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Original Article

Microbes identified from monitoring cell manipulations in 5-year life of the Cell Factory G. Gaslini

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ABSTRACT

Introduction: Quality and safety of a cell product, essential to guarantee the health of patients, depends on many factors including an appropriate environmental monitoring of the manufacturing rooms. Nonetheless, the maintenance of a controlled environment is requested to minimize the risk of contamination. Thus, a timely detection of changes in microbiological trends is important to adopt promptly effective measures against resistant strains that, in turn, may invalidate not only the sanitization procedures but also the safety of the cell product.
Methods: We analyzed microbes found in our cell processing clean room over the last 5 years. We used 10.147 plates for air sampler, passive air monitoring and for checking instruments and operators of the production unit.
Results: From these plates, 747 colonies were subjected to identification by the MALDI-TOF Vitek® MS system and the large majority of them was gram positive (97.8%) as witnessed by the finding that the most represented *genera* harvested from the classified areas were *Staphylococcus* (65%), *Micrococcus* (13%), *Kocuria* (8%) and *Bacillus* (5%). We never detected fungi. Most microbes found in the operators (both from class A and B) were collected from forearms and resulted of the *Staphylococcus genus.*
Conclusions: The observed microbial contamination is to be attributed to the personnel and no substantial microbial pitfalls in our Cell Factory has been detected.

1. Introduction

Manufacturing of cellular products refers to a standardized quality system regulated by Good Manufacturing Practice (GMP) rules [1]. The majority of the cell products cannot be terminally sterilized, thus the process should be conducted aseptically to prevent microbial contaminations and carried out in clean areas of appropriate environmental cleanliness level. The classification of clean rooms should be done in accordance to ISO 14644-1 and includes four different areas named D, C, B and A to which correspond decreasing air particles and microbial contamination [1]. Beyond the qualitative and quantitative controls necessary to guarantee the quality of the final cell product, an accurate environmental monitoring program has to be implemented to assess the effectiveness of contamination control measures adopted. This includes non-viable (<0.5 μm and <5 μm) and viable particles monitoring in all classified areas and air pressure differential between adjacent rooms [1]. Analysis of microbes present in the classified rooms are of critical importance to assess changes in trends and to adopt fruitful measures if i) resistant strains are observed, ii) problems with cleaning and/or sanitization procedures emerged or iii) bad practices are put in place by the personnel.

The most widely microbiological monitoring methods are represented by the use of different types of agar plates for air, surface

Abbreviations: GMP, Good Manufacturing Practice; mo, micro-organisms; RODAC, Replicate Organism Detection and Counting; HEBAS, High Efficiency Biological Air Sampler; TSA, Tryptic soy agar; CFU, Colony Forming Unit; MALDI-TOF, Matrix Assisted Laser Desorption Ionization Time-of-Flight.

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and personnel monitoring. In particular, settle plates may be positioned i) in the appropriate location of an active air sampler [2–4] that allows to measure the micro-organisms (mo) present in the air per m^3 and ii) in defined points of the classified rooms (number of plates depends on room dimension) to count microbes that deposit by gravity onto the surface plate in 4 h. Indeed, Replicate Organism Detection and Counting (RODAC) contact plates are usually utilized to detect and enumerate microbes on surfaces, textiles or Tyvek suits.

Aim of the study was to perform a qualitative and quantitative environmental analysis of the microbiological monitoring carried out in our pharmaceutical manufacturing rooms during the cell manipulations performed (n = 85) in the last five years. Due to the few data available in the literature on clean room microflora [5–13], we provide extensive and detailed data on microbes harvested in the classified D, C, B and A rooms by active air sampler, passive air, surface and personnel monitoring. Thus, we report *genera* and *species* of mo collected from the microbiological monitoring (10.174 plates) carried out, pinpointing to the issue that the microbes found in our clean rooms, as reported by others, are of human origin, as witnessing by the consistent presence of the *Staphylococcus genus* that is integral part of normal human flora.

2. Methods

2.1. Classified areas in the G. Gaslini Cell Factory

The production site of our Cell Factory, a GMP certified laboratory, is approximately 40 m^2 and is composed, from the entry to the cell processing lab of i) a class D changing room, ii) a class D area with 4 pass-boxes (2 for passing materials from/to unclassified area and class D, additional 2 for passing materials from/to class D and C), iii) a second changing room (grade C), iv) a class C room, with a refrigerator +4 °C and −20 °C and two additional pass-boxes for passing materials from/to class B and C areas, v) a third changing room divided in a space for non-sterile clothing and one for sterile clothing (grade B) and vi) a cell processing laboratory classified as grade B where is placed a flow cabinet (grade A). In the cell processing lab are present two incubators, a centrifuge, one CliniMACS plus and, from 2018, a CliniMacs Prodigy (Miltenyi Biotec, Bergisch Gladbach, Germany). All these classified areas were selected for microbiological monitoring. Here we report the results from routine aseptic monitoring applied to cell manufacturing.

2.2. Active air sampling

Microbiological monitoring system consists of a High Efficiency Biological Air Sampler (HEBAS) impactor (Rigel Life Sciences, Rome, Italy), which aspirate a total of 1 m^3 of environmental air and dispense particles contained in air samples on microbiological plates. In details, HEBAS uses impact technology inertial to maximize the effectiveness of standard culture media. HEBAS head is designed to make a direct and precise sample air at a low velocity onto the culture surface for ensure high collection efficiency (validated according to ISO14698/1) minimizing serious stress and possible trauma to each micro-organism.

Furthermore, contamination that forms outside the controlled environment can be immediately and easily identified, reducing the risk of false positives. HEBAS is made of AISI 316L stainless steel, autoclavable. The system of locking is based on magnets. The particle-bearing airflow is directed onto standard Tryptic soy agar (TSA) 90 mm settle plates (BD, New Jersey, USA). After finishing the collection cycle, the plates are incubated and the total colony count determined. Air samples were aspirated by the impactor with a velocity of 28 L/min. The results obtained from active air sampling are presented as Colony Forming Unit (CFU)/m^3 of air. Three HEBAS are present in the production site and positioned in class C room, in the cell processing class B lab and inside the class A flow hood.

2.3. Passive air sampling

TSA plates were located in all the classified areas as follows: one in class D room with the 4 pass-boxes, ii) one in the class C changing room, iii) 3 in the class C room, iv) 2 in the third changing room, v) 3 in the cell processing lab and vi) 3 under the laminar hood with active flow. Plates were opened, agar were exposed directly to the ambient air and placed for 4 h. Every 4 h plates were replaced by new ones. The micro-organisms transported by particles were deposited on the surface of the agar. The results are reported as CFU/4 h.

2.4. Surface sampling

To monitor surfaces, floors, pass-boxes, instruments and personnel, we used TSA with Lecithin and Polysorbate 80 with Penase, 65 mm RODAC plates (BD). Lecithin and Polysorbate 80 are specifically included to neutralize surface disinfectants. Thus, a direct contact method was used and a 10 s pressure was applied by the QC operator. Subsequently, the surfaces from which samples were collected were adequately cleaned using alcohol. Such monitoring was performed at the end of the manipulation process and on the instruments used. Eleven contact plates were used in the following classified areas: 4 under the flow cabinet, 4 in class B, 3 in class C and 1 in class D. The results are reported as CFU/plate.

2.5. Personnel

Operators (n = 3) were monitored during cell manipulation in class A (gloves and forearms) and at the end of the process in class B (gloves, forearms, forehead, mask and chest). For gloves, we used 90 mm TSA plates with the same composition of the RODAC ones. All the other samples were collected using the contact plates. Operators underwent to 3 round of consecutive dressing that are i) taking off clothes, wearing low particle release overalls, overshoes or sterile shoes, gloves and mask in class D iii) a second wearing non-sterile gown and overshoes with changing of gloves in class C changing room and iii) the sterile dressing with Tyvek comprising diving suit and shoes and wearing sterile gloves.

2.6. Incubation conditions and microbial identification

Plates were incubated for 3–5 days at 25 °C, CFU counted, and subsequently moved in a 35 °C incubator for additional 2–3 days. After the second CFU count, all plates previously located in class A and B stored at 4 °C until microbial identification. Micro-organisms grown in plates from class C and D areas were identified, from manipulations performed every two-three month, by selecting representative colonies based on their different color and shape.

Identification was performed by collecting each CFU, using a sterile loop, and distributing it on the surface of a plate (i.e. Columbia 5% blood for bacteria and Sabouraud + gentamicin for fungi) that was then placed in an incubator at 35 °C (for bacteria) or 25 °C (for fungi) for 24 h. Afterwards, a small aliquot of each individual colony was placed in a Vitek® MS target slide and VITEK MS-CHCA MATRIX was added. Next, samples were analyzed using the (Matrix Assisted Laser Desorption Ionization Time-of-Flight (MALDI-TOF) Vitek® MS system (Biomerieux, Marcy-l'Étoile, France) which is a Mass Spectrometry System allowing to identify over 15.000 distinct strains and 1.000 species using a database that accounts for diversity within a species for greater accuracy.

2.7. Measurement points

The study was carried out by analyzing microbes grown on the plates located in the production site during the manipulation processes conducted from June 2019 to July 2023. The total number of plates used was 10.174 from which 747 colonies were subjected to identification.

3. Results

3.1. Overview of the micro-organisms identified in the classified areas

We collected and identified the mo grown on plates, both settle and contact, during all the microbiological monitoring in the classified areas of the production site from 2019 to 2023. We subjected a total of 747 colonies to identification by the MALDI-TOF Vitek® MS system. The details for each year and for individual classified area, number of plates used in each type of sampling and number of microbes harvested for identification are reported in Table 1. Less than 20% of all colonies tested resulted unidentified from the database. As shown in Fig. 1 (upper panel), the large majority of microbes was gram positive (97.8%) as witnessed by the finding that the most represented *genera* were *Staphylococcus* (65% of all mo), *Micrococcus* (13%), *Kocuria* (8%) and *Bacillus* (5%). The other *genera*, representing only the 6% of the total micro-organisms identified, are reported in details in Table 2. The bacterial *species* found (Fig. 1, lower panel) were: i) *Staphylococcus Epidermidis* (24%), *Micrococcus Luteus* (12%), *Staphylococcus Capitis* (11%), *Staphylococcus Hominis* (9%) and *Staphylococcus Cohnii* (7%). Fungi were never detected.

3.2. Microbes in class A

In class A, only a couple of microbes grew in the plates, thus few were collected (n = 12) and successfully identified (n = 10). These mo derived from settle plates by gravity and, more marginally, by contact monitoring of the laminar flow cabinet as reported in details: i) at the edge by settle (colonies n = 5) or contact plates (n = 1), ii) at the center by settle (n = 2) or contact (n = 1) plates and iii) in the internal side of the front glass by contact plates (n = 1). Among them, *Staphylococcus* was the most *genus* found (60%), but we observed also the *Bacillus* (20%), *Paenibacillus* and *Kocuria genera* (both 10%) (Fig. 2A, left panel). Similar to that observed in the class A area, the most represented *species* (20%) were the *Staphylococcus* Epi*dermidis*, *Staphylococcus Capitis* and *Bacillus Cereus*, whereas *Staphylococcus Haemolyticus*, *Kocuria Rhizophyla*, *Paenibacillus Pabuli* and *Staphylococcus Cohnii* represented together 10% of all the *species* identified (Fig. 2A, table on the right).

3.3. Micro-organisms harvested in class B area: rooms and instruments

As expected, a higher number of microbes (n = 252) were collected in classified B area and analyzed (n = 220). Eighty-eight micro-organisms were isolated from the settle plates positioned in the third changing room (n = 88), in particular in the pre- (n = 22) and post- (n = 66) dressing areas. In the cell processing laboratory, we identified 132 colonies grown on: i) settle plates placed on the floor near air intake (n = 9), in front of the incubators (n = 7) and at the center of the room (n = 9), ii) active air sampler (n = 28), iii) contact plates used for checking microbial contamination in the floor (n = 18), pass-box (n = 20), window to C room

Table 1
Plates, type of sampling and number of microbes harvested for identification.

Year	Type of sampling	Classified area							
		A		B		C		D	
		n. plates	n. mo	n. plates	n. mo	n. plates	n. mo	n. plates	n. mo
2019	**Active**	30	–	30	6	30	–	–	–
	Passive	74	2	193	55	156	–	53	–
	Contact	63	1	105	9	84	–	42	–
2020	**Active**	39	–	39	1	39	4	–	–
	Passive	78	3	198	10	170	32	44	19
	Contact	117	–	162	8	141	11	42	4
2021	**Active**	36	–	36	5	33	5	–	–
	Passive	72	2	159	31	137	27	43	5
	Contact	112	–	152	3	98	6	43	2
2022	**Active**	40	–	40	4	40	3	–	–
	Passive	80	–	138	19	190	13	46	2
	Contact	116	1	141	5	82	7	29	2
2023	**Active**	76	1	76	15	76	2	–	–
	Passive	152	1	252	69	304	4	79	2
	Contact	224	1	521	12	124	1	54	1
Total		1309	12	2242	252	1704	113	475	37

Year	Instruments				Operators			
	Class B		Class C		Class A		Class B	
	n. plates	n. mo	n. plates	n. mo	n. plates	n. mo	n. plates	n. mo
2019	160	3	40	–	108	–	343	–
2020	112	5	32	1	296	11	462	23
2021	88	1	19	4	148	17	329	71
2022	133	9	25	1	156	3	420	28
2023	315	12	73	2	279	25	915	115
Total	808	30	189	8	978	58	2469	237

n = number; n. mo = number of microbes harvested for identification.

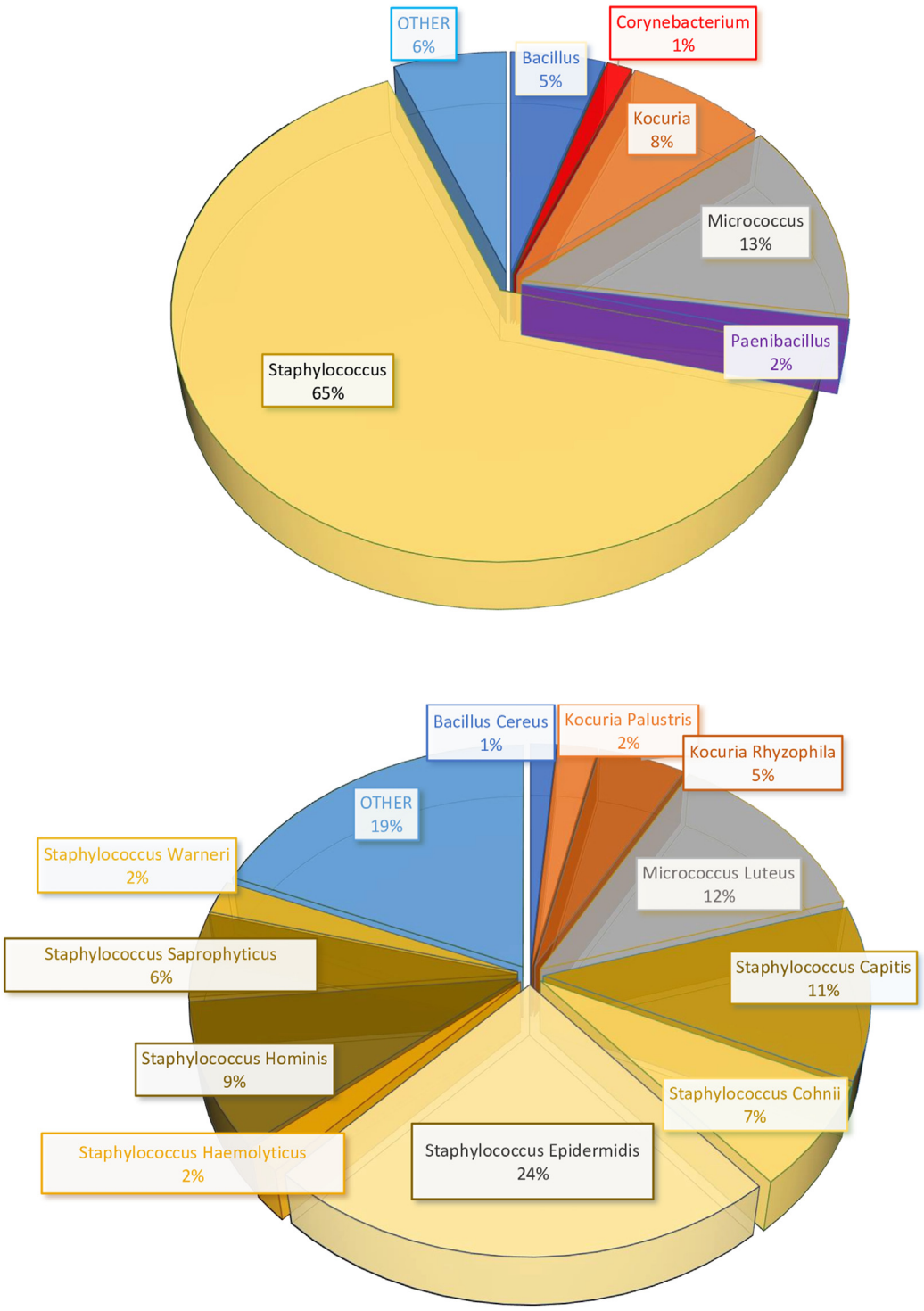


Fig. 1. Overview of all micro-organisms identified in the Cell Factory. Upper diagram shows the *genera* related to microbes found from July 2019 and June 2023. Percentage of each individual *genus* is shown. Lower panel shows the *species* identified in the same period. *Genera* and *species* included in "OTHER" are reported in details in Table 2.

(n = 10), door handles (n = 5) and external part of the flow cabinet glass (n = 2) and iv) contact plates used for monitoring instruments that are the CliniMACS Prodigy (n = 8), CliniMACS Plus (n = 5), incubators (n = 4), centrifuge (n = 2) and plasma press (n = 5).

As shown in Fig. 2B, left panel, *Staphylococcus* was the most represented *genus* (60%), followed by *Micrococcus* (18%), *Kocuria* (10%) and *Bacillus* (6%) (Fig. 2B). As reported in Fig. 2B (table on the right), a wide panel of *species* were identified such as *Staphylococcus Epidermidis* (17%), *Micrococcus Luteus* (16%), *Staphylococcus Hominis* (10%), *Staphylococcus Capitis* (8%), and *Staphylococcus Cohnii* (7%).

3.4. Microbes identified in classified C and D areas

In class C a total of 101 mo were identified, mainly from the floor and *Staphylococcus* was the most represented *genus* (52%). *Micrococcus* (19%), *Kocuria* (15 %) *Bacillus* (4%) and *Moraxella* (2%) were consistently present (Fig. 2C, left panel). *Micrococcus Luteus* was the

Table 2
Individual *genus* representing together the 6% of the total micro-organisms identified.

Genus	% identification/all mo	Species	Classified area
Acinetobacter	0.59	*Acinetobacter Iwoffii*	B
		Acinetobacter radioresistens	D
		Acinetobacter Ursingii	D
Aerococcus	0.198	*Aerococcus Viridans*	C
Aeromonas	0.198	*Aeromonas Salmonicida*	C
Aneuribacillus	0.198	*Aneuribacillus Aneurilyticus*	Op. class A
Bacteroides	0.198	*Bacteroides Ovatus*	B
Bifidobacterium	0.198	*Bifidobacterium*	B
Brevibacterium	0.198	*Brevibacterium Casei*	B
Citrobacter	0.198	*Citrobacter Koseri*	B
Dermacoccus	0.198	*Dermacoccus Barathri*	B
Franconibacter	0.198	*Franconibacter Helveticus*	C
Lactococcus	0.198	*Lactococcus Lattis*	C
Legionella	0.198	*Legionella Londinensis*	C
Lysinbacillus	0.395	*Lysinbacillus fusiformis*	Op. class B
Microbacterium	0.198	*Microbacterium Flavescens*	B
Moraxella	0.988	*Moraxella Osionensis*	B and C
Pseudomonas	0.593	*Pseudomonas fluorescens*	B
		Pseudomonas Oryzihabitans	
Rhizobacterium	0.198	*Rhizobacterium Radiobacter*	B
Rhodococcus	0.198	*Rhodococcus erythropolis*	Op. class A
Roseomonas	0.198	*Roseomonas mucosa*	Op. class A
Rothia	0.198	*Rothia mucillaginosa*	C
Solibacillus	0.198	*Solibacillus Silvestris*	C
Streptococcus	0.198	*Streptococcus Parasanguis*	Op. class B

Mo = micro-organism.

main *species* (18%) found, followed by *Staphylococcus Epidermidis* (11%), *Kocuria Rhizophyla* (10%), *Staphylococcus Saprophyticus*, *Staphylococcus Hominis* and *Staphylococcus Cohnii* (each 8%) (Fig. 2C, table on the right).

In class D a total of 23 different microbes were present (Fig. 2D). Again, *Staphylococcus* was the most observed *genus* (55%), whereas *Kocuria* (14 %), *Acinetobacter*, *Bacillus*, *Micrococcus* (9% each), and *Corynebacterium* (5%) were consistently present (Fig. 2D, left panel). *Staphylococcus Cohnii*, *Staphylococcus Hominis* and *Staphylococcus Epidermidis* were the most represented *species* (14%), followed by *Micrococcus Luteus* and *Kocuria Rhizophyla* (9%) (Fig. 2D, table on the right).

3.5. Micro-organisms harvested on production operators

3.5.1. Class A

The three operators of the production unit have been monitored in class A, immediately after system opening procedures, using glove plates (for right and left hands) and contact plates (for forearm, head, mask, and chest). Of note, op#3 ended her activity in the Cell Factory on February 2021.

From the fifthy-eight mo subjected to identification from class A monitoring (Fig. 3), 44 were successfully recognized and, among them, the majority were collected from right forearm (n = 21), although the presence of microbial contamination was also observed in the right glove (n = 13), left glove (n = 7) and left forearm (n = 3). As shown in Fig. 3, *Staphylococcus* was the most represented *genus* in each of the three operators (op#1 in panel A, op#2 in panel B and op#3 in panel C, respectively). All *species* found in each individual operator are reported in the right panels of Fig. 3. Microbiological monitoring of op#1 in class A revealed the presence of only two *genera* (left panel) that are *Staphylococcus* (92%) and *Corynebacterium* (8%). On op #2, *Staphylococcus* represented 45% of all of the mo collected, followed by *Micrococcus* (25%), *Bacillus* (10%), *Aneuribacillus*, *Rhodococcus*, *Paenibacillus* and *Kocuria* (5% each). Again, *Staphylococcus* represented 82% of all mo collected in class A from op #3, and the remaining microbes were represented by *Roseomonas* and *Kocuria* (9% each). When we analyzed the *species* identified from each single operator (Fig. 3, right tables), we found that *Staphylococcus Epidermidis* was the most represented in op #1 (panel A), then we detected the *Staphylococcus Hominis* and *Staphylococcus Warneri* (15%), *Staphylococcus Caprae*, *Staphylococcus Capitis* and *Corynebacterium Pseudodiphteriticum* (8%). In contrast, *Micrococcus Luteus* was the most represented mo (25%) found on op #2 (panel B), although other *species* were found and reported in details in the right panel. In the op #3 we predominantly found that *Staphylococcus Cohnii*, *Staphylococcus Hominis* and *Staphylococcus Warneri* (18%).

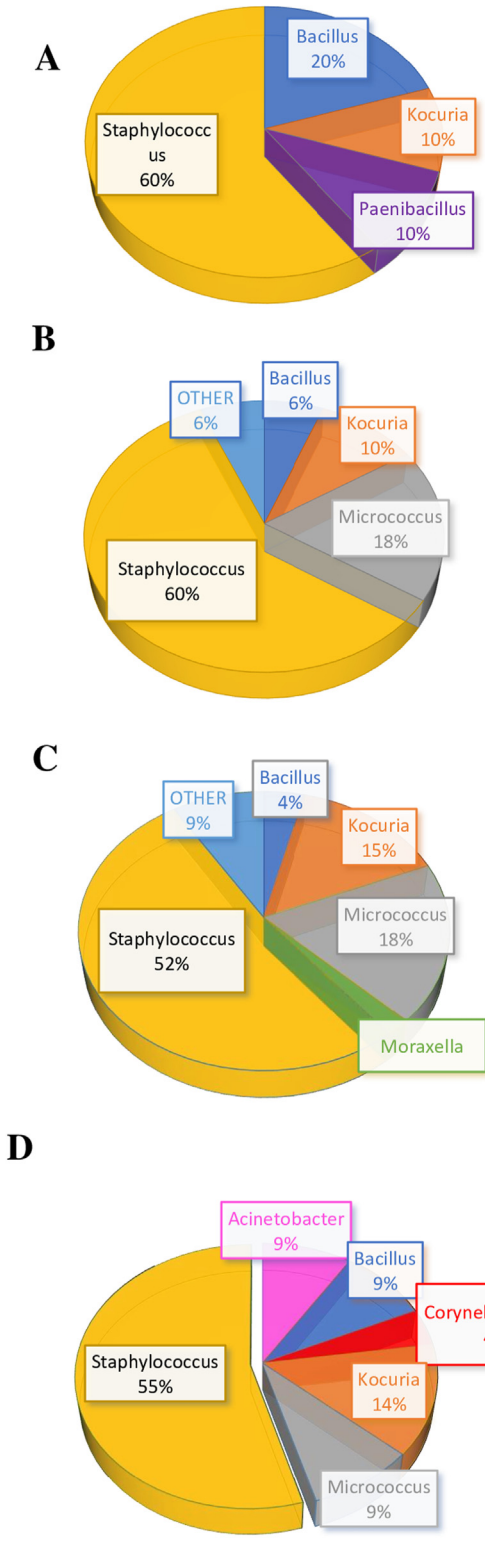
3.5.2. Class B

All the operators were also monitored in classified area B at the end of each procedure (gloves, forearms, forehead, mask and chest). We collected 237 mo and identified 186, most of them from the forehead (n = 55), followed by mask (n = 46), chest (n = 37), left forearm (n = 17), right forearm (n = 15), left glove (n = 10) and right glove (n = 6). As shown in Fig. 4, *Staphylococcus* was the most represented *genus* found in op #1 (74%, panel A), #2 (79%, panel B) and #3 (68%, panel C). Of note, *Micrococcus*, *Kocuria* and *Bacillus* were found in all the three operators, whereas *Pseudomonas* was detected only in op #1.

In terms of *species* (Fig. 4 tables on the right), the *Staphylococcus Epidermidis* was the most represented in op #1 (40%, panel A), op #2 (35%, panel B) and op #3 (47%, panel C). As expected, the large majority of the species found were bacteria that physiologically resides on human tissues and includes *Staphylococcus Capitis*, *Staphylococcus Hominis*, *Micrococcus Luteus*, *Corynebacterium Mucifaciensis* and *Tuberculostearicum*. Nonetheless, *Kocuria Rhizophila* and *Palustris* which normal habitat is the mammalian skin, soil and rhizoplane were found in op#1 and #2 or op#2 and #3, respectively. Other *species* are environmental microbes such as *Bacillus Simplex* (op#1 and #2), *Bacillus Licheniformis* (op#3), *Paenibacillus Lactis* (op#1).

4. Discussion

The environmental microbiological monitoring of a Cell Factory includes air, surfaces and personnel and is of crucial importance to promptly detect any change in microbiological trends and to adopt



Class A species	number
Bacillus Cereus	2
Kocuria Rhyzophila	1
Penibacillus Pabuli	1
Staphylococcus Capitis	2
Staphylococcus Cohnii	1
Staphylococcus Epidermidis	2
Staphylococcus Haemolyticus	1

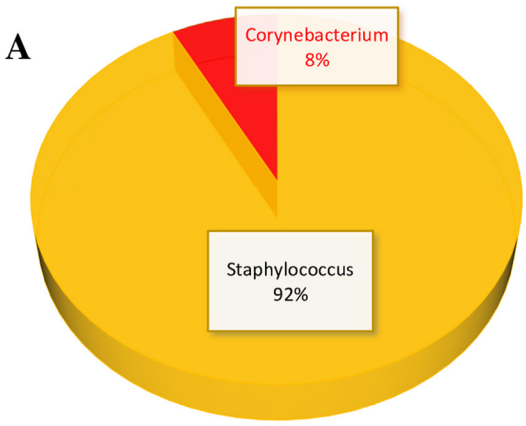
Class B species	number
Bacillus Altitudinis	3
Kocuria Palustris	8
Kocuria Rhyzophila	10
Kocuria Rosea	3
Micrococcus Luteus	36
Micrococcus Lylae	4
Staphylococcus Capitis	17
Staphylococcus Caprae	3
Staphylococcus Cohnii	16
Staphylococcus Epidermidis	38
Staphylococcus Haemolyticus	10
Staphylococcus Hominis	23
Staphylococcus Saprophyticus	10
Staphylococcus Warneri	6
OTHER	33

Class C species	number
Kocuria Palustris	4
Kocuria Rhyzophila	10
Micrococcus Luteus	18
Moraxella Osionensis	2
Staphylococcus Arlettae	2
Staphylococcus Capitis	4
Staphylococcus Cohnii	8
Staphylococcus Epidermidis	11
Staphylococcus Haemolyticus	4
Staphylococcus Hominis	8
Staphylococcus Hominis	5
Staphylococcus Saprophyticus	8
OTHER	17

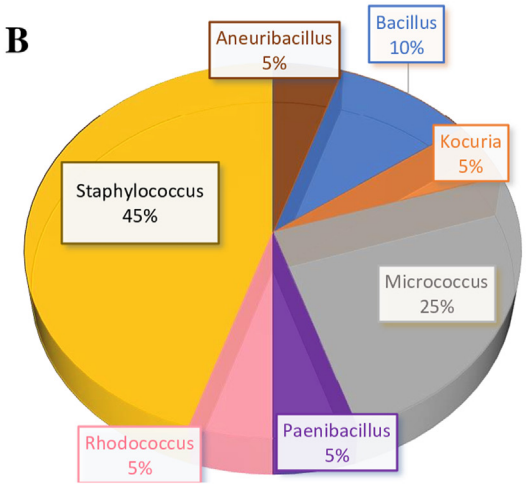
Class D species	number
Acinetobacter radioresistens	1
Acinetobacter Ursingii	1
Bacillus Cereus	1
Bacillus Simplex	1
Corynebacterium pseudotubercolosis	1
Kocuria Rhyzophila	2
Kocuria Rosae	1
Micrococcus Luteus	2
Staphylococcus Cohnii	3
Staphylococcus Epidermidis	3
Staphylococcus Haemolyticus	1
Staphylococcus Hominis	3
Staphylococcus Saprophyticus	1
Staphylococcus Warneri	1
Acinetobacter radioresistens	1

Fig. 2. Microbes identified in each class of contamination in the last five years. Micro-organisms defined for *genus* (left diagrams) and *species* (right tables) found in class A (panel A), class B (panel B), class C (panel C) and class D (panel D) are reported.

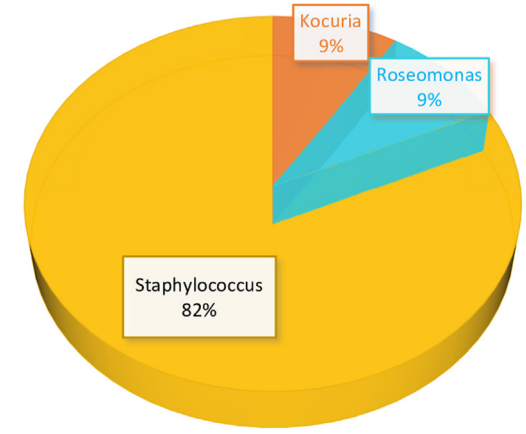
measures effective to prevent any contamination of the cell product, thus avoiding potential risk for patients. In this view, we implemented a microbiological monitoring program that includes, among others, the setting up of the cleaning validation and microbial monitoring at rest in the production site. These procedures allowed us to demonstrate that our manufacturing site is virtually free of microbial contamination after cleaning of the rooms and in the absence of operators inside (not shown). Thus, we



Species, Operator #1 class A	number
Staphylococcus Epidermidis	6
Staphylococcus Caprae	1
Corynebacterium Pseudodiphteriticum	1
Staphylococcus Capitis	1
Staphylococcus Warneri	2
Staphylococcus Hominis	2



Species, Operator #2 class A	number
Micrococcus Luteus	5
Aneuribacillus Aneurilyticus	1
Staphylococcus Capitis	2
Kocuria Rhyzophila	1
Rhodococcus erythropolis	1
Staphylococcus Epidermidis	3
Bacillus Cereus	1
Paenibacillus Provenciensis	1
Bacillus circulans	1
Staphylococcus Hominis	1
Staphylococcus Warneri	1
Staphylococcus Pasteuri	1
Staphylococcus Saprophyticus	1

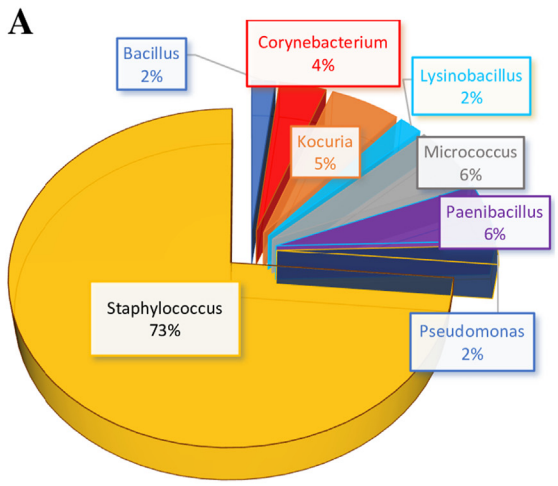


Species, Operator #3 class A	number
Staphylococcus Hominis	2
Staphylococcus Capitis	1
Staphylococcus Saprophyticus	1
Staphylococcus Epidermidis	1
Staphylococcus Warneri	2
Kocuria Rhyzophila	1
Roseomonas mucosa	1
Staphylococcus Cohnii	2

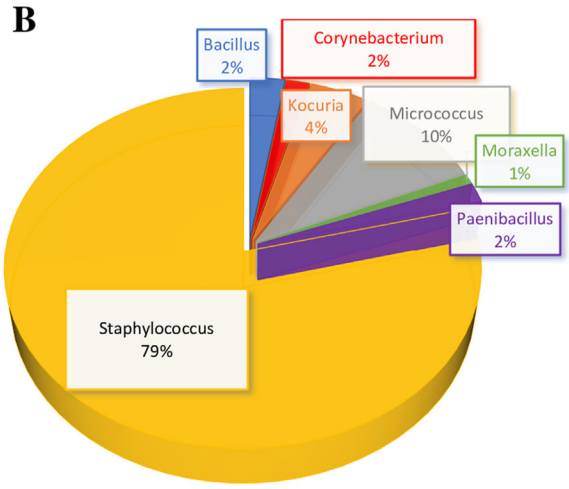
Fig. 3. Micro-organisms harvested from operators of the production Unit (class A). *Genus* (left diagrams) and *species* (right tables) of microbes harvested from microbiological monitoring of operator #1 (panel A), operator #2 (panel B) and operator #3 (panel C) are shown.

analyzed the microbe flora harvested from the plates used for monitoring environment, instruments and personnel involved in the processes of cell manipulation carried out in the entire producing life of our Cell Factory (2019–2023).

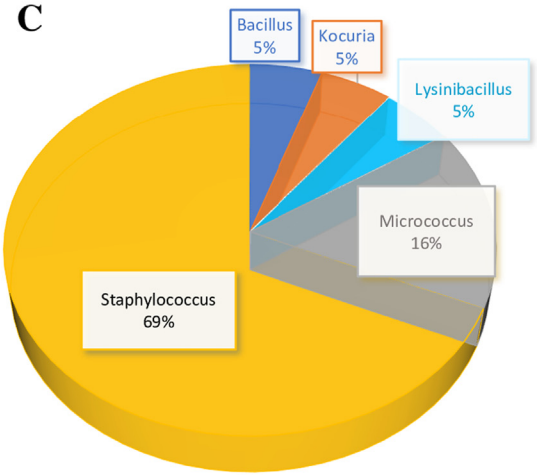
Consistently with that observed by others [5,6,8,14], the vast majority of microbes identified was gram positive (97.8%) mainly represented by the *Staphylococcus genus*, whereas the most present gram-negative *genus* is the *Acinetobacter*. Of note, we never isolated fungi. In all areas studied the sampled points with microbiological growth correspond to the floor and center of the rooms, where all air streams converge and the mobility of the personnel is greater. Furthermore, higher number of microbes was harvested for identification from plates used for monitoring the environment and instruments in class B area (14.7%), as compared to class A (0.9%),



Species, operator #1 class B	number
Staphylococcus Epidermidis	21
Staphylococcus Capitis	7
Staphylococcus Hominis	6
Bacillus Simplex	1
Micrococcus Luteus	3
Corynebacterium Mucifaciensis	2
Staphylococcus Saprophyticus	2
Kocuria Rhyzophila	3
Paenibacillus Lactis	1
Paenibacillus glicanolyticus	1
Staphylococcus Haemolyticus	1
Lysinbacillus fusiformis	1
Pseudomonas Oryzihabitans	1
Staphylococcus Pasteuri	1
Penibacillus Provenciensis	1
Streptococcus Parasanguis	1



Species, operator #2 class B	Number
Staphylococcus Hominis	12
Staphylococcus Epidermidis	40
Kocuria Rhyzophila	2
Staphylococcus Cohnii	7
Micrococcus Luteus	11
Kocuria Palustris	3
Staphylococcus Capitis	21
Corynebacterium tubercolostearicum	2
Staphylococcus Warneri	2
Staphylococcus Saprophyticus	5
Paenibacillus Pabuli	2
Bacillus Simplex	2
OTHER	5



Species, operator #3 class B	number
Lysinibacillus Fusiformis	1
Bacillus licheniformis	1
Staphylococcus Epidermidis	9
Micrococcus Luteus	3
Staphylococcus Ludgunensis	1
Staphylococcus Capitis	1
Staphylococcus Hominis	1
Staphylococcus Warneri	1
Kocuria Palustris	1

Fig. 4. Microbes identified for monitoring of operators (production unit) in class B. *Genus* (left diagrams) and *species* (right tables) of microbes harvested from microbiological monitoring of operator #1 (panel A), operator #2 (panel B) and operator #3 (panel C) are shown.

class C (10.8%) or D (7.7%). This is related to the *routine* behavior of operators during cell processing, that involves the continuous presence of the production operators in class B room and the entrance of QC personnel only occasionally for checking producers in class A or picking up samples for qualitative analysis. Thus, the class B rooms are the busiest area during cell manipulation processes. In this view, a consistent number of colonies was collected and identified from operators (9.5% of all plates) in class B.

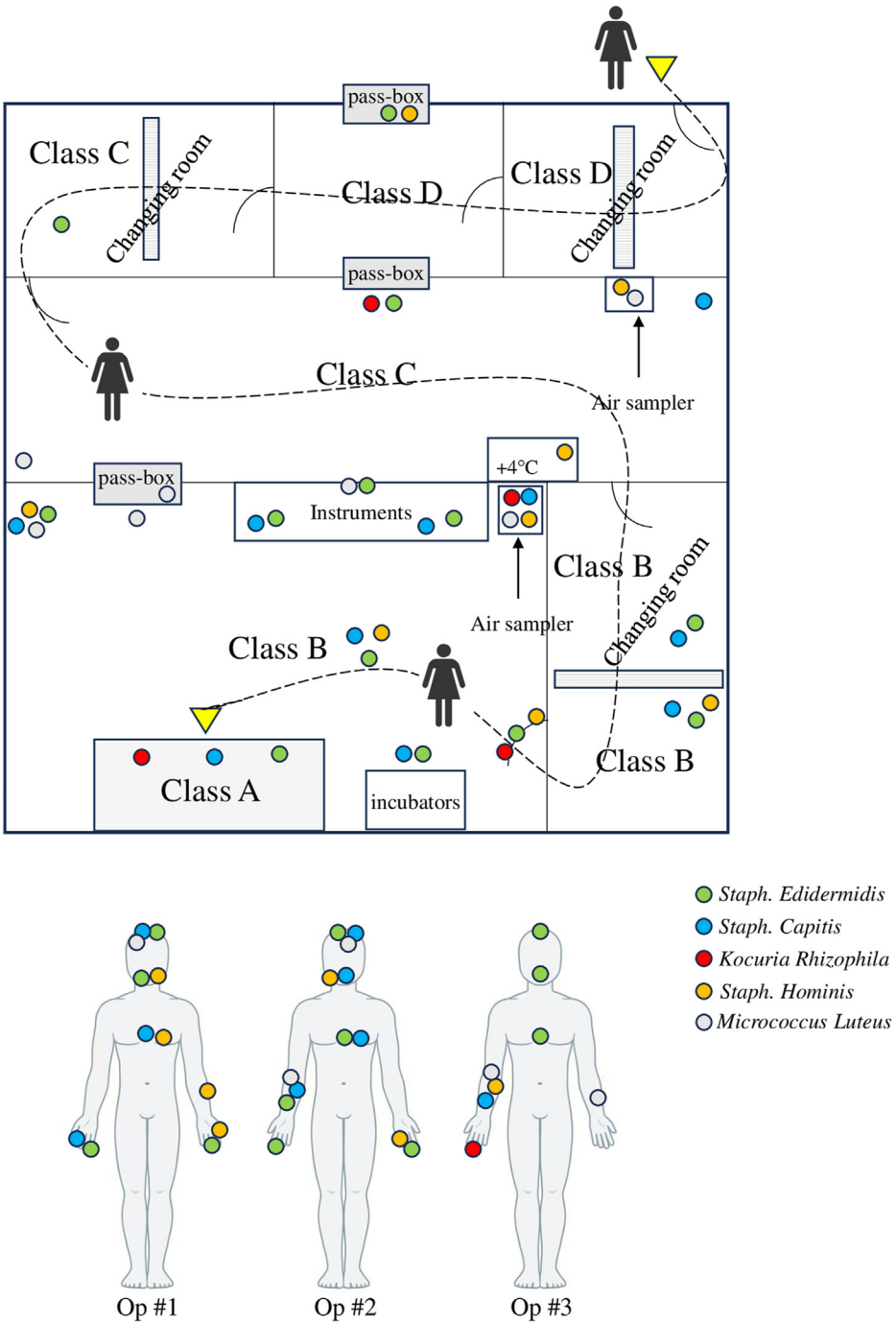


Fig. 5. The itinerary of personnel from unclassified area to the cell processing lab (class B). Yellow triangles show the start and the end point, whereas dashed line the itinerary performed by the operators of the production unit. Four representative microbes (colored circles) collected from operators (op#1, #2 and #3) have been found in the manufacturing site.

In class A, 58% of microbes were found in passive sampling plates and were mainly (60%) of the *Staphylococcus genus*, of which the *Epidermidis* and *Capitis species* were the most represented. These microbes are integral part of normal human flora, especially skin and scalp respectively, thus supporting the concept that the operator is the main source of contamination. Noteworthy, the same *species* were isolated from all the three operators monitored in both class A and B. Other *genera* found are *Kocuria* that live on human skin and oral cavity, *Paenibacillus* and *Bacillus* that normally home in a variety of environments. Such microbes were consistently isolated from operators, especially when monitored in class B. In terms of *genera*, the microflora of classified B area is similar to that observed in class A, with the exception of *Paenibacillus* that is not present whereas *Micrococcus* grows. The predominant *species* (59.1%) harvested in class B rooms, beyond those already found in classified A area (25%), were *Micrococcus Luteus*, *Staphylococcus Hominis* and *Cohnii*. Again, these micro-organisms are physiologically resident on the skin and epidermidis, indeed have been collected from all operators, with exception of *Staphylococcus Cohnii* that has never been isolated form op #1.

Different from all the other classified areas, *Moraxella* was found in class C, whereas *Acinetobacter* and *Corynebacterium*

were harvested from class D. *Moraxella* is a commensal gram-negative resident of the rhino-pharynx tract, whereas the other two *genera* are widely present in nature and commensals on the skin.

Taken together, we here reported extensive data on microbes grown in our Cell Factory, pinpointing to the *genera* and *species* identified. The most represented *genus* is consistently *Staphylococcus* that does not represent a serious complication in consideration of the issues that i) is not sporogenous and ii) the sanitization procedures applied in cleanrooms (sporicidal and alcohol) are effective for a complete elimination of the microbe.

Taken in mind the itinerary of personnel from unclassified area to the cell processing lab (class B), and main *species* found on the three operators involved in the manufacturing process, it is indisputable that the microbial contamination is to be attributed to the personnel. As shown in Fig. 5, the four most *species* found in operators (class A and B) are spread in the entire B room during cell manipulation, including flow cabinet (classified A area), floor, instruments, air and doors. Such microbial contamination of human origin, confirmed by others in manuscripts already published, may be related to i) the mo emission capacity of each operator that may reach air with more than hundreds of microbes every minute, ii) the strategy used to sanitize and introduce materials and ii) clothing procedures.

5. Conclusions

In conclusion, although we do not envisage any substantial microbial pitfalls in our Cell Factory, we are aware that we have to maintain a positive trend of contamination, avoiding resistant strains, and to improve continuously the skill training of personnel, the risk analysis, sanitization procedures and the monitoring program.

Declaration of competing interest

The authors of this manuscript declare no conflict of interest.

Acknowledgment(s)

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