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Towards the drug factory microbiome: microbial community variations in an antibiotic-producing manufacturing plant

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ABSTRACT

Microbiome projects are currently booming around the globe, enabled by advances in culture-independent microbial community analysis and high-throughput sequencing. One emerging application of microbiome science involves exploring microbial diversity in *built environments*, and one unexplored built environment is the pharmaceutical factory, notably factories producing antibiotics, as they could be enriched in antibiotic-resistant microbes. To examine the drug factory microbiome, we launched this interdisciplinary, hypothesis-generating study to benchmark culture-independent microbiome analysis in drug-manufacturing units producing antibiotics and non-antibiotic drugs, against traditional microbial identification and quantification techniques. Over a course of four months, we prospectively collected 234 samples from antibiotic (kanamycin and amoxicillin) and non-antibiotic (acetaminophen) production areas within a pharmaceutical factory in Egypt. All samples were analyzed by traditional culture-based methods, and microbial communities of representative samples were profiled by16S rRNA gene sequencing. Additionally, antibiotic resistance profiles of some samples were determined, and representative resistance genes were screened. The 16S rRNA analysis revealed a typical predominance of Proteobacteria (36%), Firmicutes (31%), and Bacteroidetes (17%). The microbial composition of the samples was highly affected by the use of water, environmental conditions during the production process, the presence of personnel, and the type of the product. The effect of these factors was confirmed by total aerobic microbial counts and identification of biomarker microbes. In conclusion, these observations can aid in the future for optimal design and management of pharmaceutical manufacturing units and speak to a greater need for implementing microbiome research in the quality assurance of built environments.

Keywords: Microbiome variation, industry design, pharmaceutical manufacturing, built environments, diagnostic innovation, interdisciplinary research.

INTRODUCTION

The sequencing revolution at the start of this millennium opened the way to culture-independent diagnostics and to metagenomics-based microbial community analysis (Riesenfeld et al., 2004b; Schloss and Handelsman, 2003). The microbiome concept emerged to describe the total microbial community within a particular ecosystem, whether human-associated or inanimate (Lederberg, 2000; Relman and Falkow, 2001; Rizkallah et al., 2010). Microbiome projects have boomed to explore the microbial dark matter within humans, animals, extreme environments, and eventually all earth habitats, under the Earth Microbiome Project (Gilbert et al., 2010). Among the important, yet less studied, habitats are those in built environments, as they represent a unique ecological interface between human and nature (King, 2014; Lax et al., 2015; Leung and Lee, 2016). One such unexplored environment is the drug factory, a built environment in which there is interplay between different factors, such as the raw material, pharmaceutical product, workers, and different environmental factors (e.g., water, aerosol, dust, humidity). Of particular interest are drug factories that produce antibiotics, as they are possible hot spots for selection of resistant microbes.

Antimicrobial resistance is becoming one of the top threats to human health (Centers for Disease Control and Prevention (CDC), 2013; Levy and Marshall, 2004), and deaths by multi-resistant bacteria are expected to exceed those caused by cancer in 2050 (O'Neill, 2014). The misuse and overuse of antibiotics by humans exert pressure on bacteria, which already have extraordinary genetic capacities to gain and exchange resistance genes through mutagenesis and horizontal gene transfer. Consequently, bacteria have developed resistance to almost every produced antibiotic within months to a few years of its discovery or introduction in the market (Schmieder and Edwards, 2012). Based on this century-long observation, we hypothesized here that continuous exposure of instruments, environment, and personnel—within a drug factory—to antibiotics might not only alter their microbiomes, but would also lead to enrichment/accumulation of microbes that are resistant to the produced antibiotic or its analogs.

To develop and test this hypothesis, we launched this pilot study with the goal of analyzing the microbiome of a drug-manufacturing plant, with emphasis on antibiotic-producing machines, and compare these to non-antibiotic-producing ones. Whereas determining microbial counts and identification of indicator bacteria in pharmaceutical factories are essential quality

control steps, DNA sequence-based approaches for microbial identification and quantification remain in early research stages. Even in the absence of any bacterial growth on conventional media, uncultured bacteria were reported in considerable numbers in pharmaceutical clean rooms (Nagarkar et al., 2001; Sheraba et al., 2010). Therefore, our focus was to investigate the pharmaceutical factory microbiome by culture-independent microbial profiling. As a pilot study, this work mainly aimed to benchmark culture-independent microbiome analysis against traditional culture-based microbial identification and quantification techniques

MATERIALS AND METHODS

Sample collection. All samples were collected from different production rooms in a drug factory in Egypt (Table 1). Sixty environmental samples were collected once per month for a period of four successive months (May through August 2014)—except that at the first sampling time only 54 samples were collected. The samples were collected under normal operation condition from (i) a non-antibiotic manufacturing area (NA), where acetaminophen tablets and simethicone chewable tablets are produced; (ii) a beta-lactam antibiotic manufacturing area (BL) which produces amoxicillin capsules and dry suspensions; and from (iii) a kanamycin wet suspension production department (which will be described as the *aminoglycoside production area,* AG).

Table 1: List of all sampled factory rooms and the main activity therein

Area	Room#	Process
Non-antibiotic area (Acetaminophen)	S04	Wet granulation mixer room
	S12	Dry V-mixer room
	S08	Bed fluidized drier room
	S06	Dry milling room
Beta-lactam area	P04	Capsule V-mixer room
	P08	Bed fluidized drier room for dry suspension
	P03	Capsule filling room
	P20	Dry suspension filling room
Aminoglycoside area (Kanamycin)	SL08	Preparation room
	SL09	Filling room

In industrial terms, all these areas are classified as Class D (ISO 8) as their air input is supplied with H14 HEPA filters. In the acetaminophen tablet department, four preparation rooms were sampled (wet granulation mixer room, S04, dry V-mixer room, S12, bed fluidized drier room, S08, and dry milling room, S06). The sampling of beta-lactam production area was conducted in two preparation rooms (Capsule V-mixer room, P04 and bed fluidized drier room, P08, for dry suspension), and two filling rooms (capsule filling room, P03, and dry suspension filling room, P20). In the aminoglycoside antibiotic production area, two rooms were sampled, in which preparation and filling processes take place separately (SL08 and SL09, respectively).

At least one air sample was collected from each tested room by passive air sampling (settle plate). An automated air sampler (SAS Duo 360, BioScience International, USA) was also used for air sample collection (USP 797, 2015). It was programmed to collect 1000 L of air in three minutes. From every room, three surface samples (wall, the outer and the inner surfaces of the machine present) were collected either by contact plates (RODAC plates) or swabs. From each department, fingerprints of two workers were sampled on settle plates.

Determination of total aerobic microbial count (TAMC) in collected samples. The Petri dishes of settle or RODAC plates or the plates of the air sampler were incubated at 30-35˚C for 24-48 hours. The resulting colonies were manually counted, and the total count was expressed as colony-forming units (CFU)/plate for surface RODAC plates, CFU/m^3 in case of air sampler, and CFU/plate/time period for passive air samples.

After their enumeration, the colonies were harvested in 5 mL of Tryptone Soya Broth and incubated overnight. The resulting cultures were then stored in glycerol at -80°C until further used.

Determination of the antimicrobial susceptibility of collected samples. The antibiotic susceptibility of subcultures, from the stored glycerol samples, was determined by the Kirby Bauer disk diffusion method against the three antibiotics produced in the factory (amoxicillin, kanamycin, and erythromycin). Additionally, their susceptibility to nitrofurantoin (Oxoid, UK, 300 ug), an antibiotic never produced in this factory, was determined. This antibiotic was used as a control, since it is not expected to exert any selection pressure on bacteria in the factory environment.

The amoxicillin (25 ug), kanamycin (30 ug), and erythromycin (15 ug) disks were prepared in-house with antibiotic powder of the same batches used in the production process. Results were interpreted according to the Clinical Laboratory Standard Institute guidelines (CLSI, 2011).

Biochemical identification of isolated bacteria. Biochemical identification, to at least the genus level, was performed following Bergey's manual for Identification (Bergey and Holt, 1994).

DNA extraction and microbial community profiling by Illumina sequencing of 16S rRNA genes. Swabs were collected from the machine inner surfaces of the three tested areas within the pharmaceutical factory (two from each area: one during the first sampling and the other during

the last sampling). The collected swabs were from rooms S04, P04 and SL09 of non-antibiotic, beta-lactam and aminoglycoside production areas, respectively. DNA was extracted from each swab with the PowerSoil DNA extraction kit (MO-BIO laboratories, USA) and the MO-BiO vortex adapter. The manufacturer's instructions were followed without modifications.

Universal bacterial degenerate primers 8F– AGAGTTTGATCMTGGCTCAG and 926R-CCGTCAATTCMTTTRAGT, which encompass the hypervariable regions V1-V5 of the 16S rRNA gene were used to pre-amplify the extracted DNA, a process that increases total nucleic acid yield. AB-Gene DNA polymerase (Thermo Scientific, Waltham, Mass., USA) was used for amplification, at an annealing temperature of 52°C and just ten cycles of amplification, so that amplification biases are kept at a minimum (Sipos et al., 2007). PCR products were purified with Nucleofast 96 PCR filter plates (Macherey-Nagel, Düren, Germany).

DNA was sequenced at Centros FISABIO, Valencia, Spain (courtesy of Dr. Alex Mira) in an Illumina MiSeq Sequencer, per manufacturer's instructions (Illumina), by the use of the 2x300 bp paired-end protocol. The sequencing library was generated by the Illumina amplicon library protocol (Part #15044223 Rev. A). Gene-specific primer sequences used in this protocol were selected from (Klindworth et al., 2013) to target the V3 and V4 regions of the 16S rRNA gene, resulting in a single amplicon of approximately 450 bp.

Bioinformatics analysis of 16S sequence data. QIIME version 1.9.1 (Caporaso et al., 2010) was used for downstream analysis using the closed reference method for operational taxonomic units (OTUs) picking based on 97% identity with the Greengenes database version 13.8 (DeSantis et al., 2006).

For microbiome comparisons, 16S sequence data were obtained from public databases (e.g., HMP and EMP) and re-analyzed with QIIME for beta-diversity by the unweighted and weighted UniFrac distance metric for taxa composition and relative abundance, respectively. Three-dimensional principal coordinate analysis (PCoA) plots were used for clustering different samples according to their weighted UniFrac distances and visualized with Emperor (Lozupone and Knight, 2005). BugBase (URL: https://bugbase.cs.umn.edu (Ward et al., 2017)) was used for higher-level analysis and for attempts to correlate microbial taxa with phenotypes.

Detection of selected resistance genes using polymerase chain reaction (PCR). Twenty-four samples were selected for the detection of resistance genes. These samples were of the same

sample types in the three tested areas (air, fingerprint, and inner and outer machine surfaces), taken at the first and the fourth sampling months. Three primer sets (ThermoScientific) were used for amplifying an internal region of the selected genes by PCR: (bla_{TEM} and bla_{SHV} were selected as examples of beta-lactam resistance genes (Dallenne et al., 2010) and *aphA* as an example of a common aminoglycosides resistance gene (Noppe-Leclercq et al., 1999). PCR products were analyzed by gel electrophoresis on 1.5% agarose gel in Tris-Acetate-EDTA buffer and stained with ethidium bromide (Sigma).

PCRs were either conducted in a miniPCR (Marx, 2015) thermal cycler by Amplyus (Cambridge, MA, USA) or a Veriti Thermocycler (Applied Biosystems, Foster City, CA), and positive PCR products were then purified with Gene JET PCR Purification Kit (Thermo Fisher Scientific, Lithuania) and sequenced using ABI 3730 XL DNA sequencer (Applied Biosystems, Foster City, CA, USA).

BLASTN (URL: https://blast.ncbi.nlm.nih.gov/Blast.cgi, (Altschul et al., 1997)), under default settings, was used for comparison of sequencing products to resistance gene variants.

Statistical analysis. Statistical tests were performed in different software tools. Data Desk, Version 6.3 (Ithaca, NY, USA) was used for data visualization, some descriptive statistics, and some preliminary hypothesis testing. Additionally, some freely available online tools were used for analysis of variance (ANOVA) and Chi-Square test: the Free Statistics Calculators version 4.0 (URL: http://www.danielsoper.com/statcalc/calculator.aspx) was used for one-way ANOVA on numeric data of bacterial counts. Chi-square calculator was used for simple contingency tables (available at the Social Science Statistics website, URL: http://www.socscistatistics.com/tests/chisquare/Default2.aspx). Significance of paired groups of microbial count results was assessed by the Student t-test. Unless otherwise stated, significance level to reject the null hypothesis was at P-value of < 0.05.

Sequence deposition. All raw sequence reads were submitted to the Sequence Read Archive (SRA) and were all deposited under BioProject No. PRJNA391006 and assigned Biosample No. SAMN07259341 through SAMN07259346.

RESULTS

The pharmaceutical factory represents a unique built environment where there is interplay between different factors such as the product, personnel, and the environment, e.g., water supply, filtered air, humidity, and dust (mostly associated with personnel movement). This work focused on clean rooms in which solid dosage forms, or powder for suspension, are produced. Samples were obtained from several locations within production areas of (i) beta-lactam antibiotic (BL); (ii) aminoglycoside antibiotic (AG); and (iii) a non-antibiotic (NA) drug. For each sample, a total aerobic microbial count (TAMC) was determined, and main microbial types were determined by culture-based microbial identification typically used for microbial quality control. From each of the three main areas, representative samples were sequenced in a pilot microbiome profiling effort. Additionally, antimicrobial susceptibility of collected environmental samples to the antibiotics produced in the factory was determined.

TAMC of collected samples

In terms of quality standards, the applied action limits for TAMC are 100 CFU/plate /4 h. for passive air samples and 200 CFU/m^3 for active air samples. The surface samples action limit is 50 CFU/plate, while there are no recommended count limits for fingerprint samples in Class D rooms (European Commission, 2008; USP 797, 2015).

In this study, most samples were within the allowed TAMC limits, except six air samples from rooms S08, S04 and S12 in the non-antibiotic area and rooms SL09 (two samples) and SL08 in the aminoglycoside area, three controller RODAC plate samples from room P04 and five samples from the machine outer surface RODAC plate from rooms P08 (three samples) and two samples in room SL09 (Table S1).

Overall, there was no significant difference in the average TAMC of passive air samples and surface samples in different rooms within each area. The highest TAMC for passive air samples was observed in rooms S08 and P08 of non-antibiotic and beta-lactam areas, respectively. In the aminoglycoside area, the TAMC of passive air samples was comparable. The beta-lactam area samples had a significantly lower mean TAMC than the aminoglycoside and non-antibiotic areas (P = 0.003 and 0.004, respectively).

The highest TAMC of surface samples was detected in machine outer surface samples of rooms P08 and SL09 of beta-lactam and aminoglycoside areas, respectively. Tested surface

samples in non-antibiotic area were of door lock samples, and their TAMC was comparable in the four tested rooms. In fingerprint samples, two workers from two different rooms were sampled from each area. No significant difference was detected between the fingerprint TAMC within each area (Tables S2-S4).

Table 2: Types and number of isolates identified by culture-based and biochemical methods.

Microorganism detected		Number of isolates (%)		
		NA area (41 isolates)	**BL area (14 isolates)**	**AG area (20 isolates)**
Gram-positive bacteria	*Staphylococcus* spp.	18 (43.9%)	5 (35.7%)	11 (55%)
	Staphylococcus aureus	5 (12.2%)	1 (7.14%)	1 (5%)
	Micrococcus spp.	5 (12.2%)	2 (14.28%)	1 (5%)
	Corynebacterium spp.	4 (9.76%)	2 (14.28%)	–
	Bacillus or *Clostridium* spp.	4 (9.76%)	–	1 (5%)
	Enterococcus or *Streptococcus* spp.	2 (2.44%)	–	1 (5%)
	Lactobacillus spp.	–	1 (7.14%)	1 (5%)
Gram-negative bacteria	Non-lactose-fermenting Enterobacteriaceae	1 (4.88%)	–	3 (15%)
Fungi	*Candida* spp.	2 (2.44%)	3 (21.43%)	1 (5%)

Biochemical identification of isolated bacteria

Seventy-five colonies, which represented distinct colony morphologies, were selected from settle and RODAC plates of air and surface samples. They included 41 isolates from the non-antibiotic area, 14 from the beta-lactam area and 20 from aminoglycoside area. Most of the identified isolates were *Staphylococcus* species (54%), from which 9% were identified as *Staphylococcus aureus*. In addition, a few isolates were identified as *Micrococcus* spp. (11%), *Corynebacterium* spp. (8%), *Candida* spp. (8%), *Bacillus* spp. (7%) and followed by a few (~5%) Gram-negative non-lactose-fermenting Enterobacteriaceae. Fewer species were identified as *Enterococcus* or *Streptococcus* (4%) and *Lactobacillus* (3%) (Table 2).

Results of high-throughput sequencing of 16S rRNA genes in representative samples

The high-throughput sequencing data yielded 397,452 reads, after 1,879 sequences were filtered out for either short length or low quality. The average number of reads was 66,242 per sample and the average read length was 463 bp. After OTU assignment and clustering, using QIIME (Caporaso et al., 2010) and Greengenes (DeSantis et al., 2006), the sequencing reads were mapped to 2,114 OTUs.

To validate our sequencing depth and its coverage of microbiome diversity in all samples, we generated rarefaction curves for each sample using phylogenetic distance/diversity (Fig. 1A). The results showed adequate coverage and comparable sequencing depth for all samples, except for BL4, which had higher alpha diversity. This was confirmed when other alpha diversity indices were compared i.e., Shannon, ACE and Chao1 (Table 3). Interestingly, the overall community diversity increased between the first and fourth month (Fig. 1B), and while the difference in Chao1 index (expressing richness) did not reach statistical significance, the increase in Shannon diversity index in month 4 was significant ($P = 0.043$, ANOVA).

Table 3: Alpha diversity measures of the different sequenced microbiomes.

Sample	Shannon	ACE	Chao1
BL1	5.252	752.764	752.32
BL4	7.672	1813.634	1826.583
AG1	5.784	352.562	340.5
AG4	6.329	608.584	600.525
NA1	6.115	843.074	847.038
NA4	6.634	987.565	996.131

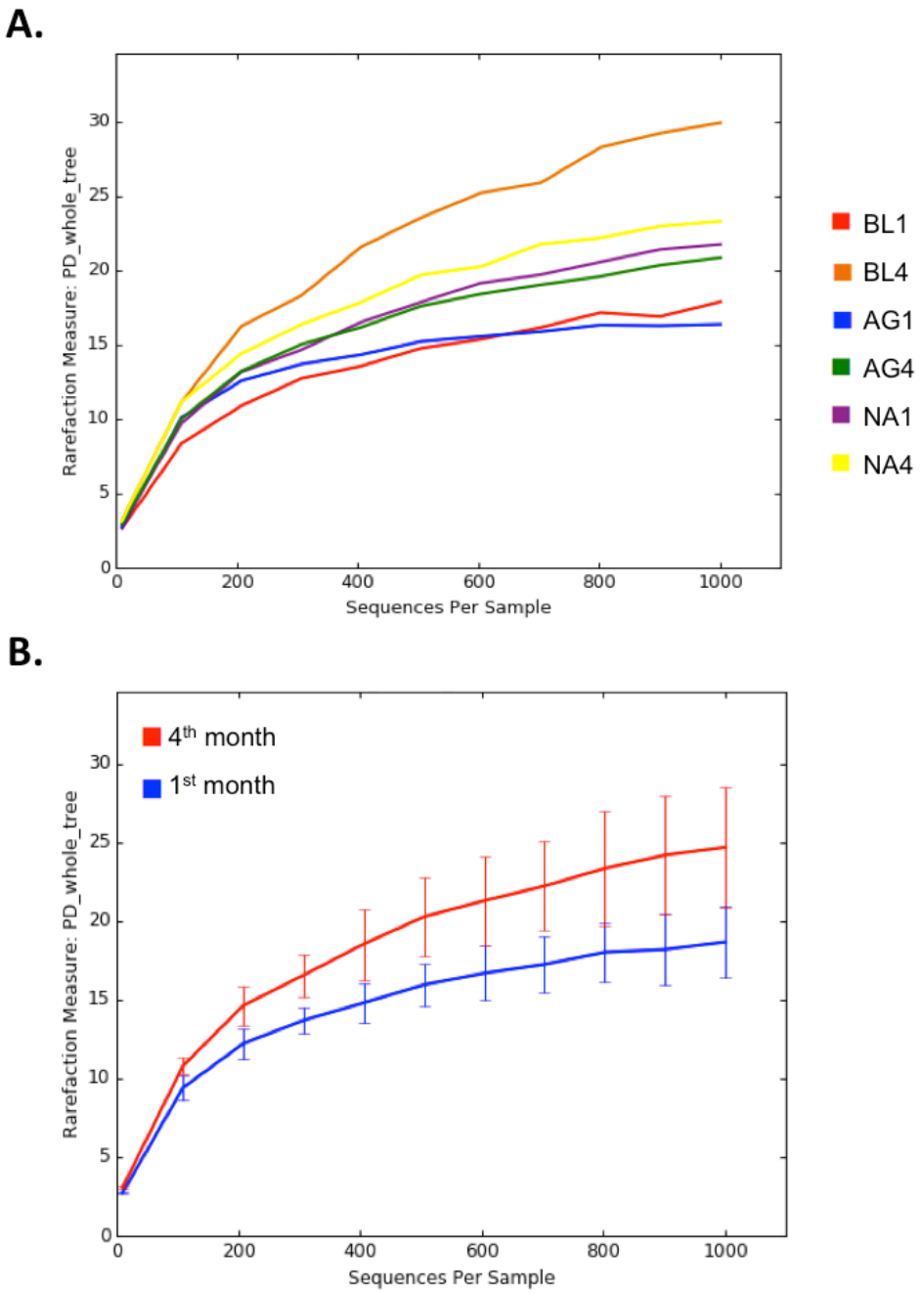

Figure 1: Rarefaction plots showing estimations of alpha diversity within the 6 samples (A) and the combined samples from first vs. fourth month (B). The plots show the coverage of each sample represented as phylogenetic distance/diversity (PD_whole_tree) vs. number of sequences, for the superiority of PD_whole_tree over simple OTU counts.

Microbiome composition across different rooms in the pharmaceutical factory

Overall, 29 microbial phyla were identified, and the samples were dominated by Proteobacteria, Firmicutes, Bacteroidetes, Thermi, and Actinobacteria with relative abundance 36%, 31%, 16%, 7%, and 4%, respectively (Fig. 2).

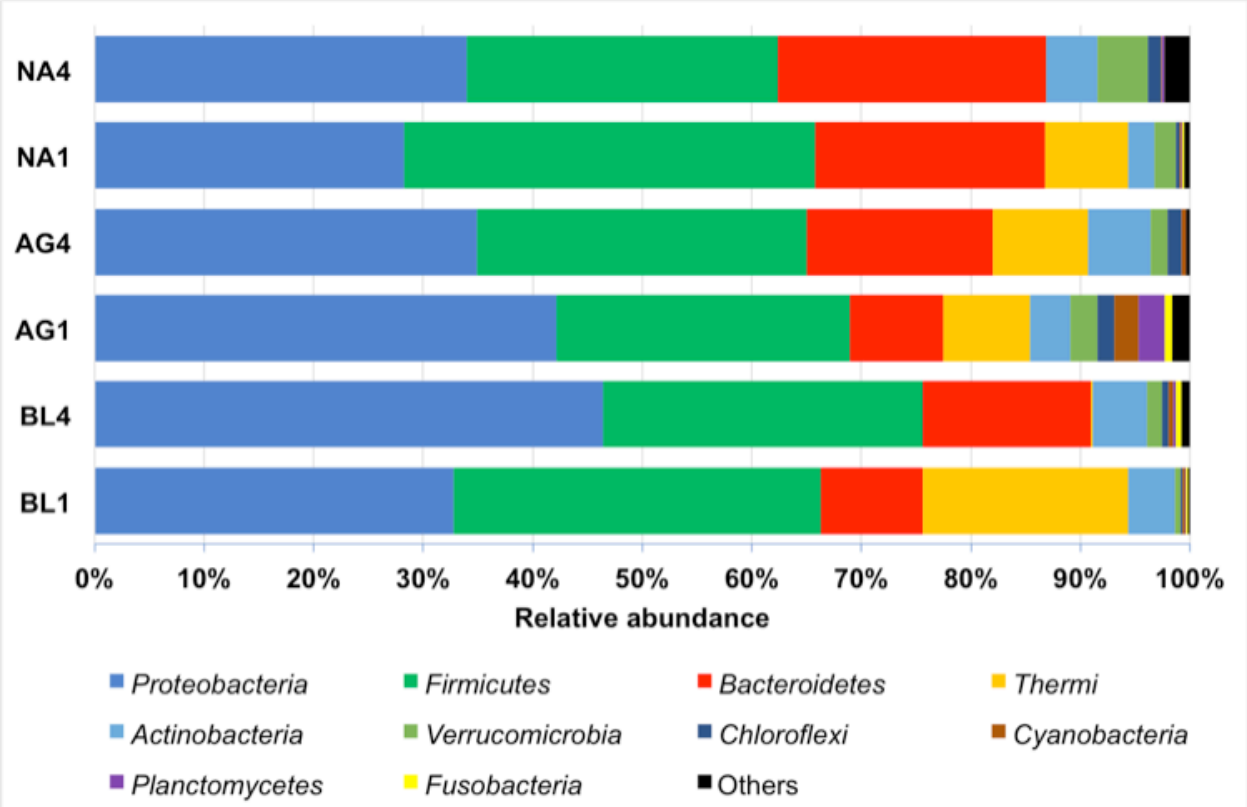

Figure 2: Taxonomic composition of the microbial communities associated with the tested machine inner surface samples

Genus-level analysis picked 418 genera in the six samples. Among the top genera (with a mean relative abundance > 1%), *Bacteroides* was the most abundant (highest mean) within all samples (Tables 4 and S5). *Staphylococcus* and *Acinetobacter* increased up to 7% and 15% in the beta-lactam room after 4 months, while *Legionella* increased to 9% in the aminoglycoside room. *Acinetobacter* increased in all fourth month samples, with the highest increase (~18%) in the non-antibiotic room. Several other OTUs increased significantly between the first and fourth month, notably *Bacteroides eggerthii*, which underwent the most statistically significant increase ($P = 0.0006$), Family Nitrosomonadaceae (F), whose mean abundance increased most dramatically (~21 fold), and *Clostridium difficile*, which reached the highest abundance (while $P < 0.05$) after four months (Table 5).

Table 4: Genus-level analysis showing abundance values for all taxa with mean abundance > 1%. In some cases entire families (F) or orders (O) are shown, when all genera within these families or orders are combined.

Taxon	BL1	BL4	AG1	AG4	NA1	NA4
Bacteroides	4.61	10.30	7.20	9.54	15.29	18.99
Planococcacea (F)	18.77	2.42	6.89	10.48	19.64	0.06
Meiothermus	18.78	0.00	7.96	8.75	7.61	0.03
Acinetobacter	1.06	15.69	1.06	2.98	0.20	18.63
Hydrogenophilus	7.51	0.15	5.63	3.97	5.51	0.06
Comamonadaceae (F)	2.42	1.03	7.96	1.42	3.30	2.27
Beijerinckiaceae (F)	3.61	0.00	5.94	3.43	2.67	0.00
Enterobacteriaceae (F)	1.00	1.57	0.37	1.36	4.56	1.79
Legionella	0.00	0.40	0.00	9.09	1.07	0.00
Staphylococcus	0.61	7.02	2.30	0.00	0.00	0.07
Clostridiales (O)	0.64	1.61	2.44	1.77	1.33	2.16
Clostridium	0.65	1.97	0.00	2.67	1.14	2.94
Streptococcus	4.10	1.14	1.27	0.49	0.73	1.21
Ruminococcaceae (F)	0.38	0.94	0.03	2.91	1.81	2.47
Eubacterium	0.70	1.48	0.00	1.82	1.17	2.59
Enterococcus	0.54	1.43	2.78	0.34	0.99	1.62
Faecalibacterium	0.39	1.32	0.00	0.56	1.64	3.56
Akkermansia	0.39	0.59	1.97	0.45	1.45	2.06
Lachnospiraceae (F)	0.26	0.30	1.74	2.32	0.38	0.89
Enhydrobacter	4.67	1.03	0.01	0.00	0.02	0.00
Saprospirales (O)	0.03	1.03	0.52	1.29	1.12	1.69
Others	28.88	48.57	43.94	34.36	28.36	36.94

Table 5: Comparing OTUs between samples collected the first and the fourth months (Sorted by *P-value*)

Taxon	1st month (mean)	4th month (mean)	*P*-value
Bacteroides eggerthii	0	3.333	0.0006
Oscillospira (G)	15.333	124	0.0027
S0208 (O)	0	3	0.0065
Bacteroides uniformis	0	1.667	0.0075
Bifidobacterium	35.333	243	0.0156
Diaphorobacter (G)	1.333	0	0.0161
Clostridiales (O)	0	82.667	0.0164
Acinetobacter (G)	0	3.333	0.0194
Sphingomonadaceae (F)	7.333	135.333	0.0202
Bifidobacterium	0.667	2.333	0.0241
Sphingomonas (G)	0	2	0.0257
Clostridium difficile	297.333	1742.333	0.0351
Kocuria rhizophila	0	316.667	0.0416
Saprospirales (O)	0	20	0.0447
Bacteroides (G)	0.333	1.667	0.0474
Nitrosomonadaceae (F)	16.333	345	0.0499

Regardless of the taxonomic level, a core set of 32 non-redundant taxa were consistently found across all six samples (Fig. 3). The relative abundances of these core taxa corroborate the past results, and highlight some dramatic changes, such as the striking increase of *Acientobacter* in the fourth month, although it was not as pronounced in the aminoglycoside room, and the dramatic variations in the abundance of members of family Planococcaceae.

Interestingly, all significantly changed OTUs between the two groups of samples from rooms dealing with antibiotics and the room not dealing with antibiotics had only increased in the room not involving antibiotics (Tables 6 and S5). Most significant OTUs in that condition were *Propionibacterium acnes, Faecalibacterium prausnitzii,* genus *Sutterella* and family Lachnospiraceae.

Taxon	BL1	BL4	AG1	AG4	NA1	NA4	Mean
Bacteroides (G)	4.61%	10.30%	7.20%	9.54%	15.29%	18.99%	10.99%
Planococcaceae (F)	18.77%	2.42%	6.89%	10.48%	19.64%	0.06%	9.71%
Acinetobacter (G)	1.06%	15.69%	1.06%	2.98%	0.20%	18.63%	6.60%
Hydrogenophilus (G)	7.51%	0.15%	5.63%	3.97%	5.51%	0.06%	3.80%
Comamonadaceae (F)	2.42%	1.03%	7.96%	1.42%	3.30%	2.27%	3.07%
Enterobacteriaceae (F)	1.00%	1.57%	0.37%	1.36%	4.56%	1.79%	1.77%
Clostridiales (O)	0.64%	1.61%	2.44%	1.77%	1.33%	2.16%	1.66%
Streptococcus (G)	4.10%	1.14%	1.27%	0.49%	0.73%	1.21%	1.49%
Ruminococcaceae (F)	0.38%	0.94%	0.03%	2.91%	1.81%	2.47%	1.42%
Enterococcus (G)	0.54%	1.43%	2.78%	0.34%	0.99%	1.62%	1.28%
Akkermansia (G)	0.39%	0.59%	1.97%	0.45%	1.45%	2.06%	1.15%
Lachnospiraceae (G)	0.26%	0.30%	1.74%	2.32%	0.38%	0.89%	0.98%
Saprospirales (O)	0.03%	1.03%	0.52%	1.29%	1.12%	1.69%	0.95%
Sutterella (G)	0.34%	0.88%	0.94%	0.38%	1.43%	1.34%	0.88%
auto67_4W (F)	0.17%	0.70%	0.44%	1.12%	0.50%	2.36%	0.88%
Pseudomonas (G)	0.03%	2.45%	0.01%	1.17%	1.05%	0.46%	0.86%
Corynebacterium (G)	0.90%	1.38%	1.21%	0.27%	0.53%	0.54%	0.81%
Rhizobiales (O)	0.36%	0.23%	1.87%	0.96%	0.25%	0.66%	0.72%
Bifidobacterium (G)	0.23%	0.46%	0.77%	0.72%	0.48%	1.55%	0.70%
Bacillus (G)	0.58%	0.47%	0.41%	0.12%	1.83%	0.14%	0.59%
Dialister (G)	0.06%	0.34%	1.10%	0.53%	0.30%	0.78%	0.52%
Sphingomonadaceae (F)	0.46%	0.75%	0.26%	1.37%	0.01%	0.09%	0.49%
Prevotella (G)	0.79%	0.79%	0.10%	0.30%	0.44%	0.41%	0.47%
Propionibacterium (G)	0.34%	0.14%	1.17%	0.19%	0.47%	0.43%	0.46%
ASSO-13 (O)	0.28%	0.57%	0.23%	0.11%	0.85%	0.62%	0.45%
SHA-98 (O)	0.04%	0.18%	0.70%	0.32%	0.04%	1.24%	0.42%
Delftia (G)	0.17%	0.16%	1.21%	0.16%	0.01%	0.29%	0.33%
Rhodospirillaceae (F)	0.39%	0.15%	1.05%	0.09%	0.12%	0.12%	0.32%
Betaproteobacteria (C)	0.32%	0.00%	0.01%	0.51%	0.01%	0.00%	0.14%
Rhizobiaceae (F)	0.01%	0.00%	0.01%	0.49%	0.00%	0.01%	0.09%
Agrobacterium (G)	0.12%	0.26%	0.03%	0.04%	0.00%	0.01%	0.08%
Peptostreptococcaceae (F)	0.01%	0.12%	0.04%	0.01%	0.04%	0.06%	0.05%

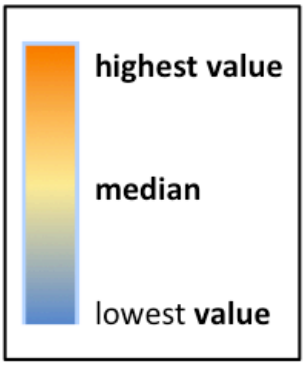

Figure 3: Heatmap of relative abundance of the core 32 taxa. Orange color represents the highest relative abundances while dark blue represents the lowest abundance of core OTUs. Some striking differences are emphasized with dashed borders (e.g., variations in abundance of family Planococcaceae and genus *Acinetobacter*).

Table 6: Comparing OTUs between samples collected from rooms related to antibiotics (2 rooms) and non-antibiotics (1 room). G= Genus, F = Family.

OTU	Taxonomy	Rooms involving antibiotics (mean)	Rooms not involving antibiotics (mean)	*P*-value
972955	*Propionibacterium acnes*	0	1.5	0.0081
1088265	*Propionibacterium acnes*	111	261	0.0273
213813	*Bacteroides (G)*	0	1.5	0.0081
339013	*Bacteroides (G)*	23.5	220	0.0472
187324	*Bacteroides caccae*	68.5	222.5	0.0205
276149	*Parabacteroides (G)*	51	211.5	0.0472
291090	*Parabacteroides distasonis*	1.75	10.5	0.0296
588929	*Prevotella copri*	0	1.5	0.0081
759751	Lachnospiraceae (F)	0	3.5	0.0003
311820	Lachnospiraceae (F)	0	2	0.0309
361811	Ruminococcaceae (F)	18.75	137	0.0144
525215	*Faecalibacterium prausnitzii*	0.5	4.5	0.0078
190169	*Faecalibacterium prausnitzii*	0	1.5	0.0081
340219	*Faecalibacterium prausnitzii*	0	1.5	0.0081
1046997	*Dialister (G)*	0.5	2	0.0257
548587	*Eubacterium dolichum*	0.25	2	0.0095
112891	*Sutterella (G)*	0	2.5	0.0012
363731	*Akkermansia muciniphila*	2	10.5	0.0103

Factory microbiome samples vs. the global microbiome

In an attempt to explore potential origins of the different taxa detected in the factory rooms, and to better situate those microbiome samples in a larger context, we reanalyzed publicly available 16S microbiome sequences from 1,689 samples, representing 17 ecosystems, five of which are the five HMP sites (gut, skin, vagina, and nasal and oral cavities) and the remaining ones represent various EMP habitats. The patterns obtained, visualized on a 3-dimensional PCoA plot, were intriguing. The six factory microbiome samples clustered at the intersection of human and earth microbiome samples, with a close proximity to oral, aerosol, non-saline surface and soil microbiomes (Fig. 4).

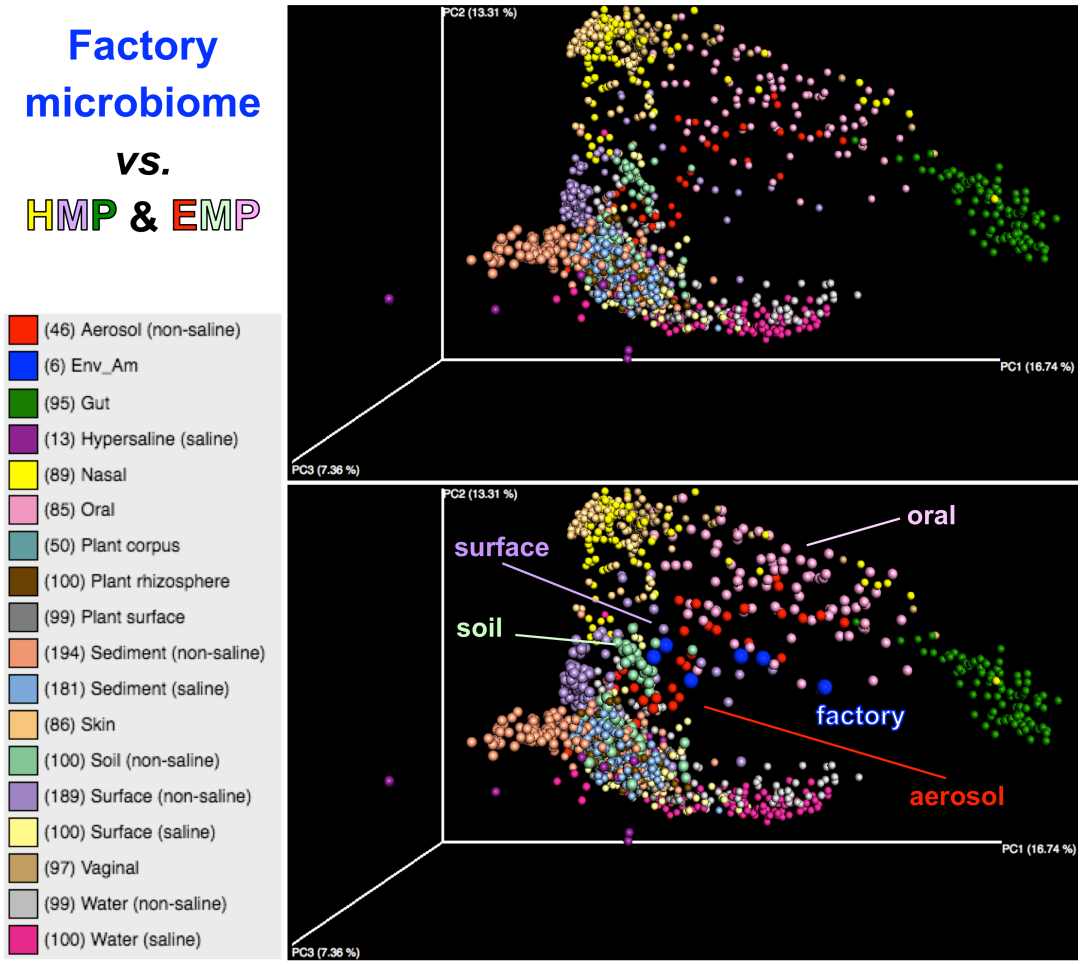

Figure 4: Principal coordinate analysis (PCoA) plots of the six factory microbiome samples in comparison with public data from the Human Microbiome Project (HMP) and the Earth Microbiome Project. Samples are color coded as shown in the legend. In the lower panel, the factory microbiome samples are shown as blue spheres, and the most closely related environments are annotated.

Antimicrobial susceptibility of collected samples

Most of the collected samples (186 samples out of the 234) were resistant to at least one of the tested antibiotics. A substantial number of samples (101 samples) were resistant to all four tested antibiotics (Table S6). In the beta-lactam area, the number of amoxicillin-resistant samples was significantly higher ($P < 0.05$) than in other areas; yet, the number of kanamycin-resistant samples was significantly lower than in other areas ($P < 0.05$). On the other hand, in the non-antibiotic and aminoglycoside areas, the only significant difference in antibiotic resistance was that of nitrofurantoin-resistant samples. In most of the tested areas, there was a significant increase in the number of resistant samples with time (Table 7).

Table 7: Number of resistant samples to each tested antibiotic during the whole sampling period in **the three areas** and the results of the corresponding Chi-square and ANOVA tests

Area	Antibiotic	Number of resistant results (%)				Total (%)	Chi square *P*-value	
		May	Jun.	Jul.	Aug.		Between months	Between antibiotics
NA area	Erythromycin	16 (66.7)	19 (79.2)	17 (70.8)	21 (87.5)	73 (76.04)	0.34	0.00914
	Kanamycin	14 (58.3)	19 (79.17)	16 (66.7)	19 (79.2)	68 (70.83)	0.30	
	Amoxicillin	15 (62.5)	19 (79.17)	18 (75)	18 (75)	70 (72.92)	0.59	
	Nitrofurantoin	11 (45.8)	15 (62.5)	14 (58.3)	13 (54.2)	53 (55.21)	0.69	
	Chi-square P-value	0.4936	0.4959	0.64418	0.571			
	Total R score (%)	56 (58.3)	72 (75)	65(67.7)	71(74)			
	Chi-square P-value	**0.049115**						
BL area	Erythromycin	16 (66.7)	10 (41.7)	19 (79.2)	22 (91.7)	67(69.8)	**0.0014**	0.00009
	Kanamycin	8 (33.3)	6 (24)	13 (54.2)	17 (70.8)	44 (45.8)	**0.0061**	
	Amoxicillin	18 (75)	13 (54.2)	20 (83.3)	22 (91.7)	73 (76.04)	**0.0167**	
	Nitrofurantoin	14 (58.3)	8 (33.3)	18 (75)	21 (87.5)	61 (63.54)	**0.0007**	
	Chi-square P-value	**0.0223**	0.1947	0.10598	0.12791			
	Total R score (%)	56 (58.3)	37 (38.5)	70 (72.9)	82 (85.4)			
	Chi-square P-value	**< 0.00001**						
AG area	Erythromycin	2 (33.3)	7 (58.3)	9 (75)	11 (91.7)	29 (69.04)	0.0630	0.00363
	Kanamycin	2 (33.3)	9 (75)	9 (75)	11 (91.7)	31 (73.8)	0.0693	
	Amoxicillin	2 (33.3)	6 (50)	9 (75)	10 (83.3)	27 (64.3)	0.1084	
	Nitrofurantoin	1 (16.7)	5 (41.7)	3 (25)	7 (58.3)	16 (38.1)	0.2416	
	Chi-square P-value	0.8952	0.3973	**0.0229**	0.1175			
	Total R score (%)	7 (29.17)	27 (56.25)	30 (62.5)	39 (81.3)			
	Chi-square P-value	**0.000267**						

Detection of selected resistance genes:

The selected rooms for this analysis were the V-mixer room of non-antibiotic area (S12), the filling room of aminoglycoside area (SL09), and the V-mixer room of beta-lactam one (P04). These samples were selected because they had several sensitive isolates in the first month but more resistant ones in the fourth.

Most tested samples contained beta-lactamase genes (18 out of 24). The genes were either of bla_{TEM} or bla_{SHV} families (encoding extended-spectrum beta-lactamases, ESBLs). All bla_{TEM} genes were $bla_{\text{TEM-1}}$, except two samples that were $bla_{\text{TEM-2}}$ and $bla_{\text{TEM-116}}$, and the only detectable SHV resistance gene was bla_{SHV12}. The aminoglycoside resistance gene, *aphA-1*, was not detectable in any of the aminoglycoside production areas, and only beta-lactamase genes were detected there; however, *aphA-1* was detected in all tested samples of the non-antibiotic area in the fourth month. In that area, $bla_{\text{TEM-1}}$ was the only detectable gene in the first month, while $bla_{\text{TEM-1}}$ and *aphA-1* were co-detected in the fourth month from both air and fingerprint samples.

DISCUSSION

It is a common practice to conduct routine microbial cultures in different production areas of pharmaceutical factories to assess the environmental microbial burden, and track the source of any emerging or spreading microbial contamination. In this context, strict guidelines regulate microbial count limits in different locations of production clean rooms for non-sterile products. Although DNA-based methods have been well developed in many other research areas and have been applied to pharmaceutical production facilities (Sandle, 2011; Sheraba et al., 2010), studies on microbiome profiling or metagenomics of the built factory environment are still in their infancy. Solely relying on culture results as a measure of environmental bioburden is no longer sufficient, as culturable bacteria represent only a small fraction of biodiversity (Riesenfeld et al., 2004a), and many bacteria retain a viable but nonculturable (VBNC) state, as they fail to grow on routine bacteriological media but are fully capable of renewed metabolic activity, gene expression, and rRNA synthesis (Oliver, 2005). Given the importance of correlating factory microbiota—including less abundant and VBNC members—with the manufactured product and to study selection dynamics of different bacteria when the produced drug is an antibiotic, we launched this pilot study as a first step towards a national drug factory microbiome project.

In our study, the predominance of Proteobacteria, Firmicutes, and Bacteroidetes is in accordance with what has been previously reported on Class D room microbial communities (Park et al., 2014). Despite the small number of samples, the effect of the production environment and condition on microbial community composition in the machine inner surface can be clearly observed. The presence of water-inhabiting bacteria, such as Legionellaceae, Lachnospiraceae, Clostridiales, and the genus *Chelatococcus* (McLellan et al., 2013) was pronounced in the aminoglycoside area, in which there is enormous water usage during production of kanamycin suspension. These bacterial types are known for their biofilm-forming ability even in purified water, and thus can resist disinfection or filtration processes they have been trough. Many of these bacteria are oligotrophic, i.e., able to grow in low nutrient environment. These taxa also need longer contact time and higher concentration of chloramine disinfectants, actually used in water systems. The detection of family Saprospiraceae, one of Bacteriodetes families present in our samples, may also be attributed to water usage. Yet, their detection in higher percentage in the non-antibiotic area may be due to their ability to utilize

complex carbon sources (Kekacs et al., 2015), which are abundant in the components of the non-antibiotic (acetaminophen) tablet additives.

The high predominance of family Hydrogenophilaceae and genus *Meiothermus* in microbial communities of both beta-lactam and aminoglycoside production areas may be attributed to the thermophilic nature of these bacteria (optimum growth at 50°C, Bulat et al., 2004). They were detected in samples from the powder-mixing machine in the beta-lactam area, where friction often generates heat. They were also detected in the machine of the aminoglycoside area, which operates at high temperature. In addition, *Meiothermus* has the ability to form biofilms on stainless steel and other surfaces (Kolari et al., 2003), which could explain its presence on machine surfaces. The higher abundance of this genus in the beta-lactam area may also be related to its alkaliphilic nature, given that amoxicillin is formulated as an alkaline salt (sodium amoxicillin salt) (Lopez-Lopez et al., 2015). Another potential physicochemical effect is the use of sodium chloride as flocculating agent in some suspensions formulas, which could have selected for a halophilic bacterial family such as Planococcaceae, which has some member that can withstand up to 17% NaCl (De Vos et al., 2009). These organisms are also known to be resistant to quaternary ammonium compound (QAC) disinfectants (Struchtemeyer and Elshahed, 2012), which was the disinfectant used during the sampling period.

The presence of organisms that constitute members of normal human microbiota such as *Staphylococcus, Streptococcus, Micrococcus, Lactobacillus* and *Bacteroides* (Human Microbiome Project Consortium, 2012) highlight the contribution of the personnel to the analyzed microbiome. This contribution has been strikingly demonstrated when the factory samples were compared to HMP and EMP data (Fig. 4).

It was somehow worrisome to detect a few genera with known pathogenic members, such as *Staphylococcus* and *Clostridum* as the factory workers can transfer these organisms to the public, perhaps even after selection of more resistant members. In other terms, the personnel would bring some contaminant microbes to an antibiotic-producing area, then the surviving resistant variants of these microbes will be carried back to the community, posing more risk.

The influence of the personnel on the factory microbiome is not unprecedented for a built environment. An extensive longitudinal home microbiome study clearly demonstrated that

humans had the major effect on the built environment (Lax et al., 2014). Humans were also demonstrated to be the main source of microbial communities on their mobile phones (Meadow et al., 2014). Yet, it was shown here that the pharmaceutical factory is a bit more complex because of the chemical nature of raw materials and final products, as well as other environmental factors that are combined in shaping the microbial assemblages.

The effect of these different factors on the microbiome composition was confirmed by the other performed analyses. For instance, the types of organisms identified by biochemical methods tend to agree with 16S rRNA profiling. The identification of bacterial genera that are indigenous to human, such as *Staphylococcus, Micrococcus* and *Corynebacterium*, especially from the personnel-related samples (fingerprints and door lock contact plates) confirms the contribution of workers to the analyzed microbiomes. These bacteria usually colonize the skin and mucous membranes, and can be easily transmitted to the environment by airborne particulates or aerosol (Otto, 2009; Sandle, 2011). The high percentage of *Staphylococcus* species among cultured samples, and their presence in the contact plate samples, may also be attributed to the used disinfection policy. The use of quaternary ammonium compounds (QAC) may be providing an environment that selects for biocide-resistant staphylococci (Panel on Biological Hazards of the Norwegian Scientific Committee for Food Safety, 2009). Moreover, the effect of soil, highlighted by the comparison with EMP samples (Fig. 4) was pronounced by the detection of spore-forming Gram-positive rods (*Bacillus* and *Clostridium* species), especially in air samples. These are often considered as indicators for soil/dust contamination that could have been transported into clean areas through dust, equipment, and personnel footwear (Halls, 2004; Sandle, 2011).

Water usage likely affected the TAMC, as the highest TAMC of air samples was detected in the aminoglycoside area, since water is involved in most manufacturing processes in that area where production of a wet suspension formula takes place. The presence of water may have contributed as a source of contamination or growth support (Sandle, 2011). The same water effect was reported when comparing the TAMC of workers' fingerprints. Workers whose fingerprint samples had high TAMC were from rooms with water usage (wet granulation room, S04, and filling room, SL09). In addition, the effect of personnel was pronounced in the significantly high TAMC of surface samples in sites that were in direct contact with personnel

(i.e., machine outer surfaces of rooms, P08 and SL09, and machine controller samples from room P04).

The results of antimicrobial susceptibility tests are alarming. Most samples showed resistance towards at least one of these antibiotics (79.5%). In addition, about half of collected samples (43.16 %) were resistant to all four tested antibiotics. This indicated the expected spread of resistant bacteria, even if harmless to human, in the pharmaceutical factory, similar to what has been reported by Sarker *et al.* (2014). As hypothesized and expected, the highest percentage of resistant bacteria was against the three antibiotics produced in the factory: erythromycin (73.12%), amoxicillin (72.45%), and kanamycin (63.6%). This enrichment of resistant microbes is likely to be related to the selective pressure exerted by the produced antibiotics on residing bacteria in each antibiotic production area (O'Brien, 2002; Sarker et al., 2014; Tacconelli et al., 2008). On the other hand, resistance against the control antibiotic, nitrofurantoin, was not particularly high.

The increase of *Acinteobacter* in most areas in the fourth month is a neat depiction of the introduction of a population of highly resistant bacteria in the factory environment. Interestingly, *Acinetobacter* is also a great example of a potential pathogen and potential multi-resistant microbe that is neither among those analyzed by traditional methods nor among those analyzed by routine quality control protocols. The incidental spread of *Acinetobacter* in the factory makes a strong case for the use of unbiased, culture-independent microbiome profiling at least to update quality control measures with new emerging microbes that may pose public health threats.

In general, a detectable increase was observed in the total percentages of resistant microbes with time. Many studies suggested that excessive contact between antimicrobial agents and germs enhance the trafficking of resistance genes (Corno et al., 2014; O'Brien, 2002; Relman and Falkow, 2001; Tacconelli et al., 2008), and therefore microbial resistance is strongly associated with the exposure time to an antibiotic (Nazaret and Aminov, 2014)

It remains to acknowledge that the results of this pilot study are preliminary. They just provide enough evidence to warrant pursuing a more systematic, more comprehensive prospective microbiome survey in different factories. The main limitation of this work is the small number of samples analyzed by high-throughput sequencing and the lack of a total metagenomic survey that will provide a full resistome profile (Elbehery et al., 2016).

CONCLUSION

This pilot study offers a snapshot of a drug factory microbiome under normal operation condition. The work focused on rooms and machines involved in manufacturing antibiotics in comparison to a control non-antibiotic drug product. Several factors were suggested to affect the cultured and uncultured microbiome of this factory, including the personnel, use of water, prevailing environmental condition within the machines (e.g., temperature), the chemicals used, and the type of product (pH and antimicrobial activity of the products). Future studies of pharmaceutical plant microbiomes are therefore required to determine all possible factors affecting the microbial composition, as well as the resistome, and the possible effects of these microbial assemblages on workers and subsequently on the entire community. Such systems-level analysis can also help to modify or optimize good manufacturing practice measures to avoid the unwanted effects.

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