microorganisms (total microorganisms, bacteria, or fungi). Considering the rain conditions in 2019, the data were divided into two periods: the dry period (April to September, mean precipitation ≈ 44.65 mm) and the rainy period (October to March, mean precipitation ≈ 131.6 mm) (CEPAGRI, 2019).

The normality of the data distribution was evaluated using the Shapiro-Wilk test and the data were considered nonparametric. The dependent variable, CFU counts, was analyzed considering the type of sampling, monthly periods, and seasonal periods as independent variables. These comparisons employed the Mann-Whitney or Kruskal-Wallis tests. The significance level adopted was 5% ($\alpha = 0.05$). All the calculations were performed using GraphPad Prism v. 8.0 software (GraphPad, San Diego, California, USA).

RESULTS

Microbial levels in air and surface samples

The number of air and surface samplings varied during the year, with 27 to 33 monthly collection points for air sampling and 50 to 65 points for surface sampling. This numerical variation was due to the non-use of these areas, since unused areas were not sampled to quantify viable particles and/or for microbial identification.

Over the year, 684 surface samples and 361 air samples were collected. Comparing the medians for the sampling types (air and surface), statistically significant differences were found between them for total counts (bacteria + fungi) ($p < 0.0001$), bacteria counts ($p < 0.0001$), and fungi counts ($p < 0.0001$) (Mann-Whitney test). In the case of the total counts (Figure 1A), the median and interquartile range (IQR) for the air samples (median = 2 and IQR = 8) were higher than for the surface samples (median = 0 and IQR = 2). The same was observed for the bacteria counts (Figure 1B), with both parameters presenting higher values for the air samples (median = 1 and IQR = 4.5), compared to the surface samples (median = 0 and IQR = 1). For the fungi counts (Figure 1C), the median for the air samples (median = 0 and IQR = 1) was similar to the surface samples (median = 0 and IQR = 0), with a difference only in the IQR value.

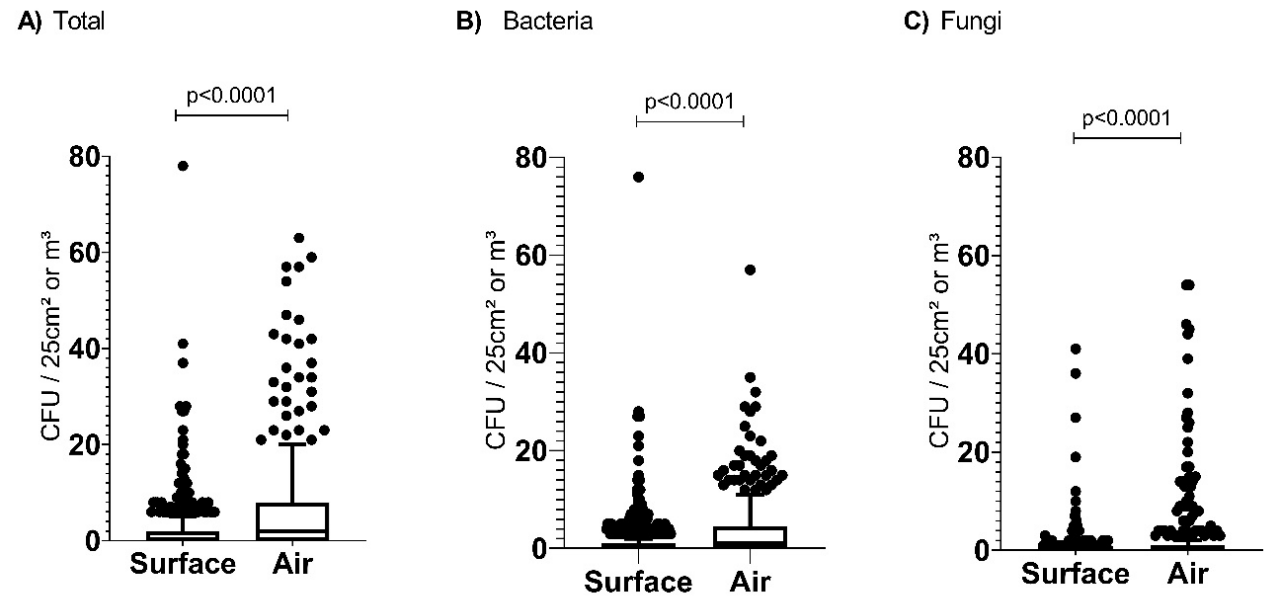

FIGURE 1 - Distribution of microorganism counts for sampling of surfaces (CFU/25 cm²) and air (CFU/m³) in the factory environments throughout the year (684 surface samples and 361 air samples): (A) total microorganisms, (B) bacteria, and (C) fungi. Data were compared using the Mann-Whitney test ($p > 0.05$).

For the air sampling, considering the total CFU/m³ data (bacteria and fungi) over 12 months (Figure 2A), the monthly median values ranged from 0.5 to 4, with the lowest value in December and the highest values in March and May. There was a slight variation among the medians, with some discrepant points, but no statistical difference was generally observed among the months ($p = 0.1596$; Kruskal-Wallis test).

When the groups of microorganisms were analyzed individually, the medians for the total quantification of bacteria (Figure 2B) ranged between 0 and 3, with the lowest value in December and the highest values in March, April, and May. No significant differences were detected during the period analyzed ($p = 0.0506$; Kruskal-Wallis test). For fungi counts (Figure 2C), the median of all months was 0 and no significant differences were detected during the period analyzed ($p = 0.8318$; Kruskal-Wallis test).

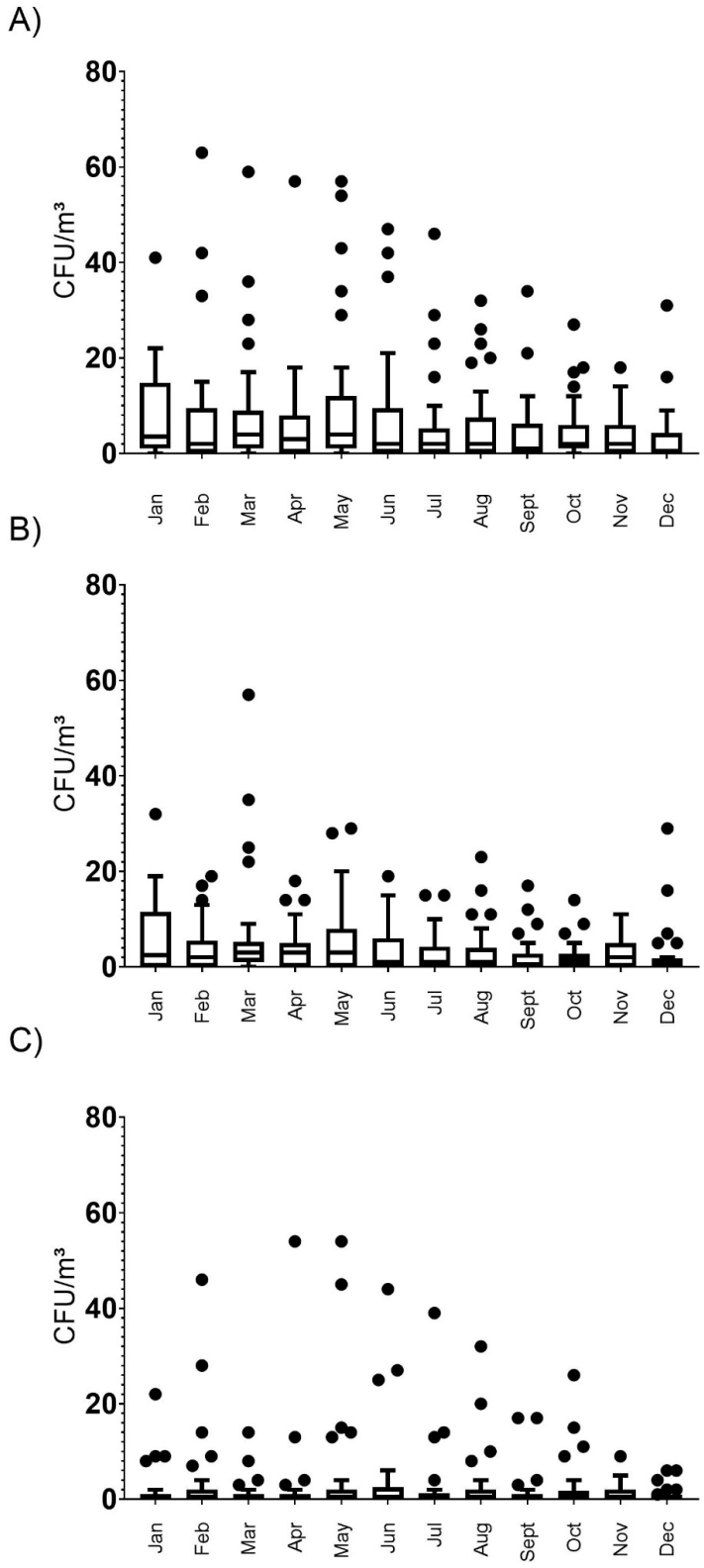

FIGURE 2 - Distributions of counts of (A) total microorganisms, (B) bacteria, and (C) fungi sampled in the air (CFU/m³) of the factory environments in the different months. Box plots representing the median, 1st and 3rd quartiles, upper and lower limits, and outliers. Data were compared using the Kruskal-Wallis test ($p > 0.05$), obtaining the values (A) $p = 0.1596$, (B) $p = 0.0506$, and (C) $p = 0.8318$, respectively. The numbers (n) of samples obtained per month were 28 (January), 33 (February), 30 (March), 31 (April), 33 (May), 29 (June), 30 (July), 32 (August), 28 (September), 32 (October), 27 (November), and 28 (December).

In the case of the surface monitoring, the monthly median values (total CFU/25 cm²) were 1 for July and 0 for the other eleven months (Figure 3A). No statistically significant differences were detected (p = 0.1396; Kruskal-Wallis test). Individually, bacteria (Figure 3B) and fungi (Figure 3C) showed a median of 0. For the total quantification of fungi, the 3rd quartile of all months was equal to zero, indicating that the presence of this group of microorganisms was not detected in 75% of the surface samples. No significant differences were detected between the bacteria group (p = 0.1521; Kruskal-Wallis test) and the fungi group (p = 0.2835; Kruskal-Wallis test) during the period analyzed.

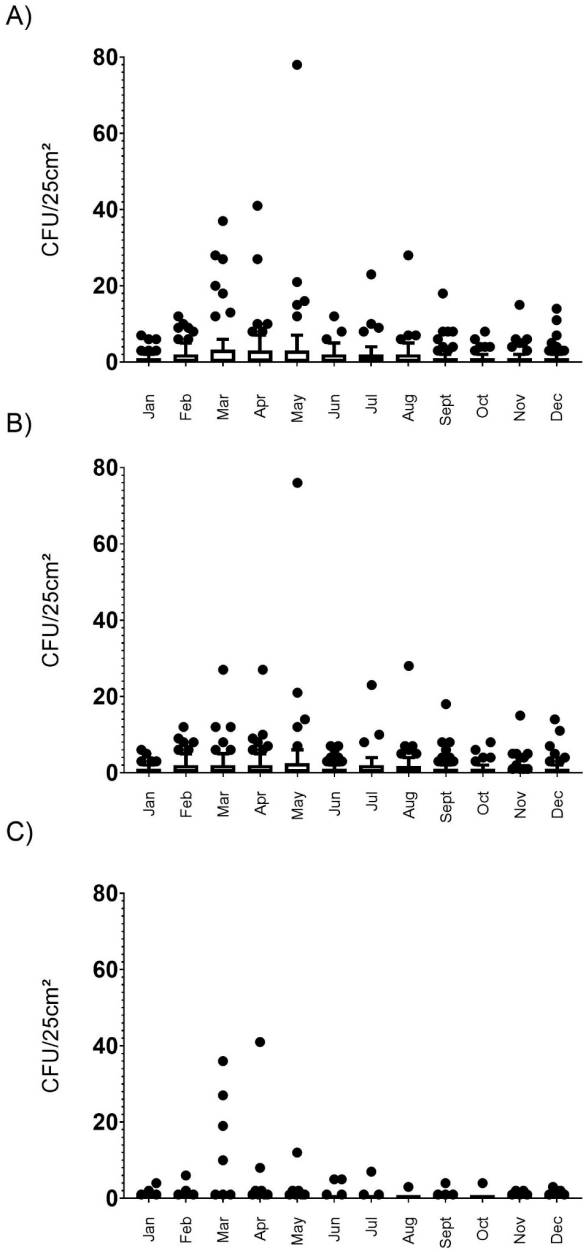

FIGURE 3 - Distributions of counts of (A) total microorganisms, (B) bacteria, and (C) fungi sampled on the surfaces (CFU/25 cm²) of the factory environments in the different months. Box plots representing the median, 1st and 3rd quartiles, upper and lower limits, and outliers. Data were compared using the Kruskal-Wallis test (p > 0.05), obtaining the values (A) p = 0.1396, (B) p = 0.1521, and (C) p = 0.2835, respectively. The numbers (n) of samples obtained per month were 52 (January), 64 (February), 54 (March), 60 (April), 65 (May), 57 (June), 59 (July), 60 (August), 51 (September), 60 (October), 50 (November), and 52 (December).

Influence of seasonality on the culturable environmental microbiota

Investigation was made of whether seasonal variability (rainfall conditions) might influence the pattern of microbial levels. For this, the air and surface sampling data were grouped according to the rainy period (October, November, December, January, February, and March) and the dry period (April, May, June, July, August, and September). For surface measurements, a total of 352 samples were obtained during the dry period and 332 during the rainy period (Figure 4). For air monitoring (Figure 5), 183 samplings were performed during the dry period and 178 during the rainy period.

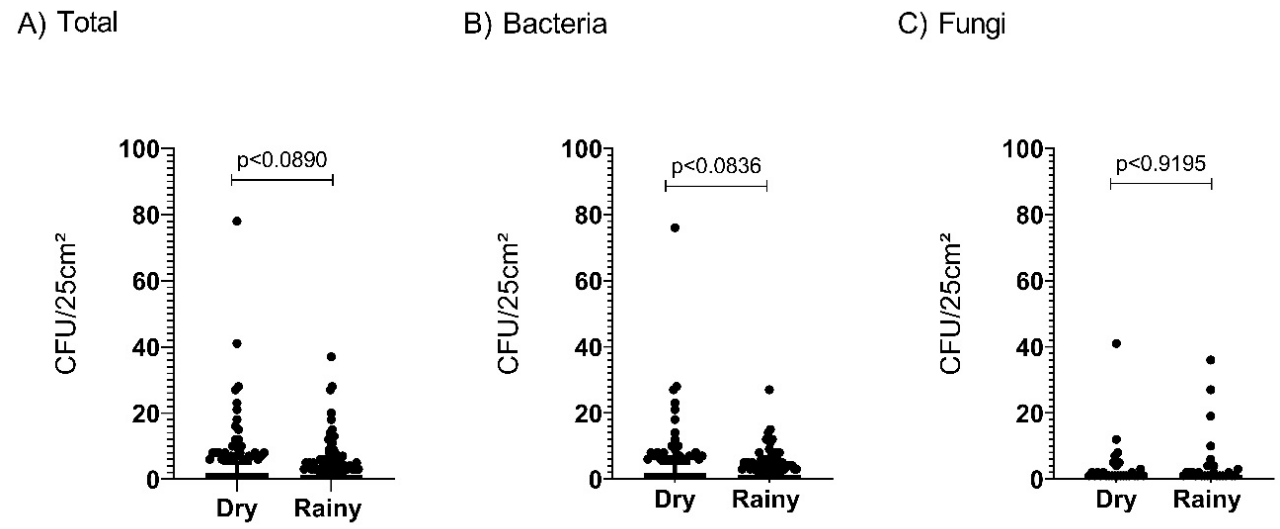

FIGURE 4 - Distributions of the total counts of microorganisms sampled on the factory environment surfaces (CFU/25 cm²) during the dry period (April, May, June, July, August, and September) and the rainy period (January, February, March, October, November, and December). A total of 352 samples were obtained for the dry period and 332 for the rainy period. The data were compared using the Mann-Whitney test ($p > 0.05$). For both periods, the median of the three groups was 0.

Analysis of the air sampling data showed that the medians for the dry and rainy periods were not statistically different for any of the groups evaluated, considering the total count ($p < 0.0890$), bacteria count ($p < 0.0836$), and fungi count ($p < 0.9195$) (Mann-Whitney test). The same was found for the surface sampling data, with no differences between seasonal periods for total count ($p < 0.8505$), bacteria count ($p < 0.6170$), and fungi count ($p < 0.5854$) (Mann-Whitney test).

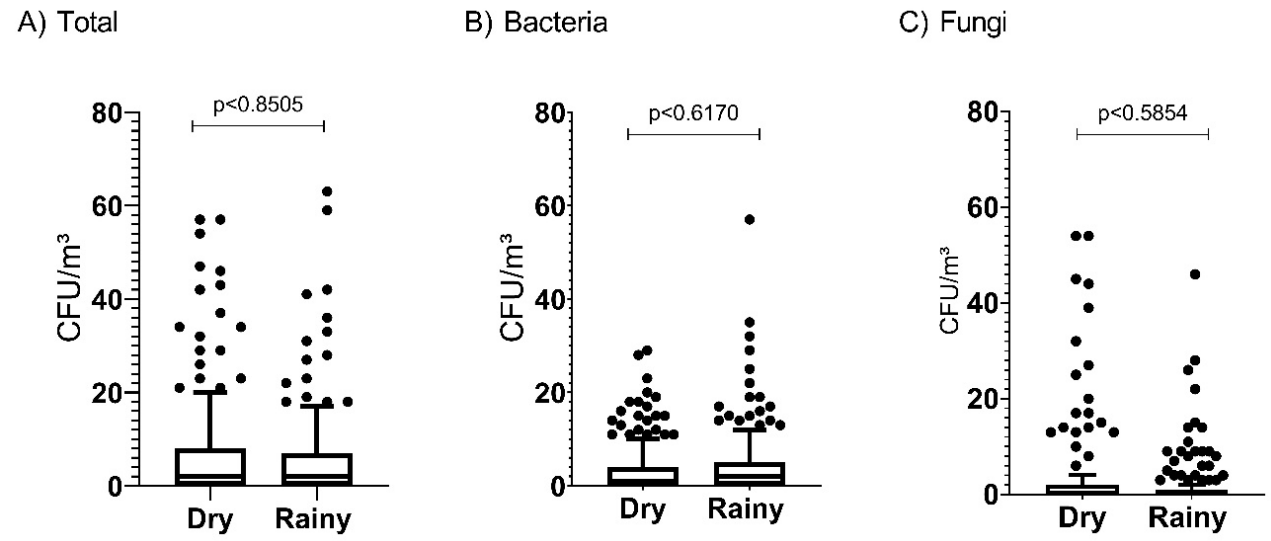

FIGURE 5 - Distributions of the total counts of microorganisms sampled in the factory indoor air environment (CFU/m³) during the dry period (April, May, June, July, August, and September) and the rainy period (January, February, March, October, November, and December). A total of 183 samples were obtained for the dry period and 178 for the rainy period. The data were compared using the Mann-Whitney test ($p > 0.05$). (A) Median of 2 for both periods. (B) Medians of 1 and 2 for the dry and rainy periods, respectively. (C) Median of 0 for both periods.

Identification of cultured microorganisms

During the entire period analyzed, a total of 3558 CFUs, including both bacteria and fungi, were counted for the two sampling methods. From this total, 1339 microorganisms were identified, where 975 were bacteria and 364 were fungi, consequently providing information for approximately 37.6% of the culturable microbiota of the environments tested (Table I). Based on these data, the most frequently occurring microorganisms in the industrial plant were ranked by group (Figures 6 and 7). The main bacteria genera found were *Staphylococcus*, *Micrococcus*, and *Bacillus* (Figure 6A), with the most abundant representative species being *Staphylococcus epidermidis* (174; 15%), *Micrococcus* spp. (131; 13%), and *Bacillus megaterium* (48; 5%) (Figure 6B). For the fungi group (Figure 7), the identifications were mostly at the genus level, with the most frequent being *Cladosporium* sp. (85; 23%), *Penicillium* sp. (76; 21%), and *Curvularia* sp. (52; 14%). Finally, the five microorganisms of highest incidence throughout the year were ranked for both groups. In the bacteria group (Table II), *Staphylococcus epidermidis* was found in all months, together with *Micrococcus* spp. (except for March). In the fungi group (Table III), *Penicillium* spp. was found in all months except December, while *Alternaria* spp. was present in all months except June and September.

TABLE I - Percentage of identifications in relation to the total number of CFUs counted

	Total			Bacteria			Fungi		
	CFUs	**IDs**	**%**	**CFUs**	**IDs**	**%**	**CFUs**	**IDs**	**%**
January	265	100	37,7%	202	79	39,1%	63	21	33,3%
February	351	106	30,2%	223	74	33,2%	128	32	25,0%
March	453	132	29,1%	321	88	27,4%	132	44	33,3%
April	373	105	28,2%	235	96	40,9%	138	9	6,5%
May	562	138	24,6%	385	115	29,9%	177	23	13,0%
June	292	126	43,2%	159	82	51,6%	133	44	33,1%
July	279	111	39,8%	189	76	40,2%	90	35	38,9%
August	282	130	46,1%	192	97	50,5%	90	33	36,7%
September	188	112	59,6%	133	75	56,4%	55	37	67,3%
October	200	126	63,0%	118	85	72,0%	82	41	50,0%
November	156	88	56,4%	118	63	53,4%	38	25	65,8%
December	157	65	41,4%	127	45	35,4%	30	20	66,7%
TOTAL	3558	1339	37,6%	2402	975	40,6%	1156	364	31,5%

IDs: number of identifications. %: percentage of identifications in relation to the number of CFUs counted in the period.

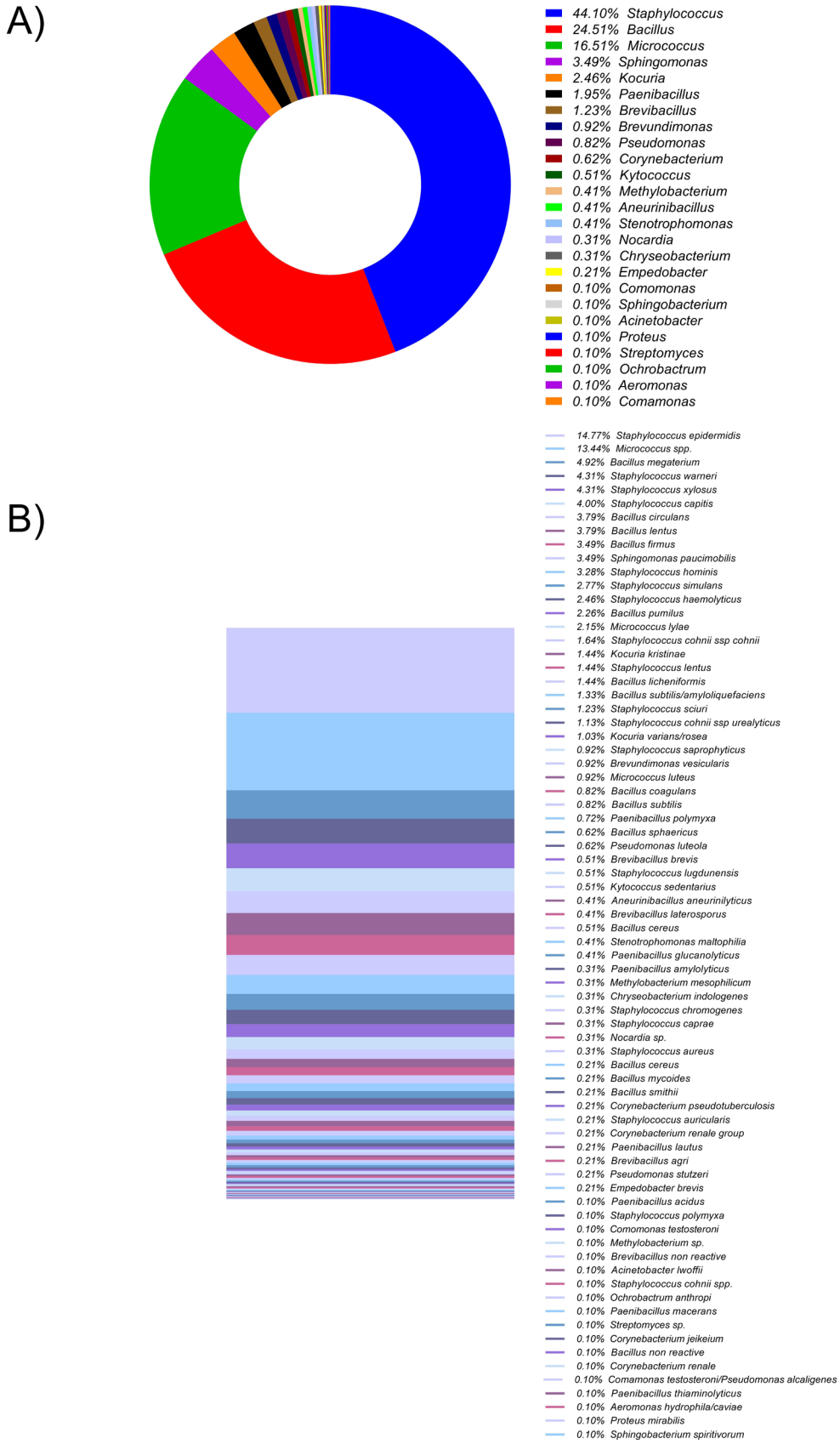

FIGURE 6 - Diversity of bacteria identified throughout the year: (A) genera and (B) species. The data are shown as percentages in relation to the total number of bacteria colonies identified (975).

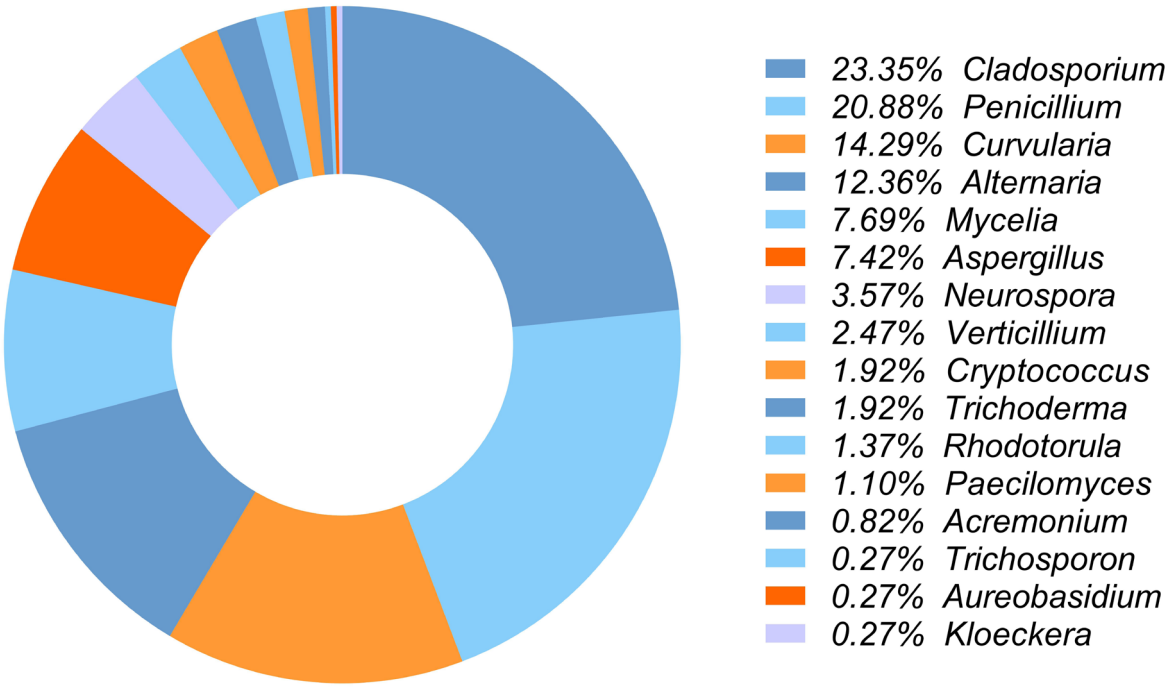

FIGURE 7 - Diversity of fungi genera identified throughout the year. The data are shown as percentages in relation to the number of fungi colonies identified (364).

TABLE II - Bacteria with higher incidence during the months

Month	Microorganism	IDs	%	Month	Microorganism	IDs	%
January	*Micrococcus spp.*	13	16,5%	July	*Micrococcus spp.*	16	21,1%
	Staphylococcus hominis	7	8,9%		*Staphylococcus epidermidis*	12	15,8%
	Staphylococcus haemolyticus	7	8,9%		*Staphylococcus warneri*	6	7,9%
	Staphylococcus epidermidis	7	8,9%		*Bacillus firmus*	4	5,3%
	Staphylococcus capitis	5	6,3%		*Bacillus megaterium*	4	5,3%
	6th onwards	40	50,6%		6th onwards	34	44,7%
February	*Staphylococcus simulans*	12	16,2%	August	*Staphylococcus epidermidis*	21	21,6%
	Bacillus pumilus	10	13,5%		*Bacillus firmus*	11	11,3%
	Micrococcus lylae	9	12,2%		*Micrococcus spp.*	9	9,3%
	Staphylococcus epidermidis	6	8,1%		*Staphylococcus warneri*	8	8,2%
	Staphylococcus warneri	4	5,4%		*Staphylococcus xylosus*	6	6,2%
	6th onwards	33	44,6%		6th onwards	42	43,3%

TABLE II - Bacteria with higher incidence during the months

Month	Microorganism	IDs	%	Month	Microorganism	IDs	%
March	*Staphylococcus epidermidis*	13	14,8%	September	*Staphylococcus epidermidis*	13	17,3%
	Micrococcus lylae	11	12,5%		*Micrococcus spp.*	13	17,3%
	Staphylococcus simulans	6	6,8%		*Bacillus circulans*	8	10,7%
	Bacillus megaterium	5	5,7%		*Sphingomonas paucimobilis*	5	6,7%
	Bacillus sphaericus	4	4,5%		*Bacillus lentus*	5	6,7%
	6th onwards	49	55,7%		6th onwards	31	41,3%
April	*Micrococcus spp.*	20	20,8%	October	*Micrococcus spp.*	14	16,5%
	Staphylococcus epidermidis	12	12,5%		*Staphylococcus epidermidis*	13	15,3%
	Staphylococcus capitis	4	4,2%		*Staphylococcus xylosus*	8	9,4%
	Bacillus megaterium	4	4,2%		*Sphingomonas paucimobilis*	6	7,1%
	Staphylococcus cohnii ssp cohnii	4	4,2%		*Staphylococcus hominis*	5	5,9%
	6th onwards	52	54,2%		6th onwards	39	45,9%
May	*Staphylococcus epidermidis*	18	15,7%	November	*Staphylococcus epidermidis*	11	17,5%
	Micrococcus spp.	16	13,9%		*Micrococcus spp.*	10	15,9%
	Bacillus circulans	10	8,7%		*Bacillus megaterium*	6	9,5%
	Staphylococcus xylosus	10	8,7%		*Staphylococcus hominis*	5	7,9%
	Bacillus lentus	9	7,8%		*Bacillus circulans*	5	7,9%
	6th onwards	52	45,2%		6th onwards	26	41,3%
June	*Staphylococcus epidermidis*	13	15,9%	December	*Micrococcus spp.*	11	24,4%
	Bacillus megaterium	11	13,4%		*Staphylococcus epidermidis*	5	11,1%
	Staphylococcus warneri	5	6,1%		*Bacillus lentus*	5	11,1%
	Micrococcus spp.	4	4,9%		*Staphylococcus capitis*	4	8,9%
	Staphylococcus xylosus	4	4,9%		*Bacillus firmus*	3	6,7%
	6th onwards	45	54,9%		6th onwards	17	37,8%

IDs: number of identifications. %: percentage of identifications in relation to the number of CFUs counted in the period.

TABLE III - Fungi with higher incidence during the months

Month	Microorganism	IDs	%	Month	Microorganism	IDs	%
January	*Cladosporium sp.*	7	33,3%	July	*Cladosporium sp.*	7	20,0%
	Penicillium sp.	5	23,8%		*Alternaria sp.*	6	17,1%
	Aspergillus sp.	3	14,3%		*Penicillium sp.*	5	14,3%
	Alternaria sp.	2	9,5%		*Curvularia sp.*	5	14,3%
	Neurospora sp.	1	4,8%		*Mycelia sterilia*	3	8,6%
	6th onwards	3	14,3%		6th onwards	9	25,7%
February	*Alternaria sp.*	7	21,9%	August	*Penicillium sp.*	9	27,3%
	Neurospora sp.	5	15,6%		*Curvularia sp.*	7	21,2%
	Curvularia sp.	5	15,6%		*Cladosporium sp.*	6	18,2%
	Mycelia sterilia	5	15,6%		*Alternaria sp.*	3	9,1%
	Penicillium sp.	4	12,5%		*Mycelia sterilia*	2	6,1%
	6th onwards	6	18,8%		6th onwards	6	18,2%
March	*Cladosporium sp.*	14	31,8%	September	*Curvularia sp.*	7	18,9%
	Penicillium sp.	8	18,2%		*Cladosporium sp.*	7	18,9%
	Mycelia sterilia	6	13,6%		*Penicillium sp.*	5	13,5%
	Aspergillus sp.	4	9,1%		*Aspergillus sp.*	5	13,5%
	Alternaria sp.	4	9,1%		*Mycelia sterilia*	5	13,5%
	6th onwards	8	18,2%		6th onwards	8	21,6%
April	*Aspergillus sp.*	2	22,2%	October	*Penicillium sp.*	15	36,6%
	Verticillium sp.	2	22,2%		*Cladosporium sp.*	8	19,5%
	Alternaria sp.	2	22,2%		*Curvularia sp.*	8	19,5%
	Penicillium sp.	1	11,1%		*Alternaria sp.*	6	14,6%
	Curvularia sp.	1	11,1%		*Aspergillus sp.*	2	4,9%
	6th onwards	1	11,1%		6th onwards	2	4,9%
May	*Cladosporium sp.*	10	43,5%	November	*Penicillium sp.*	9	36,0%
	Penicillium sp.	4	17,4%		*Cladosporium sp.*	6	24,0%
	Aspergillus sp.	2	8,7%		*Alternaria sp.*	5	20,0%
	Verticillium sp.	2	8,7%		*Trichoderma sp.*	2	8,0%
	Alternaria sp.	1	4,3%		*Aspergillus sp.*	1	4,0%
	6th onwards	4	17,4%		6th onwards	2	8,0%

TABLE III - Fungi with higher incidence during the months

Month	Microorganism	IDs	%	Month	Microorganism	IDs	%
June	*Penicillium sp.*	10	22,7%	December	*Cladosporium sp.*	7	35,0%
	Cladosporium sp.	10	22,7%		*Alternaria sp.*	4	20,0%
	Curvularia sp.	9	20,5%		*Curvularia sp.*	4	20,0%
	Mycelia sterilia	5	11,4%		*Trichoderma sp.*	2	10,0%
	Aspergillus sp.	3	6,8%		*Verticillium sp.*	1	5,0%
	6th onwards	7	15,9%		6th onwards	2	10,0%

IDs: number of identifications. %: percentage of identifications in relation to the number of CFUs counted in the period.

DISCUSSION

This study monitored the microbiological contamination of pharmaceutical industry cleanrooms (Grade D / ISO 8) over 12 months in 2019. The CFU count distributions for bacteria and fungi evidenced a low and constant bioburden in the production environments, using both sampling methods. The medians below 5 CFU/m³ for the air sampling and below 5 CFU/25 cm² for surface sampling complied with the requirements of Anvisa (2019), ISO (2015), the European Commission (2008), and the USP Convention (2019), considering the maximum limits of 200 CFU/m³ for active air sampling and 50 CFU/plate for surface sampling in Grade D / ISO 8 cleanrooms.

Higher microorganism levels were obtained in the air sampling, compared to the surface sampling, in agreement with previous studies using the active air sampling method (Gebala and Sandle, 2013; Park *et al.*, 2014). In the present work, the fact that the cleanrooms were in use during the sampling process may have contributed to air displacement and particle carryover to the sampler, which might not have affected the surface sampling. In contrast, other studies did not observe the same profile for the sampling methods. Rooms of an antibiotic and nonantibiotic drug factory were monitored for four consecutive months by active air sampling and surface sampling, with no differences found between the sampling methods for total aerobic microbial counts (Hamdy *et al.*, 2018). Another study found that surface sampling bacteria counts were higher than active air sampling counts, for the same pharmaceutical manufacturing facility room (Kawai *et al.*, 2019). Therefore, air and surface microbial levels may vary according to the sampling conditions and the pharmaceutical production environments, which reinforces the need for constant monitoring and evaluation of these areas.

In the present study, it was hypothesized that microbial levels could be higher during rainy than dry periods, but this hypothesis was not supported by the data. However, previous findings showed seasonal influences on the air microbiota in pharmaceutical production environment rooms (Pacchioni *et al.*, 2018), as well as higher incidence of microorganisms during the rainy season in Grade D clean areas used for vaccine production (Utescher *et al.*, 2007). The non-detection of seasonal impacts on the microbiota of the industry analyzed here may have been related to stringent biological control in the clean areas, due to the Heating, ventilation and air conditioning (HVAC) system, sanitization procedures, restricted access of people, correct use of personal protective equipment (PPE), and personnel training, which together acted to reduce contamination.

Identification, at least to the genus level, was made of the culturable microbiota present in the air and on the indoor surfaces of the Grade D / ISO 8 rooms sampled in the manufacturing environment. The main limitation of the present work was related to the non-culturable microorganisms that might have been present in the environments, which could only be identified using independent culturing techniques. Previous studies have found that this portion of the microbial diversity is

quite rich and can provide valuable information about the environmental microbial ecology of pharmaceutical industrial production environments (Andrade *et al.*, 2018; Kawai *et al.*, 2019; Pacchioni *et al.*, 2018; Park *et al.*, 2014). Furthermore, only cultured colonies with different morphologies were selected to be identified, which could lead to a low observed diversity. On the other hand, this approach optimizes microbial screening, enabling a fast and economically feasible global analysis of the culturable microbiota. In summary, despite these limitations, the merits of culture-dependent microbial counts and identifications should not be ignored, since (i) they meet the requirements of current legislation (Anvisa, 2019; ISO, 2015) and have historically contributed significantly to the microbiological safety of pharmaceutical products; (ii) they are consistent and enable reproducible results (Kawai *et al.*, 2019), which is not always possible in studies that use different bioinformatics approaches for microbial meta-taxonomy and metagenomics work (Callahan *et al.*, 2017); and (iii) they allow comparison among the results of different studies published in the literature, since the data are expressed as CFU.

The profiles of the local microbiota identified showed that the bacteria with the highest incidence were the gram-positive cocci *Staphylococcus epidermidis* and *Micrococcus* spp., representing approximately 28% of the group identifications. The most frequent fungi were *Cladosporium* sp. and *Penicillium* sp., accounting for about 44% of the designations for this group. Overall, the culturable profile for the environments evaluated in this work resembled those found previously in other pharmaceutical production environment rooms (Ashour *et al.*, 2011; Guinet *et al.*, 2017; Hamdy *et al.*, 2018; Utescher *et al.*, 2007). On the other hand, in hybrid studies, where pharmaceutical industry environmental monitoring was performed using both culture-dependent and culture-independent methods (Kawai *et al.*, 2019; Pacchioni *et al.*, 2018; Park *et al.*, 2014), the phyla *Proteobacteria*, *Firmicutes*, and *Actinobacteria*, in the case of bacteria, and the genera *Malassezia* and *Aspergillus*, for fungi, have been consistently reported. In these cases, the specimens with the highest incidence of these groups were the same as those found in the studies where identification was based on biochemical and culture-dependent methods.

Overall, the Grade D / ISO 8 cleanrooms investigated here and used to produce non-sterile drugs presented a constant profile and complied with the established CFU limit values for bacteria and fungi. No seasonal climatic influences were observed. During the months of the year, the environmental microbial contamination showed a core of identified species, with only slight variation, especially for fungi. Pacchioni *et al.* (2018) also highlighted the resilience of the microbial community present in the air and water of a pharmaceutical industry, during two years of follow-up. Although air sampling has a higher rate of microorganism recovery than surface sampling, the use of both methodologies is essential to ensure efficient control of the bioburden deposited on surfaces and suspended in the air of the factory environment.

Taken together, the data supported the hypothesis that the primary source of contamination of the cleanrooms was the outdoor environment, mediated by external agents such as the entry of supplies and people into the rooms. The source appeared to be mainly of human origin, since the most frequent microorganisms identified are found on human skin and mucous membranes (Andrade *et al.*, 2018; Kawai *et al.*, 2019). This reinforces the need for good manufacturing practices, the correct use of personal protection equipment, and appropriate clothing inside the pharmaceutical production environment, in addition to controlling access to cleanrooms and performing preventive maintenance of the HVAC systems used in classified environments, to reduce the risk of contamination throughout the production chain.

CONCLUSIONS

In conclusion, seasonal variations such as rainfall may not affect the microbial levels in Grade D clean rooms, if strict control quality conditions are met. The most prevalent microbiota in these areas appear to be mainly influenced by humans and the external environment. The findings of this work contribute to a better understanding of the patterns of environmental contamination inside the cleanrooms of pharmaceutical industries, which should assist in improving preventive measures to control contamination and ensure the quality of the industrial production environment.

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Received for publication on 30th August 2023
Accepted for publication on 19th October 2023